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Opponent's Review of Doctoral Thesis by **Alessandro Panattoni**

„Synthesis and studies of modified DNA: (i) development of DNA targeting molecular scissors and (ii) competitive enzymatic incorporation of base-modified nucleotides "

The thesis has been elaborated at the Institute of Organic Chemistry and Biochemistry of the CAS, v.v.i., in Prague as a part of interdisciplinary project performed by Prof. Michal Hocek team. It consists of two major parts: a) development and proof-of-principle application of targetable chemical nucleases based on clamped phenanthroline copper complexes attached to triplex forming oligonucleotides; and b) systematic study of competitive polymerase incorporation of base-modified nucleotides in the presence of corresponding natural nucleotides. Results have been published in three research papers in renowned impacted journals (*ACS Chem Biol, Org Lett* and *ChemBioChem*; in last two of them the Candidate is the first author).

The Thesis itself is traditionally structured. Introduction chapter (22 pages) provides general background to the work and its purpose, bringing basic information on principles of DNA structure and function, *in vitro* DNA synthesis using various replication/amplification strategies, restriction cleavage, chemical synthesis of DNA, DNA modification via application of base-modified deoxynucleoside triphosphates and techniques of post-synthetic decoration of DNA. Special attention is devoted to genome editing strategies that are based on targetable nucleases, and to DNA triplexes with respect to utilization of triplex-forming oligonucleotides used in the development of site-specific DNA cleaving agents in this work. After this introductory part, aims of the work are systematically and lucidly formulated. Chapter "Results and Discussion" (~70 pages) is divided into two subchapters according to the two main themes of the Thesis mentioned above. Each contains introductory paragraphs to explain the purpose and strategy of experiments performed within the given topic, followed by description and discussion of the experimental work well documented by reasonable number of illustrations. Conclusions (3 pages) offer summarization of key results and discussion in terms of advantages and limitations of approaches and tools developed. Next 66 pages is devoted to Experimental chapter summarizing material and methods used throughout the Thesis,

List of abbreviations and References. Overall, the Thesis is written in reader-friendly, good English with minimum typos.

The following queries and comments are intended to provoke discussion during the viva defense:

1) It is stated on page 23: *Base-modified DNA finds several applications in biotechnology, diagnostics and therapy, as well as in material science*. Can you briefly present several examples of routine applications of base-modified nucleic acids in the above areas?

2) Text from p. 29: "*As a consequence of higher stability and electron-donation by ether oxygen atoms, the oxidative nuclease activity of their* Cu^{I} *complexes is higher than the one of* $[Cu(Phen)_2]^+$ *(two times higher for Clip-Phen, and higher by a factor of 60 for 3-Clip-Phen)*". Can you explain how the ether oxygens increase the "nuclease activity". What is increased – is it the rate of ROS production? Cannot other factors, such as increased stability of the Clip-phen complexes (compared to $[Cu(Phen)_2]$) or their stronger interaction with DNA, contribute to the apparent higher nuclease activity?

3) Both chemical and enzymatic strategies of DNA bearing the Clip-phen Cu complexes are presented. In addition, in both approaches base modified with a reactive group (ethynyl or azide) is introduced in the oligonucleotide, followed by click reaction with azide or ethynyl derivative of the copper complex, respectively. Are there reasons to prefer any of these approaches (chemical or enzymatic, "azide first" or "ethynyl first") in specific cases?

4) In figures 17 and 18, changes (decrease) in the intensity of band corresponding to the full length substrate oligonucleotide are measured to evaluate the extent of cleavage. Wasn't it possible to design the substrate so that its truncated fragment(s) could be seen (using end labelling similarly as in PAGE experiments to characterize the PEX products)? This should be sufficient to check site specificity of the cleavage. (I am aware that in Fig. 19 labelling inside the substrate is used to see all cleavage products).

5. Text from page 86: *"For this purpose,* ***the type of cleavage obtained by local radical formation*** *induced by CuI can be superior to CRISPR-Cas systems, which cleavage is relatively easy to be repaired".* What exactly is meant here by ***type of cleavage***?

6. Table 3 on page 97 and other tables thereafter: I wonder whether statistical evaluation of the data has been performed.

As conclusion, based on the above evaluation, this reviewer considers dissertation thesis of **Alessandro Panattoni** ready for the viva defense.

Brno, 7. September 2020 Miroslav Fojta