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„Modified nucleotides as tools to study proteins involved in mRNA 5' end metabolism”

PhD thesis abstract

Messenger RNA (mRNA) is the biomolecule responsible for carrying genetic information from the cell nucleus to the cytoplasm, which enables protein biosynthesis. The information encoded in mRNA is the sequence of amino acids that build a functional protein. The process of protein biosynthesis on the template of mRNA is called translation. At the 5’ end of eukaryotic mRNA is a structure called a cap. Cap, typically, is a positively charged 7-methylguanosine (m^7G) linked to the first transcribed nucleotide by a 5’,5’-triphophate bridge ($m^7GpppN_1pN_2$). Cap is involved in many important biological processes in eukaryotic cells, such as mRNA maturation and transport. The cap also protects mRNA from premature degradation by 5’-exonucleases, regulates mRNA translation, and plays a key role in the distinguishing of cellular and foreign mRNA by the immune system. Because of such important functions, the structure of the cap and its derivatives has become a very interesting target for chemical modifications. Chemically modified analogs of cap and its derivatives are currently used to monitor mRNA-related cellular processes, as molecular tools to study proteins that recognize cap structure, as reagents to produce mRNA vaccines, and have many other potential therapeutic applications, including anti-cancer therapies.

The main goal of my PhD thesis was to synthesize new chemically modified nucleotide analogs, including cap analogs, which could be used for studying selected processes related to the metabolism of the 5’ end of mRNA. During my Ph.D. project, I focused on the synthesis of cap analogs and their derivatives, which can be used to study the process of mRNA translation and the degradation of its 5’ end metabolites.

In the first part of my thesis, I used analogs of 7-methylguanosine 5'-monophosphate (m^7GMP) to study the role of human cytosolic 5'-nucleotidase IIIB (cN-IIIB). Cytosolic 5'-nucleotidase IIIB (cN-IIIB), is a relatively recently discovered enzyme belonging to the 5'-nucleotidase family. The role of 5'-nucleotidases is to control the concentrations of nucleoside 5'-monophosphates (NMPs) in cells by catalyzing their dephosphorylation. The cN-IIIB enzyme is a unique 5'-nucleotidase because of its substrate specificity, as it catalyzes the reaction of dephosphorylation of 7-methylguanosine 5'-monophosphate, which is one of

the metabolites of mRNA 5' end. To this day, the role of the cN-IIIB enzyme is not fully understood, but its substrate preferences suggest that it may play a key role in one of the final steps of 5' end mRNA degradation. The cN-IIIB enzyme may also be involved in the inactivation of active forms of nucleoside-derived therapeutics. In the first part of my thesis, I was looking for inhibitors of the cN-IIIB enzyme that can act as molecular tools and be used in studies that could further the knowledge of this enzyme. This project involved both the synthesis of modified nucleotides and the investigation of their substrate and inhibitory properties towards the cN-IIIB enzyme. Based on my research, I selected two m^7GMP derivatives out of dozens of compounds as the most potent inhibitors of the cN-IIIB enzyme. The selected compounds were used as tools in studies in cell lysates to verify the role of the cN-IIIB enzyme in the degradation of cap structure metabolites. The most potent inhibitors of the cN-IIIB enzyme were also used for structural studies, which yielded the first crystallographic structure of the human cN-IIIB enzyme together with a ligand. I also transformed the selected cN-IIIB enzyme inhibitors into pronucleotides, which can get into the cell interior by crossing the cell membrane and can be used for *in vivo* studies. Finally, I investigated the inhibitory properties of a new class of compounds possessing a modification that allows, under the right conditions, the spontaneous formation of a covalent bond with a protein. I tested these compounds for their inhibitory properties against the cN-IIIB enzyme. From the pool of tested compounds, I was able to identify a compound that is a covalent nanomolar inhibitor of the cN-IIIB enzyme.

The studies in the second part of my PhD thesis investigated the cap-dependent translation process of mRNA, in which the eIF4E protein is involved. Trinucleotide analogs of cap are used in an *in vitro* transcription (*IVT)* reaction to obtain functional mRNA, including mRNA used in anticancer and antiviral therapies. Unfortunately, the synthesis of trinucleotide analogs of cap is difficult and time-consuming. For this reason, in my PhD project, described in the second part of this thesis, I was developing a new pathway for the synthesis of trinucleotide cap analogs. The synthetic pathway I designed used click chemistry to accelerate and simplify the synthesis of tri- and tetranucleotide cap analogs, and additionally introduced modifications within the cap structure to potentially improve the

translational properties of mRNA. The synthetic pathway I developed reduced the time for the final step of trinucleotide cap analogs synthesis to 1 h, whereas the synthesis under standard conditions can take up to 48 h. Using the developed synthetic pathway, I obtained more than a dozen new cap analogs, which I used to study cap-dependent mRNA translation. The obtained compounds were expected to efficiently co-transcriptionally incorporate into mRNA strands and to initiate translation of such mRNA more efficiently than mRNAs terminated with naturally occurring caps. The *in vitro* transcription reaction yielded mRNAs terminated with various cap analogs, which I then used to study translation in cell lysates. The mRNA prepared in this way was also used to study the efficiency of the protein production process in dendritic cells. One of the obtained cap analogs, turned out to be a good mimetic of natural cap, as the translation efficiency of mRNA terminated with the modified trinucleotide was comparable to mRNA terminated with unmodified cap 1.

My Ph.D. project was interdisciplinary in nature, in which I was engaged in both the chemical synthesis of derivatives of the 5' end of mRNA and their characterization by biophysical, biochemical, and biological assays. My research contributed to the first crystallographic structure of the human cN-IIIB enzyme, through which the mechanism of action of the enzyme was understood. In turn, the trinucleotide cap analogs, designed and synthesized by me, can be used to synthesize mRNA with very good translational properties, which potentially have applications in therapeutic mRNA field.

List of publications

1. *7-Methylguanosine monophosphate analogues with 5′-(1,2,3-triazoyl) moiety: Synthesis and evaluation as the inhibitors of cNIIIB nucleotidase*; Mateusz Kozarski§, Dorota Kubacka§, Błażej Wojtczak, Renata Kasprzyk, Marek Baranowski, Joanna Kowalska, *Bioorganic & medicinal chemistry*, **2018**, 26(1), 191-199.
2. *Substrate-Based Design of Cytosolic Nucleotidase IIIB Inhibitors and Structural Insights into Inhibition Mechanism*; Dorota Kubacka§, Mateusz Kozarski§, Marek R. Baranowski, Radosław Wójcik, Joanna Panecka-Hofman, Dominika Strzelecka, Jerome Basquin, Jacek Jemielity, Joanna Kowalska; *Pharmaceuticals* **2022,** 15(5), 554

3. Towards superior mRNA caps accessible by click chemistry: synthesis and translational properties of triazole-bearing oligonucleotide cap analogs; Mateusz Kozarski, Karolina Drazkowska, Marcelina Bednarczyk, Marcin Warminski, Jacek Jemielity and Joanna Kowalska; *RSC Advances* **2023**, 13, 12809-1282

4. M. Chrominski, M. Kozarski i wsp. Proximity-induced SuFEx increases the potency of substrate-derived inhibitors of cytosolic nucleotidase IIIB – in preparation

§Authors have an equal contribution