INSTITUTE OF MOLECULAR AND TRANSLATIONAL MEDICINE

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Evaluation Report on the Doctoral Thesis
Enzymatic synthesis of DNA modified in the minor groove
by Ján Matyašovský

The presented dissertation thesis is focused on the synthesis of new nucleotides modified at their position 2 and evaluation of possibilities to use them in enzymatic synthesis of minor groove modified DNA. This is a very interesting research topic since there are almost no literature precedents in which 2-modified purine nucleotides were incorporated into the DNA by polymerases. This is in a sharp contrast to major groove modified NTPs which are quite common, presumably due to much higher tolerance of DNA polymerases to bulky substituents in the major groove site in contrast to the minor groove. This thesis shows that a small substituent may be tolerated by some DNA polymerases and if the substituent is a reactive moiety (such as alkyne, alkene etc.), it may be used for post synthetic modifications with much less limits to the bulkiness of the newly introduced substituent. This possibility opens a completely new space for the labelling of the DNA with fluorescent dyes or other useful labels.

It is interesting, that dATP analugues substituted at the position 2 with chloro, amino, methyl, vinyl, and ethynyl group are well tolerated by multiple DNA polymerases and even the synthesis of DNA containing several modified A-nucleotides is possible when they are separated by natural nucleotides. On the other hand, the alkylamino substituents in the same position are tolerated by Therminator DNA polymerase that only performs a single nucleotide extension. It is great, however, that the polymerase is able to extend this modified primer by natural NTPs and synthesize the full length products. In addition, it was shown that there is a possibility to make a site-specifically minor groove-modified DNA molecules. To further explore the opportunities for minor groove DNA modifications, dITP analogues were synthesized and studied in order to mimic the 2-substituted dGTP from which 2-methyl and 2-vinylderivatives were good substrates for Therminator DNA polymerase. More information has been obtained from the experiments with restriction endonucleases. Some of the modifications did not affect them in the process of the DNA cleavage but some did and this was different in various endonucleases. This work shows that not only the structures in the major groove of the DNA but to some extent even in then minor groove play important role in the cleavage process.

The thesis has a traditional structure with an introduction part followed by specific aims and rationale of the thesis, then results and discussion, conclusion, experimental part, and references. The thesis is written in a very clear language and it is easy to follow the main ideas, hypotheses, experiment arrangements and results.

Questions:

1. How did you select all of the DNA polymerases for this work?
2. How the selection of the template sequence was done? Does the most successful Therminator DNA polymerase prefer some sequences (before and after the modified NTP) over another? In other words, is the effectivity of the incorporation sequence-specific?
3. In the experiments with $d^{R}ATP$ analogues that may be incorporated four times, have you tried several (2-4) consecutive incorporations?

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4. In Figure 23, why is the intensity of the bands in line 2, 7, and 12 (addition of a natural dATP) so much lower than in other lines? Where did the label go? Did you use different concentration of the labelled primer? Also, is KOD XL polymerase cleaving the last nucleotide from the primer (bands formed below the primer in lines 8 and 9)?
5. Figure 24b. In all lines, even at high concentrations, there is a visible band at the position of a primer (no d^RATP added). How did you prove that your reaction achieved the full conversion? What if your final full length DNA was a mixture of DNA containing modified d^RA and natural dA?
6. If dITP derivatives were considered as dGTP analogues, why did not you prepare alkylaminoderivatives that would contain the 2-aminogroup similarly to your dA analogues? These would be closer to the structure of dGTP (with remaining H-bond donor) and you show a precedent in the introduction to this chapter, that polymerase κ can incorporate such NTPs into the new DNA strand.

Despite of all the comments and questions mentioned above, I must state that all specific aims were completely fulfilled. This work is highly novel and it is very interesting to finally learn, how minor groove substituents influence the incorporation of a NTP into new DNA strand. The design of both site-selective modifications followed by post synthetic introduction of bulky labels is smart. The thesis shows that the author did a lot of experimental work which resulted in three publications in respected journals as the first author and in addition, he is a co-author of another three publications. The thesis is a very nice multi-disciplinary piece of work and the author had to master many different 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 16.3.2020

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