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Review of the PhD thesis by Zuzanna Pakosz-Stępień

Characterisation of nonbacterial gyrases – from biochemical analysis, through compound-enzyme interactions to structural biology

Zuzanna Pakosz-Stępień's PhD thesis, titled "Characterisation of Nonbacterial Gyrases – From Biochemical Analysis to Structural Biology" focuses on unraveling the structures and mechanisms of action of a group of enzymes known as DNA gyrases. This work was conducted under the supervision of Professor Jonathan Heddle at the Małopolska Centre of Biotechnology, Jagiellonian University.

DNA gyrases, belonging to the type II topoisomerase family, are key enzymes in regulating the twisting of DNA, which is crucial for controlling DNA supercoiling during processes like DNA replication. Importantly, DNA gyrases are found in bacteria, archaea, and some eukaryotic parasites but not in humans, making them attractive targets for antibiotic drug development. In the first part of her Thesis, Zuzanna describes studies aimed at understanding the mechanism of action of new small molecular compounds that specifically target the DNA gyrase from *Plasmodium falciparum.* Her goal was to provide a blueprint for the development of new drugs to combat malaria. To achieve this, Zuzanna undertook the challenging task to express, purify, and reconstitute the complete *Plasmodium falciparum* DNA gyrase (*Pf*Gyr). DNA gyrase is made up of two subunits, GyrA and GyrB, and it functions as a heterotetrametric complex, GyrA2GyrB2. Zuzanna's first significant hurdle arose when numerous attempts to express active *Pf*GyrA proved unsuccessful. Challenges persisted as the purification protocol for *Pf*GyrB yielded an enzyme that was not sufficiently pure. To address this challenge, Zuzanna optimized the purification process, successfully obtaining a pure *Pf*GyrB. She also discovered that *Pf*GyrB

exhibited activity when combined with *E. coli* GyrA, forming a hybrid complex known as *Ec*GyrA*Pf*GyrB. Using this setup, she screened a chemical library consisting of 98 compounds to identify any that could preferentially inhibit the supercoiling activity of the *Ec*GyrA*Pf*GyrB complex. Zuzanna discovered two promising candidates: alizarin red S (ARS) and purpurogallin (PPG), both exhibiting mid-range IC_{50} values. Through a series of biochemical experiments, including DNA supercoiling inhibition assay, DNA cleavage assay, ATPase activity assays, EMSA and MST Zuzanna demonstrated that PPG inhibits interactions between *Pf*GyrB and DNA. In her pursuit to understand the molecular mechanism of this newfound inhibition, Zuzanna employed structural biology methods, including attempts to analyze the *Ec*GyrA*Pf*GyrB-DNA complex using cryo-EM. Unfortunately, these attempts have proved unsuccessful. Subsequently, Zuzanna had the effectiveness of PPG tested *in vivo*. While growth inhibition assays conducted on *P. falciparum* cultures revealed a modest inhibitory effect, and it remains uncertain whether observed effect is a result of the demonstrated *in vitro* activity against the DNA gyrase, PPG exhibited similar level of activity to quinolones, which are currently in clinical use. In conclusion, Zuzanna discovered PPG that represents a promising new candidate for an anti-malarial drug. It can serve as a template for developing more effective derivatives, which would broaden the current strategies for anti-malarial therapy. In my view, the data presented in the first part of this Thesis present original and relevant findings and represent interesting advancement in the field. Some results presented in the first part of the Thesis were published in *Antimicrob Agents Chemother* (DOI:10.1128/AAC.00267-21), where Zuzanna is the first author.

In the second part of her Thesis, Zuzanna aims to uncover new insights on the structure/function of DNA gyrases from a hyperthermophile, *Archaeoglobus sulfaticallidus.* Given that this organism thrives in high-temperature and high salt environments, its DNA gyrase may exhibit unique adaptations, rendering the enzyme an intriguing subject of study with potential biotechnological significance. In this case, DNA gyrase from *Archaeoglobus sulfaticallidus* (*As*Gyr) was purified with exceptional yields and purity, enabling Zuzanna to perform comprehensive biochemical, biophysical, and structural characterization. These included thermal stability studies and DNA supercoiling activity studies of *As*Gyr at different temperatures, pH, and KCl concentrations. Next, Zuzanna utilized cutting-edge cryo-EM techniques to examine the structure of *As*Gyr, aiming to identify the structural characteristics

that endow it with remarkable thermal stability compared to its bacterial counterparts. She obtained a high-resolution map (3.19 Å) of *As*Gyr-DNA-MFX complex which allowed for an in-depth exploration of the structural elements at the core of the complex and provided a clear visualization of domain positions in relation to previously published structures. Significantly, the structure obtained by Zuzanna represents the first full-length DNA gyrase with an extended DNA fragment and a fluoroquinolone antibiotic. Solving the structure of full length *As*Gyr with long DNA fragment and moxifloxacin allowed Zuzanna to make several interesting observations. Zuzanna discovered a unique arrangement of *As*GyrA C-terminal domain (CTD), which is responsible for DNA binding and wrapping. Using structural and biochemical data Zuzanna has shown that distinctive highly positively charged pattern on the surface of *As*GyrA CTD plays a crucial role in stabilizing the DNA-enzyme complex. This prevents DNA slippage, especially in high-temperature and high-salt conditions. It's interesting to note that *As*GyrA CTD lacks an acidic tail. The acidic tail is thought to weaken the connection between gyrase and DNA. Zuzanna proposes that the absence of the acidic tail might be an adaptation to high temperatures. In hot environments, organisms like *Archaeoglobus sulfaticallidus* may not need the acidic tail in their DNA gyrase because the heat itself already weakens the connection between gyrase and DNA. Zuzanna also found that *As*Gyr (as well as *E. coli* DNA gyrase used in control experiments) possesses the capability to efficiently relax positively supercoiled DNA in an ATP-independent manner. This intriguing discovery challenges the prevailing understanding of DNA gyrase activity and necessitates a thorough reexamination of the current state of knowledge in this field. In my opinion, the data presented in the second part of the Thesis bring novel and significant findings, extending our understanding about the mechanism of action of DNA gyrase. As indicated by the Author (page 7), results described in the second part of the Thesis will be included in a manuscript that is currently in preparation. Zuzanna is the first author of that manuscript.

In general, the Thesis structure is clear and follows a logical order. It comprises multiple sections, including Acknowledgments, Table of contents, List of publication, Abbreviations list (missing from Table of contents), Abstract (both Polish and English version), followed by comprehensive Introduction. In this section, Zuzanna describes the main players of her Thesis and moves on to focus on the structure and mechanism of action of DNA gyrases. She proceeds

to discuss the distribution of DNA gyrase in nature, highlighting why they make interesting drug targets. Further, she provides historical background on drugs targeting DNA gyrase and their mechanism of action. This part of the introduction is nicely integrated with available structural biology data. In general, the Introduction section, while somewhat lengthy, is comprehensive and written in a clear manner. Following the Introduction, there is a well-defined section outlining the Aims and a comprehensive Materials and Methods section. In my opinion, the Results, Discussion, and Summary sections are clearly written. This work includes a reference list of 191 publications, which is appropriate. Overall, I find this Thesis easy to read, with only a few minor errors (for instance, *Plasmodium falciparum* is sometimes italicized and other times it's not). The figures are clear, mostly well-described, effectively communicate the message, and underscore the substantial research efforts undertaken by Zuzanna during her PhD.

I find Zuzanna's work fascinating for several reasons. Firstly, it addresses fundamental and pertinent biological questions. This is illustrated by the fact that, during the period when this work was conducted, another research group published a paper in *Nature Communications* describing the cryo-EM structure of the *E. coli* DNA gyrase nucleoprotein complex (reference 144). If there is any proof that the subject of investigation is significant and timely, this is certainly it. Second, the effort required to compile this Thesis is truly impressive. It is evident from the extensive Materials and Methods section (pages 52-66) that Zuzanna had to attain expertise in numerous experimental techniques to address the research questions she was investigating. Third, I am impressed by Zuzanna's attempt to validate her *in vitro* findings through *in vivo* studies. Although the *in vivo* work was conducted in collaboration and did not yield groundbreaking results, I believe it was a valuable endeavor and afforded Zuzanna valuable experience in collaborating with other scientists. Zuzanna's collaborative skills are also highlighted by the fact that she is a co-author on three additional publications in prestigious journals such as *Nature Catalysis, Sci Adv*, and *Nucleic Acids Res.*

In summary, the work presented in the Thesis maintains a very high standard, and its core findings have already been published or are in preparation for publication in prestigious international peer-reviewed journals, with Zuzanna as the first author. Throughout her PhD

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studies, Zuzanna has acquired significant expertise, and her research makes a substantial contribution to our understanding of the field. In conclusion, I firmly believe that Zuzanna Pakosz-Stępień's PhD thesis meets all the requirements for the title of Doctor of Philosophy based on Dz.U. z 2018 r. poz. 1668 z późn. zm. Therefore, I recommend that she be admitted to the subsequent stages of the doctoral dissertation proceedings. Furthermore, due to the high quality of the work presented in this Thesis, I recommend that it should be considered for appropriate distinctions, if permitted by the Doctoral School regulations.

Podsumowując, jestem przekonany, że praca doktorska Zuzanny Pakosz-Stępień spełnia wszystkie warunki określone w artykule 187 ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz.U. z 2018 r. poz. 1668 z późn. zm.). Dlatego też rekomenduję, aby Pani Zuzanna Pakosz-Stępień została dopuszczona do kolejnych etapów postępowania doktorskiego. Ponadto, ze względu na wysoką jakość pracy przedstawionej w tej rozprawie, rekomenduję rozważenie możliwości przyznania wyróżnienia, jeśli pozwala na to regulamin Szkoły Doktorskiej.

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