Institute of Molecular and Translational Medicine / Faculty of Medicine and Dentistry Palacky University Olomouc
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Evaluation Report on the Doctoral Thesis
Synthesis and studies of modified DNA: (i) development of DNA targeting molecular scissors and (ii) competitive enzymatic incorporation of base-modified nucleotides
by Alessandro Panattoni

The presented dissertation thesis is focused on the synthesis and studies of modified DNA.

In the first part a new type of artificial metalonucleases was designed that were supposed to cleave the DNA duplex at specific regions which would be very useful in molecular biology along with other methods such as CRISPR/Cas or Zinc Finger Nucleases. Each of these methods have its advantages and disadvantages and it is clear, that a complementary procedure would be very helpful. This work is based on the knowledge that copper (I) phenantroline complexes are able to cleave the DNA non-specifically *via* generation of various reactive species at the site where they bind to the DNA. The main idea was to equip these normally non-specific scissors with a component that would allow for their attachment to the DNA in specific regions, creating scissors that cleave the DNA specifically. Triplex Forming Oligonucleotides (TFO) were selected as the component that would be responsible for the sequence recognition. In the thesis, the synthesis of both, Cu^{I} Phenantroline complexes and TFOs is well described as well as many attempts for their connection. Several approaches were tried to synthesize the designed molecules and although most of the approaches did not work well, at the end a set of scissors was successfully prepared and tested for their ability to cleave the DNA selectively. The newly designed scissors were proven to bind to the DNA, they form stable triplexes and cleave the DNA at its specific regions, however, it was found that it is difficult to fully cleave the target DNA and retain the selectivity to a single nucleotide which is probably due to the fact, that linkers connecting of the TFO and the cleaving complexes are flexible and in addition, the radical species may also cause cleavage within larger area of the DNA. Despite that, this new type of molecular scissors might be very useful for degradation of a selected gene and creating knock-out phenotypes.

The second part is dedicated to the synthesis of modified nucleosides and to the study of their incorporation into the DNA by various DNA polymerases in primer extension experiments. This work is a significant broadening of a previous study in Angewandte Chem. **2014**, *53*, 7552 and it brings a lot of new data about the influence of various types of the substituents on the enzymatic reaction. The enzymatic assays are correctly designed and all important quantities that define the enzymatic reaction were measured (K_M, K_{cat}/K_M, etc.). This is very important for the prediction of how the polymerases would incorporated a modified nucleoside triphosphate in the presence of natural NTPs which would happen in living cells.

The thesis has a traditional structure with an introduction part that contains the majority of the most relevant references of the related work which is followed by specific aims and rationale of the thesis, then results and discussion, conclusion, experimental part, references, and appendices. The experimental design is flawless, almost all of my questions that I had during the reading were consequently answered in the following chapters. The thesis is written in a very clear language and it

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is easy to follow the main ideas, hypotheses, experiment arrangements and results. There is only a small amount of typos and other mistakes.

General comments to the text and content:

- The description of the figures and tables is incomplete, it is sometimes difficult to find the experimental details, the reader has to try to find them in the text or in the appendices which makes the reading a little complicated. It would be great if the bands in gel pictures would be assigned what they are.
- In the introductory part to the competitive incorporation of modified nucleotides, only work Angewandte Chem. **2014**, *53*, 7552 is cited but there are a few earlier precedents (e.g. Biochemistry **2009**, *48*, 7547; Anal. Chem. **2009**, *81*, 9079) that could have been cited as well although they are not as comprehensive and sophisticated.

Questions and topics for the discussion:

1. How stable is the molecular scissor itself in solution containing ascorbate? The complex creates highly reactive species; don't they cleave their own ssDNA part (the TFO)?
2. How would you design the experiment to get the scissors to the DNA of a target cell to destroy the gene of interest *in vivo*?
3. Do you have any idea how to improve the selectivity of your system in order to get the cleavage at a specific site?

Despite of all the comments and questions mentioned above, I must state that all aims of this thesis were completely fulfilled. All of the conclusions are fully supported by the experiments. The amount of work is enormous, both the chemistry and biochemistry part. This thesis resulted in two first-author publications in respected journals and in addition, co-authorship of another publication on the topic of this thesis and three publications that are not related to the topic of this thesis but this confirms high ability of the author to collaborate with other researchers. The thesis is a very nice multi-disciplinary piece of work and the author had to master a variety of techniques to achieve all of the proposed goals.

In conclusion, the presented results are original and of a high scientific value and therefore I recommend the Thesis for the defense and further proceedings for obtaining the PhD degree.

In Olomouc 30.8.2020

Doc. RNDr. Milan Urban, Ph.D.