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Report on the dissertation thesis of Pedro Güixens Gallardo

Synthesis and delivery of novel fluorescently-labelled nucleotides and their nucleic acids for bio-analytical applications

Reviewer: **Prof. PharmDr. Petr Zimčík, Ph.D.**, Faculty of Pharmacy in Hradec Králové, Charles University

The presented work is focused on the synthesis and subsequent thorough studies of novel fluorescently-labeled nucleotides. The work is composed of several chapters starting from introduction, followed by aims, results with detailed discussion, conclusion and experimental section. First, the author introduced the readers into the topic of nucleic acids, their artificial synthesis using various enzymes, their fluorescent modification and possible problematic incorporation *in cellulo*. The whole work is divided into several chapters where the author synthesized several fluorescent dyes, prepared the corresponding nucleoside triphosphates, studied the fluorescent properties *per se* or as a response to changes of surrounding viscosity or presence of proteins. Following these studies, he tried to deliver the fluorescently labeled nucleoside triphosphates into the cells and focused his work on monitoring their fate *in cellulo*, in particular on the incorporation of the fluorescent labels into genomic DNA. This last step is highly ambitious, and the successful result was obtained only in one case of methylated BODIPY dye. However, even this can be considered as an excellent result in this highly challenging aim.

The whole work is well described and discussed; the author proved that he is able to work with number of techniques not only limited to the organic synthesis. Besides this, he also performed photophysical determination of the parameters, biochemical experiments in analysis of the DNA synthesis and he learned the *in vitro* techniques while working with the cells. This proves that he is able to learn and apply wide range of new approaches focusing on the final aim of the characterization of the intended molecules. The results were published in three impacted journals with the student as the first author and one journal where he is the coauthor. This is another conformation of very good scientific work.

From the graphical and formal point of view, the work is very well prepared with only limited typing or grammar errors that do not decrease high scientific level.

Questions and comments:

- In the list of prodrugs in the introduction (p 30), I largely miss the recent most successful modification of antiviral nucleotide drugs – the “ProTide” approach combining phenol and aminoacid moieties on the phosphate.
- Are you aware of any study on how large modifications on the nucleosides are still recognized by enzymes in PEX (in PCR it is apparently more problematic)? The question

is motivated by possible red-shift of the BODIPY emission by extended conjugation using styryl moieties. Red-shifted emission would be beneficial for *in vivo* applications.

- Do you have any information on the mechanism how *o*TINA may inhibit the non-templated addition? Is it *e.g.* by interaction with the enzyme or by π - π stacking with newly synthesized dsDNA?
- Fig. 65 B, 69 - is it possible to determine the binding constant and stoichiometry from the obtained data for interaction of TBdp-modified DNA and p53 or LutR proteins?
- In the case of $27\text{DNA}^{\text{LutR}2\text{dC}^{\text{TBdp}}}$ (*i.e.* DNA modified by two TBdp dyes), do both dyes bind to the protein? If only one dye binds and the second is free, you should perhaps see biexponential decays of lifetimes. Did you observe this?
- As I understood from the experiments with dsDNA and ssDNA (chapter 3.2.7.4), the restriction of free rotation does not play any role in influencing the lifetime. Rather photo-induced electron transfer seems to lower the fluorescence lifetime. In that case, it is perhaps not necessary to use the probe with free rotation (and lower fluorescence) and it would be possible to use strongly fluorescent BDP dye with restricted rotation (*e.g.* hexamethylated BDP). Could you comment on this?
- Is there any explanation why the $\text{dC}^{\text{mBdpTP}}$ was successfully incorporated into genomic DNA while analogous $\text{dC}^{\text{TBdpTP}}$ was not?

Overall, I consider the presented dissertation work as an excellent scientific background for Ph.D. and I recommend the work for the defense.

Hradec Králové, 18.11.2020

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