Abstract

Cancers are the second most frequent cause of death in the world. Despite the continuous progress in medicine, we still do not have a fully effective anti-cancer therapy. The low-molecular-weight chemotherapeutic agents used so far show high systemic toxicity and unsatisfactory therapeutic effects. Therefore, strategies that increase the specificity of drug action by, for example, the application of targeting molecules as carriers that selectively recognize a molecular target on the surface of cancer cells are gaining an increasing interest. This approach allows to use much stronger toxins, as they are delivered directly to the cancer cells, excluding healthy tissues.

The most commonly used targeting molecules are monoclonal antibodies (mAb). After conjugation with highly-potent toxins they form Antibody-Drug Conjugates (ADCs). So far, nine ADC preparations have been approved by the FDA for patient therapy. Despite the advantages such as high molecular target affinity, stability, and long half-life in the body, monoclonal antibodies also exhibit disadvantages. Among others, the possibility of stimulating the immune response or the large size resulting in suboptimal penetration of the solid tumors. Therefore, new alternative classes of targeting molecules are being sought. The spectrum of interest of researchers groups includes such macromolecules as affibody, aptamers, DARPins, inhibitory cysteine knots (knottins), and centrins. This set of molecules also includes receptor ligands, including fibroblast growth factors (FGFs), which have been the subject of research conducted in our group for over 20 years.

Fibroblast growth factor 2 (FGF2) belongs to the FGF1 subfamily. It has a high affinity for the fibroblast growth factor receptor 1 (FGFR1), a transmembrane protein overproduced in many types of cancer cells including lung, breast, bladder, prostate, pancreas, multiple myeloma, and many types of sarcomas. In previous studies, we have shown that FGF2, when covalently bounded with a potent cytotoxin monomethyl auristatin E (MMAE), is highly toxic to FGFR1-overproducing cancer cells (EC_{50} in a nanomolar range), but is virtually non-toxic to normal cells with low level of FGFR1. This indicates that FGF2 can be used as a targeting molecule and is an interesting alternative to monoclonal antibodies. This observation is a good starting point for further work on the development of therapies targeting FGFR1 overproducing tumors.

In my research, I have developed and characterized a number of conjugates.
The first group of FGF2 conjugates contained two MMAE molecules attached to naturally occurring cysteinyl residues in the protein. To increase the loading of targeting molecule by the cytotoxics, an additional, highly reactive cysteinyl residue presents in the KCK sequence was introduced into the wild-type FGF2 sequence. The linker was introduced either at the N-terminus or at the C-terminus of the protein. As a result, it was possible to conjugate three cytotoxic molecules to one targeting molecule. Within my work, I obtained five FGF2-MMAE conjugates, characterized by purity exceeding 95%, undisturbed tertiary structure, retained high affinity to FGFR1, and high specificity and toxicity (EC_{50} at the nanomolar level) towards cells overproducing the FGFR1. The results of this work were published in 2017 in the *ACS Omega* journal and were the subject of a patent application.

The second stage of the development of FGF2 conjugates was increasing their hydrophilicity and hydrodynamic radius to extend circulation time in the body. Application of a hydrophilic auristatin derivative (auristatin Y, AY) and its PEGylated derivatives resulted in an increase in the toxicity of FGF2 conjugates towards FGFR1-positive cells, a reduction in non-specific toxicity and an increase in the hydrodynamic radius to a value that reduces the removal of the conjugate from the body during the glomerular filtration process. In this work, I synthesized three PEGylated auristatin Y derivatives and four FGF2-AY conjugates. All conjugates were characterized by high purity, no tendency to aggregation, preserved affinity for the receptor, increased toxicity towards FGFR1-positive tumor cells, and reduced toxicity towards FGFR1-negative cells. Moreover, one of the conjugates showed an increased hydrodynamic radius (~ 60 kDa), allowing to overcoming the threshold of the glomerular filtration. The work was published in 2020 in the *Molecular Pharmaceutics* journal and in the international patent application.

The most advanced FGF2-based conjugate contained two cytotoxics, differing in the mechanism of action. The first cytotoxic was the MMAE molecule, the inhibitor of microtubule polymerization (previously used). MMAE is a hydrophobic oligopeptide with the ability to passively cross biological membranes. The second toxin was the RNA polymerase II and III inhibitor - α-amanitin (αAMTN), a hydrophilic bicyclic octapeptide that does not passively cross the biological membranes, but is also not a substrate for ABC transporters. The use of two cytotoxics with different mechanisms of action, permeability through biological membranes and susceptibility to active efflux of the cell significantly increases the specific toxicity of the conjugate and may be useful in the treatment of heterogeneous tumors and demonstrate greater efficacy in relation to cancer cells with high potential to acquire drug resistance. The result of the performed works was the development of enzymatic, sortase A-dependent, efficient method of obtaining conjugates with two different cytotoxics. The obtained conjugate retained all the biophysical and biological functions characteristic of FGF2 and showed significantly increased cytotoxicity, especially against the tumor cells moderately overproducing FGFR1. The results of this part of the work were published in 2019 in the *Molecular Pharmaceutics* journal.