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Abstract

The cap structure (m^7GpppN) at the 5 'end of the mRNA determines the processes involved in gene expression through specific interaction with cap-binding proteins. Abnormal activity of cap binding proteins may lead to abnormal gene expression and, consequently, to cancer or neurodegenerative diseases. Regulation of the activity of cap-binding proteins can be accomplished by providing small-molecule ligands (cap analogs) that will bind to proteins while preventing their binding to the 5' end of mRNA. Due to the potential therapeutic importance of ligands of cap-binding protein, methods for their effective search and characterization are being developed. Standard methods used for this purpose, such as time-synchronized fluorescence titration and isothermal calorimetric titration, are tedious and time-consuming. In the search for protein inhibitors, the high-throughput approach is increasingly used, in which approximately 80 or more new chemical compounds are tested simultaneously for protein affinity. This approach significantly increases the efficiency of searching for chemical modifications that are crucial for interaction with the protein, as well as for the inhibitors themselves.

The main goal of the dissertation was to develop fluorescence-based methods for the search and characterization of protein ligands binding the 5 'end structure of mRNA, i.e. the cap structure. Two naturally coexisting parts can be distinguished in the study: the first one concerning the development of the methodology for studying the protein-ligand interaction, and the second, i.e. the application of the developed methods to characterize compounds with unknown properties towards to cap-binding proteins. In this work, great emphasis was placed on the development of high-throughput methods that enable efficient searching of compound libraries to find those with desired properties.

The first approach described in the PhD thesis to study the inhibition of the DcpS enzyme is a method based on monitoring enzymatic activity by measuring the concentration of fluoride ions released during the reaction. The method uses an unnatural substrate for the DcpS enzyme - m^7GMPF analog, which is hydrolyzed to m^7GMP and fluoride ion. To measure the concentration of fluoride ions, a fluorogenic probe is used, in this case a fluorescein derivative whose hydroxyl groups are protected with tert-butyldimethylsilyl groups. The developed method was the first high-throughput approach to search for and characterize DcpS inhibitors. It was used to search for new inhibitors of this enzyme, among libraries of commercially available compounds and those synthesized in the Laboratory of Bioorganic Chemistry. In total, it was used to test 1346 compounds. Among them, an inhibitor that interacts strongly with the DcpS enzyme (m^7GSpp$_S$pSG D2) was identified, with affinity similar to RG3039, a compound that has participated in clinical trials as a therapeutic for the treatment of spinal muscular atrophy. It is also the greatest achievement of the this project.

Another developed method was fluorescence anisotropy (FA). This approach is widely used to study protein: ligand interactions, which, in contrast to the above-presented method, is one-step, and which also means that the characterization of ligands using the fluorescence anisotropy method is faster. The aim of my work was to develop a fluorescence anisotropy method for 3 proteins: cN-IIIB, eIF4E and DcpS. A total of 23 protein:ligand systems were tested to develop a fluorescence anisotropy method. In the case of the cN-IIIB enzyme, it was not possible to develop a FA method due to the too low affinity of the fluorescent probes to the protein. In contrast, FA method was developed for DcpS and eIF4E proteins, and can be used for high-throughput search of ligands and their characterization. In the case of studies with the eIF4E protein, the applicability of the method was extended, it can also be used to study allosteric ligands of the eIF4E protein, as well as to assess the affinity of short oligonucleotides. Due to the speed and simplicity of the fluorescence anisotropy method, it is a competitive tool for ligand search.

Another issue addressed in the dissertation was the characterization of cap analogs modified at the C^8 position of Guo or m^7Guo. Fourteen compounds were characterized in terms of their usefulness as probes for studying the interaction with cap-binding proteins. Their spectroscopic properties, stability were analyzed, as well as their binding affinities towards eIF4E and DcpS. It was also checked how the fluorescence intensity changes under the influence of the hydrolytic activity of the enzymes DcpS and Nudt16. The greatest attention was paid to 6 cap analogs characterized by higher quantum yields in comparison to the natural cap structure, these were compounds containing -Py, -Ph, -CNPh and DMAPh fluorophores. These compounds act as molecular rotors (they are sensitive to changes in viscosity), which can be used to study the interaction with proteins. For each protein: eIF4E, DcpS and Nudt16, it was possible to find a cap analog that changes its emission properties due to binding to the protein or as a result of an enzymatic reaction. The presented research is the basis for the development of new fluorescence methods using analogs modified at the C^8 Guo or m^7Guo position as fluorescent probes.

The second part of the dissertation was the characterization of new cap analogs as ligands of eIF4E and DcpS proteins using the developed fluorescence based methods, as well as the standard fluorescence titration method. As a result of the work, 34 phosphotriazole cap analogs, 3 cap analogs with 1,5-anhydroaltritol modification, 15 phosphothioester cap analogs and 17 benzyl cap analogs were studied. The use of fluorescent methods provided valuable information on the influence of modifications on the stabilization of protein-ligand complexes. In addition, the research allowed to determine which positions of the cap structure are critical for effective protein: ligand interaction, as well as to set new research directions.