Final report

Investigation of the pharmacokinetics and siRNA delivery properties of cyclodextrin derivatives

FK124634

The research project had three main directions. The first objective was the synthesis and characterization of PET-labeled cyclodextrin derivatives. 6-deoxy-6-monoamino- (2-hydroxypropyl) -β-cyclodextrin and 6-monodeoxy-6-monoamino-randomly-methylated-beta-cyclodextrin hydrochloride, provided by Cyclolab Ltd. were conjugated with p-NCS-benzyl NODAGA chelator (NODAGA). The resulting NODAGA-cyclodextrins were purified by preparative HPLC and then identified by liquid chromatography (HPLC) and mass spectrometry (LCMS-IT-TOF). The purity of the NODAGA-cyclodextrins were higher than 98%. NODAGA-HPBCD and NODAGA-RAMEB were labeled with Gallium-68 (^{68}Ga), followed by the investigation of radiochemical purity, partition coefficient (logP) and *in vitro* stability. The radiochemical purity of the ^{68}Ga labeled NODAGA-HPBCD and NODAGA-RAMEB was greater than 98%. The octanol/water partition ratio of the ^{68}Ga-labeled NODAGA-HPBCD and NODAGA-RAMEB was -3.07±0.11, and −3.63±0.04 respectively, so the hydrophilic character of the cyclodextrin derivatives was not changed after conjugation with NODAGA. In the *in vitro* stability assay, the molecules were incubated with mouse serum, and the radiochemical purity of the samples was monitored by radio-HPLC at selected time points. The purity of the samples was higher, than 96% after the incubation, indicating that the labeled molecules did not suffer decomposition at the presence of serum.

In *in vivo* studies, the biodistribution of ^{68}Ga-NODAGA-HPBCD and ^{68}Ga-NODAGA-RAMEB was assayed by dynamic small animal PET imaging techniques in BALB/c or CB17 SCID mice. Based on the results, it can be said that both molecules are rapidly excreted through the urinary tract and accumulated in the urine. Similarly, high accumulation cannot be measured in other organs or tissues, as in the brain either. *Ex vivo* distribution data call attention to some interesting alteration of organ and tissue values. The radioactivity of the tissues was measured at 30, 60 and 90 minutes, and expressed as the percentage of injected dose. Beside kidneys and the bladder, interestingly higher activity could be observed in the lungs as well, which was decreased considerably for 90 minutes. Minor activity was observed after 30 minutes for example in the bone, pancreas, stomach and small intestine, but after 90 minutes, significantly lower activity can be measured in these organs as well. In the brain, after 30 minutes, the activity was much smaller than in other organs, the brain had one of the lowest activities.

^{68}Ga-NODAGA-RAMEB was also tested in BxPC-3 and PancTu-1 tumor-bearing CB17 SCID mice. BxPC-3 is a prostaglandin E2 (PGE2) positive tumor cell line, while PancTu-1 cells have lower PGE2 production and PGE2 receptor (EP2) expression. It was hypothesized earlier that prostaglandins tend to form inclusion complexes with the beta-CD's, thus we wanted to assess this theory and to find a connection between the accumulation of the radiolabelled RAMEB in the cancerous tissues. We found, that the accumulation of ^{68}Ga-NODAGA-RAMEB was significantly ($p \leq 0.01$) higher in BxPC3 tumors than in the PancTu-1 tumors. The tumor-background ratio values of PancTu-1 tumors were approximately 10-fold lower, than that of BxPC3 tumors, confirming the high PGE2 selectivity of the ^{68}Ga-labeled cyclodextrin. However, the pharmacokinetic properties of ^{68}Ga-NODAGA-RAMEB changed by the addition of PGE2 in BxPC3 tumors. This research direction was not proposed in the original research plan, but our results open new possibilities for the diagnosis and treatment of PGE2-positive tumors. The exact targeting

mechanism of ^{68}Ga-NODAGA-RAMEB is still not clear, but we are committed to reveal this process and the potential effectivity of RAMEB on PGE2-positive cancer cells.

The second objective was the examination of fluorescent cyclodextrins on *in vitro* models. The permeability of fluorescently-labeled HPBCD (FITC-HPBCD) was measured on two types of *in vitro* blood-brain barrier (BBB) model based on immortalized human brain capillary endothelial cells (hCMEC/D3) and primary rat brain endothelial cells. The measurements were carried out in the frame of the grant, in collaboration with Biological Research Center, Szeged, in the laboratory of Mária Deli. At first, HPBCD cellular toxicity was measured by impedance measurement, and then the permeability of FITC-HPBCD was measured on the blood-brain barrier model developed by the co-culture of rat primary cerebral endothelial cell, glial cells and pericytes and the human hCMEC/D3 model. HPBCD showed no toxic effect up to 100 µM, but a slight but significant decrease in cell index values above 300 µM could be observed after two hours of incubation. The permeability assays were performed with 50 µM FITC-HPBCD for 2 hours, placing the donor solutions onto the apical side of the cell layers and taking samples from the basal side (A-B) or in reverse direction (B-A). The FITC-HPBCD has a permeability coefficient of 0.50×10^{-6} cm/s in the A-B direction and 0.37×10^{-6} cm/s in the B-A direction on the primary rat barrier model, while 1.86×10^{-5} cm/s and 2.82×10^{-5} cm/s in the A-B and B-A directions respectively on hCMEC/D3 model. Based on the values, it can be concluded that FITC-HPBCD has a low permeability in the *in vitro* blood-brain barrier models. This is consistent with *in vivo* results of ^{68}Ga-NODAGA-HPBCD, where low brain activity was also measured. Fluorescence microscopic experiments were used to investigate the uptake of FITC-HPBCD in brain endothelial cells. Incubated with 50 µM FITC-HPBCD, after 2 hours the cells showed low, and after 24 hours showed significant cytoplasmic FITC-HPBCD accumulation in small vesicles of primary rat endothelial cells. However, the 24-hour treatment resulted in the disappearance of the continuous claudin-5 marking between primary rat endothelial cells, which indicates the breakdown of cellular connections. hCMEC/D3 cells showed faster and more intensive FITC-HPBCD uptake after 2 hours of incubation.

The characterization of the cellular effects of β-cyclodextrins on Caco-2 intestinal epithelial cells and HeLa cervical cancer cells were also performed. By RTCAnalysis the dose-dependent cytotoxicity and antiproliferative effects of HPBCD and RAMEB were determined. Using non-toxic (50 µM) cyclodextrin concentration autophagy, lysosome detection and NF-κB signalling pathway experiments were performed applying fluorescence microscopy. Autophagy was characterized by anti-LC3 protein antibody labelling with and without cyclodextrin pre-treatment. We concluded, that rhodamine-labelled HPBCD and RAMEB could be detected in autophagosomes, but the positive control chloroquine induced significantly higher autophagosome formation than cyclodextrins. In other experiments we detected the lysosomes by LysoTracker® fluorescent dyes. Caco-2 and HeLa cells showed different lysosomal accumulation of cyclodextrins in microscopic experiments, the lysosomes of Caco-2 cells contained more intensive cyclodextrin accumulation after cyclodextrin pre-treatment, than the lysosomes of HeLa cells. By flow cytometry the cellular lyososme formation was quantified, however the cyclodextrin pre-treatment did not induced lysosomal activity or lysosome formation. NF-κB detection experiment was based on the antibody labelling of its p65 subunit in the cytoplasm. After HPBCD and RAMEB pre-treatment we did not observe the translocation of the p65 subunit to the cell nuclei, indicating that cyclodextrins did not stimulate the main inflammatory pathway in the Caco-2 or

HeLa cells in non-toxic concentrations. It is an important finding regarding the safety and biocompatibility of these excipients.

In the second year of the research grant, a new research direction was opened by the discovery of the *in vivo* tumor accumulation of the newly synthesized ^{68}Ga-NODAGA-RAMEB cyclodextrin. We aimed to continue the elaboration of this topic, thus we started to test the cellular accumulation of fluorescently-labeled 2-hydroxypropyl-β-cyclodextrin (FITC-HPBCD) and random methylated-β-cyclodextrin (FITC-RAMEB) on nine different cell lines (Caco-2, Vinblastine-resistant Caco-2, HeLa, BxPC3, PancTu-1, hCMEC/D3, Jurkat, HL-60 and HT1080) by flow cytometry and found that there are significant differences in the internalized amount of FITC-HPBCD, FITC-RAMEB and the control FITC-Dextrane among the cell lines. The internalization could be inhibited by cooling the samples on ice and well correlated with the internalization of FITC-Dextran indicating that the mechanism of internalization is macropinocytosis. This mechanism was confirmed by our group on Caco-2 cells and on HeLa cells using inhibitors of endocytosis. This research direction was not proposed in the original research plan, but we believe that these results can reveal new drug delivery pathways into cancer cells.

The third objective of the research project was the formulation and characterization of polyplexes, based on cyclodextrin polymers. Quaternary-amino-beta-cyclodextrin polymer – siRNA (QABCDP-siRNA) polyplex was prepared and characterized. We used GAPDH-siRNA in our model system and the size distribution and average diameter (Z-average, nm) of the polyplex were characterized by dynamic light scattering (DLS). The average diameters of the siRNA (100 μM), QABCDP (100 μM), QABCDP-siRNA 1:1 polyplex, and QABCDP-siRNA 2:1 polyplex were 339±40 nm, 85±4 nm, 143±5 nm, and 78±3 nm respectively. The smaller size of polyplexes compared to the native siRNA indicates the condensation of the siRNA by the polymer and the formation of polyplexes. Increasing the polymer:siRNA molar ratio to 2:1 from 1:1 caused more effective condensation and the size of QABCDP-siRNA 2:1 polyplex is similar to QABCDP polymer. The interaction caused the changing of the zeta potential too and the polyplex formation was detected also by gel-electrophoresis. siRNA was labeled with a fluorescence dye and the cellular internalization of QABCDP-siRNA polyplexes was examined by fluorescence confocal microscopy on Caco-2 cells. siRNA alone was not able to enter the cytoplasm, but QABCDP-siRNA polyplexes delivered siRNA to the cytoplasm and could be detected in small intracellular vesicles. Neither amino-beta-cyclodextrin polymer (NBCDP) nor polyethyleneimine (PEI) could increase the intracellular accumulation of the siRNA. QABCDP-siRNA polyplexes delivered siRNA to the cytoplasm and could be detected in small intracellular vesicles, while interestingly PEI increased the attachment of the labelled siRNA to the cell membrane. NBCDP had no effect on siRNA cellular delivery. By using FITC-labeled QABCDP and Cy3-labeled siRNA their colocalization in the cytoplasmic vesicles after cellular uptake was confirmed. Interestingly the colocalization could be detected after 24 hours of incubation indicating a strong interaction.

Quantitative analysis of the cellular uptake of QABCDP-siRNA polyplex was measured by flow cytometry. QABCDP-siRNA polyplexes in 1:1 and 2:1 molar ratio increased the cellular fluorescence significantly, and the 2:1 polyplex caused an order of magnitude higher intensity than the siRNA, control or NBCDP treatment. PEI was toxic at the applied concentration in flow cytometry experiments. The effect of polyplexes on the GAPDH expression of Caco-2 cell monolayers was measured by ELISA test but no significant decrease of the protein expression could be measured. Similar results were obtained by immunofluorescence tests with fluorescence microscopy. GAPDH mRNA expression was

also tested without significant effect. Two other protein, P-glycoprotein and alpha-tubulin were also tested for gene silencing, but unfortunately without any success compared to the positive control Lipofectamine siRNA carrier.

The biocompatibility of QABCDP was tested by the Real-time cell analysis (RTCA) method and found that in the applied concentration range (50 nM-100 μM) the cyclodextrin polymer was not toxic on Caco-2 cells.

The model system based on GAPDH-siRNA was suitable to confirm the cellular uptake and internalization of QABCDP-siRNA polyplexes, but its effectiveness on gene silencing could not be confirmed. The possible explanation could be, that there is a too strong interaction between QABCDP and siRNA. In this case, the siRNA cannot liberate in the cytoplasm and cannot cause the degradation of the target mRNA. Experiments are running to measure the binding force between siRNA and the polymer and find the explanation for the lack of the gene silencing effect. In the case of too strong siRNA-polymer interaction new applications will be tested for the RNA delivery capability of QABCDP.

In conclusion, the objectives of the research project were successfully fulfilled. Some experiments on tumor cell lines and *in vivo* tumor models were not the object of the original research plan, we accomplished it beyond commitments. The project presented new results on the cellular effects, *in vitro* and *in vivo* behaviour of cyclodextrins, opening new applications of this interesting excipients. The main findings of the project are the following:

- ^{68}Ga-NODAGA-HPBCD and ^{68}Ga-NODAGA-RAMEB were synthetized and characterized for the first time and their exact biodistribution was tested by dynamic small animal PET imaging techniques in BALB/c or CB17 SCID mice.
- Both ^{68}Ga-NODAGA-HPBCD and ^{68}Ga-NODAGA-RAMEB showed rapid elimination without brain or other tissue accumulation. Only the lung showed an increased activity interestingly and the urine bladder due to the urinary excretion.
- ^{68}Ga-NODAGA-RAMEB accumulated in PEG2-positive tumors *in vivo* opening new possibilities for the diagnosis and treatment of PGE2-positive cancers.
- On *in vitro* blood-brain barrier models FITC-HPBCD showed low permeability, but cellular uptake into endothelial cells could be observed.
- Cyclodextrin enter the lysosomes, but its extent depends on the cell type. Cyclodextrins do not induce autophagy, NF-κ pathway and lysosome formation. Cyclodextrin enter the cells by endocytosis, its main mechanism is macropinocytosis, but it depends on the cell type.
- There is a cell-type dependent uptake of cyclodextrin derivatives, which can be applied in cell type specific drug delivery.
- QABCDP forms polyplex with siRNA. It delivers siRNA into the cytoplasm, but gene silencing could not be achieved by this complex.

There was only one deviation from the original workplan: due to the Covid-19 pandemic situation the whole research plan could not be finished during the four years of the project, the budget of the fourth year could not be depleted, therefore a one-year extension of the grant was requested and accepted.