Final report for the K124952 NKFIH grant entitled

"Preparation and cell membrane delivery of cholesteryl lipoproteins"

Cell surface proteins have important biological functions including signal transduction, cell adhesion or antigen presentation. The ultimate goal of this project was to develop biorthogonal methods to conjugate proteins with membrane anchor molecules and to demonstrate its usefulness by investigating engineered membrane anchored interleukin-15 (IL-15) derivatives.

A two-step procedure was designed to achieve the specific modification of synthetic or recombinant proteins with cholesterol derivatives. For that purpose cholesterol, the ubiquitous component of all animal and human tissues was modified at the 3β-hydroxyl group. A convergent synthesis strategy was developed for the preparation of a series of amphiphilic cholesterol derivatives with diverse bifunctional headgroups. The diversification of the lipid headgroup was achieved by the introduction of various reporters (fluorophores, radioactive isotopes) and protein capture functional groups. The resulting set of cholesterol anchors was then investigated as potential protein membrane anchors.

The cell membrane association of the fluorescent cholesterol anchors was investigated by confocal laser scanning microscopy. Optimal conditions for plasma membrane labelling of live cells in culture media was developed, and it was found that the introduction of hydrophilic sulfocyanine type dyes (sulfo-Cy3 or Cy5) to the headgroup resulted in anchor molecules soluble in cell culture media. These hydrophilic dyes have advantageous photophysical properties (A. Hus-Citharel et al. 2021) and they are able to solubilize the cholesterol anchors not only in cell culture media but in body fluids. It was shown that the synthesized cholesterol amphiphils rapidly associate with membrane structures including the plasma membrane of live cells. The membrane residence time of the membrane-incorporated fluorescent cholesterol anchors was determined in a fluorophore dilution proliferation assay. Cells were labelled simultaneously with a Cy5 labelled cholesterol anchor and carboxyfluorescein diacetate and the intensity of the cellular fluorescence was monitored by flow cytometry. Halving of the cellular fluorescence indicated that the Cy5 labelled cholesterol anchors were present in the plasma membrane of the daughter cells after successive divisions. Furthermore, the Cy5 labelled cholesterol anchor was found to be more suitable for following the early cell proliferation than carboxyfluorescein. Our accumulated experimental data led us to conclude that our cell surface coating method is applicable to live cells without genetic modification and it has no effect on cell viability or differentiation potential.

Then we have shown that maleimido derivatives of these cholesterol amphiphils can be used for conjugating recombinant, C-terminally Cys-extended proteins. For proteins lacking Cys residues, a general semisynthetic strategy was developed to introduce the C-terminal Michael donor Cys. For that purpose, maltose-binding protein (MBP) of E. coli was used as a model protein. The MxeGyrA intein fusion of MBP was overexpressed in a bacterial system and after chitin

affinity purification the cleavage of the intein was induced by Cys. This way the intein cleavage resulted in a mixture of Cys-extended and native MBP. A separation method was then developed for the enrichment of the Cys-extended protein. A thiol sepharose medium was found to be optimal for this purpose. The purified Cys-extended MBP was conjugated in a Michael addition with cholesterol amphiphils containing both a maleimide function and a fluorophore in the headgroup. A final size-exclusion chromatography step resulted in pure cholesteryl protein.

The limitation of this approach is that the C-terminal selectivity of the cholesterol anchor attachment cannot be achieved when further Cys residues are present – as in the sequence of the target protein, IL-15. In order to overcome this limitation, an abiotic functional group was introduced to the C-terminus of model proteins that can be selectively coupled to the complementary functional group in the cholesterol headgroup with fast kinetics. Various biorthogonal reactions were investigated for the cholesterol anchor conjugation. The CuAAC reaction was first considered and a biotinylated model peptide with a C-terminal azide group was prepared by native chemical ligation of a cysteinyl azide and a peptide thioester. The neighboring Cys thiol group (that is essential for the subsequent native chemical ligation step), however, inhibited the click product formation with the cholesteryl alkyne. In order to raise the effective Cu(I) concentration locally, picolyl azide as an internal copper chelating moiety was introduced to the azide component. In the case of Cys containing peptides, however, this derivative was found not to be an effective azide-alkyne ligation accelerator. These failures prompted us to investigate the catalyst-free, inverse electron-demand Diels-Alder cycloaddition as a biorthogonal conjugation method. This reaction was found to be exceptionally fast in our trials. However, the stability of the trans-cyclooctenyl Cys derivatives was low as the trans olefin reacted with the intra- and/or intermolecular thiol. Therefore, the trans-cyclooctene dienophile moiety was substituted with cyclooctyne that was less prone to thiol addition.

In order to generate a bioorthogonally reactive protein C-terminus, production of model recombinant protein thioesters (mCherry, MBP) was optimized in bacterial expression systems on 10 mg scale. Then recombinant IL-15 was overexpressed as the IL-15 – MxeGyrA intein fusion. The modified plasmid DNAs were prepared from a NEB commercial plasmid using the circular polymerase extension cloning approach resulting in the codon optimized plasmid DNAs encoding a longer (163 amino acids) and a shorter (114 amino acids) isoform of IL-15 both fused with MxeGyr A intein and a chitin binding domain. The bacterial overexpression of the longer and the shorter IL-15 isoforms was compared both in BL21DE3 and in BLK16 strains. It turned out that the protein yield is much lower for these model proteins using the BLK16 E. coli reduced genome expression strain. Furthermore, it was found that the longer isoform cannot be separated from the bacterial chaperon protein SlyD, but the shorter IL-15 isoform can be obtained in pure form. However, we had to conclude that the overexpression of the fusion construct consisting of IL-15, MxeGyrA intein and a chitin binding domain is not feasible, because under various conditions refolding of IL-15 failed. In order to increase the protein yield and to optimize the affinity

purification protocol, the chitin tag was substituted with a His_6 tag resulting in His_6-tagged IL-15-MxeGyrA proteins with or without a chitin binding domain. The purification of the His_6-tagged IL-15 fusion proteins from inclusion bodies was more effective, and the refolding of the denatured His_6-tagged proteins was also achieved. It resulted in a functional intein that could be cleaved off the IL-15 with thiols. The sequence of the isolated proteins was analysed by mass spectrometry that confirmed the proper sequence. The bacterial overexpression of IL-15 was investigated in M9 minimal media as well that make the preparation of N-15 labelled protein possible.

In order to introduce unnatural residues to the IL-15 sequence in a residue specific manner, a native chemical ligation-based synthetic method was developed. According to the four Cys residues, five IL-15 fragments ((88-114), and thioesters of (85-87), (42-84), (35-41) and (1-34)) were prepared by solid phase peptide synthesis. An enzymatic protection/deprotection scheme was developed for the native chemical ligation of the cysteinyl thioester fragments. In this method, the N-terminal amino group of the inner fragments was protected with a Met residue that could be removed by using a mutant methionyl aminopeptidase. The ligation of the fragments was executed on mg scale.

The difficulties with the biorthogonal function-bearing cysteinyl compounds and with the possible disulfide shuffling by the extra Cys residue introduced in the native chemical ligation step prompted us to investigate an alternative method for the C-terminal lipidation of IL-15. In order to overcome the reactivity of the C-terminal Cys residue of the native chemical ligation product, an enzymatic ligation strategy was chosen as a preferred solution. The transpeptidase sortase A (Srt A) catalyses the cleavage of the Thr-Gly bond of the C-terminal recognition sequence followed by the yield of an amide bond with the N-terminus of an oligoglycine nucleophile. Accordingly, an engineered IL-15 was prepared containing the LPETG recognition sequence of SrtA together with a His_6 tag at the C-terminus. A penta- and a heptamutant SrtA mutant were bacterially overexpressed and they were validated with model peptides. The sortagging method was optimized with the ligation of the C-terminal fragment of the engineered IL-15 and the fluorophore labelled peptide, GGG-miniPEG-K(Cy5). For practical reasons, we have used the Ca^{2+}-independent heptamutant SrtA in our experiments. In order to conjugate a cholesterol amphiphile to the C-terminus of the engineered IL-15, the carboxyl function of the lipid head group was modified with diamino-diethylene glycol and a tetraglycine moiety. The overexpression and affinity purification of IL-15 containing the SrtA recognition sequence and the His_6 tag at the C-terminus were optimized. The MS analysis revealed that the disulfide bond pattern in the purified protein is identical with the native pattern.

In order to determine the concentration of the IL-15 anchor molecules in cell membranes or in liposomes, H-3 labelling of the optimized amphiphilic cholesterol derivative is required. An earlier prepared H-3 labelled model anchor compound (Schäfer et al. 2015), containing a [^{3}H]1-aminopiperidine moiety, was found to be unstable and the radiolysis of this labelled anchor prompted us to prepare another H-3 labelled anchor, i.e. the anchor used in the SrtA mediated

ligation. An optimized iodination method (Szabó et al. 2021) and an asymmetric homogenous catalytic tritiation were compared, and Fmoc-[^{3}H]NaI was prepared as the precursor for the preparation of the H-3 labelled anchor containing a headgroup with SrtA recognition sequence.

In order to investigate tumor derived exosomes as delivery vectors of IL-15 in model animals, noninvasive positron emission tomography experiments were performed. The exosome association of the cholesterol anchors was optimized by fluorescence detected size-exclusion chromatography. An optimal condition was developed for the loading of B16F1 cell derived exosomes with low level of fluorescent cholesterol anchors and for the chromatographic purification of the loaded exosomes. For quantitative analytical experiments a tritium labelling method was also developed that is used for labelling anchor molecules to determine their membrane incorporation (Dvorácskó et al. 2019). Our cholesterol anchor set was then broadened by introducing the diene function tetrazine and also the PET radionuclide Ga-68 chelator group NODA to the lipid headgroup. These biorthogonal functions make the protein conjugation achievable by Diels-Alder reaction and do not require the presence or introduction of C-terminal Cys residue in the protein. The Ga-68 labelled cholesterol anchor was prepared from the NODA chelator containing precursor. Since the short half-life of Ga-68 limited the time-frame for the labelling, loading and imaging steps, our radiolabelling strategy was based on a rapid size-exclusion chromatography after Me-β-cyclodextrin inclusion complex formation of the Ga-68 labelled cholesterol anchor. The formation and the rapid purification of the Me-β-cyclodextrin inclusion complex was achieved under optimized incubation conditions followed by size exclusion chromatography. The subsequent exosome loading was also optimized to achieve the fastest possible method. As a result, all Ga-68 radioactivity could be assigned to the exosomes and the integrity of exosomes was preserved, and the obtained radioactivity was sufficient for in vivo PET imaging. The dispersion of the exosomes loaded with Ga-68 labelled cholesterol anchors were administered into anesthetized C57BL/6 mice, and as a control Ga-68 labelled cholesterol anchor was also administered into mice and static coronal PET images were recorded. Radioactivity was found mainly in the liver and in the bladder, therefore the composition of the exosome should be modified to increase the circulation time of the loaded exosomes.

As an extension of the scope of the applications of our cholesterol anchors in exogenous delivery into membrane species, the interaction of lipid nanoparticles (LNPs) with the cholesterol amphiphils was investigated. LNPs are the key delivery vectors of therapeutic mRNAs as it was evidenced during the SARS-CoV-2 pandemic. The adjuvant effects of the LNP particles can be increased by introducing small molecule adjuvants into the LNP surface. It was found that our cholesterol anchors are steadily associated with mRNA containing LNPs, and thus, they can be used as upstream LNP modifier tools.

The main goal of our publication strategy is to prepare a comprehensive paper in the highest possible ranking/impact journal, that is still in progress. Judit Darusi finished her MSc diploma work in 2018, and continued her research work in our lab. Szabolcs Dvorácskó has defended his

PhD thesis in 2019. Attila Ádám Dékány finished his MSc diploma work in 2019, and Csanád László Mészáros finished his BSc diploma work in 2019. Ádám Ködmön finished his BSc and MSc diploma work in 2018 and 2020, respectively, and continued his research work in our lab.

K124952 supported publications:

1. Í. Szabó, V.É. Varga, Sz. Dvorácskó, A.E. Farkas, T. Körmöczi, R. Berkecz, Sz. Kecskés, Á. Menyhárt, R. Frank, D. Hantosi, N.V. Cozzi, E. Frecska, Cs. Tömböly, I.A. Krizbai, F. Bari, E. Farkas "*N,N*-Dimethyltryptamine attenuates spreading depolarization and restrains neurodegeneration by sigma-1 receptor activation in the ischemic rat brain." *Neuropharmacol*. 192, 108612 (2021).
2. A. Hus-Citharel, N. Bouby, M. Corbani, J. Mion, C. Mendre, J. Darusi, Cs. Tömböly, M. Trueba, C. Serradeil-Le Gal, C. Llorens-Cortes, G. Guillon "Characterization of a functional V_{1B} vasopressin receptor in the male rat kidney: evidence for cross talk between V_{1B} and V_2 receptor signaling pathways." *Am. J. Physiol. Renal Physiol*. 321(3), F305-F321 (2021).
3. N. Almási, Sz. Török, Sz. Dvorácskó, Cs. Tömböly, Á. Csonka, Z. Baráth, Zs. Murlasits, Zs. Valkusz, A. Pósa, Cs. Varga, K. Kupai "Lessons on the Sigma-1 Receptor in TNBS-Induced Rat Colitis: Modulation of the UCHL-1, IL-6 Pathway." *Int. J. Mol. Sci.* 21(11), 4046 (2020).
4. F. Zádor, G. Nagy-Grócz, Sz. Dvorácskó, Zs. Bohár, E.K. Cseh, D. Zádori, Á. Párdutz, E. Szűcs, Cs. Tömböly, A. Borsodi, S. Benyhe, L. Vécsei "Long-term systemic administration of kynurenic acid brain region specifically elevates the abundance of functional CB1 receptors in rats." *Neurochem. Int.* 138, 104752 (2020).
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6. F. Zádor, G. Nagy-Grócz, G. Kekesi, Sz. Dvorácskó, E. Szűcs, Cs. Tömböly, Gy. Horvath, S. Benyhe, L. Vécsei "Kynurenines and the endocannabinoid system in schizophrenia: common points and potential interactions." *Molecules*, 24(20), 3709 (2019).

K124952 supported diploma works:

Dékány Attila Ádám (MSc 2019)

"Fehérjék sejtmembránhoz horgonyzására alkalmas szulfoindocianin festékkel jelölt koleszterinszármazék előállítása"

Mészáros László Csanád (BSc 2019)

"Interleukin-15 fragmensek szintézise és natív kémiai ligációjának vizsgálata"

Ködmön Ádám (MSc 2020)

"Exoszómák módosítása képalkotásra és fehérje bevitelre alkalmas koleszterin származékokkal"