SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the cellular effects of β- cyclodextrin derivatives

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1. Introduction

Cyclodextrins are high molecular weight, hydrophilic, cyclic, non-reducing oligosaccharides, applied as excipients in drug formulations to increase water solubility, to engineer new delivery systems and to modulate bioavailability of lipophilic drugs. The pharmaceutical and the food industry also use cyclodextrins to mask unpleasant flavor, reduce irritant effects and for stabilization. In addition to these two industries, the cosmetics, diagnostics, explosives, and plastic industries use these excipients in a variety of ways. Recently, these molecules are extensively used as active pharmaceutical ingredients and in biomedical technologies.

The research on cyclodextrins have begun in 1891 in France, when Antonie Villiers discovered, that during the fermentation of starch, an unknown substance, called "cellulosine" cyrstallized. From the beginning of 1950s, the research of cyclodextrins, their enzymatic production, isolation and characterization have begun to be intensified. Nowadays, these excipients were manufactured from partially hydrolyzed starch by cyclodextrin glycosyl transferase enzyme. Cyclodextrins can be divided into three major group according to their chemical structure. α-cyclodextrins are composed of six, β-cyclodextrins of seven and even γ-cyclodextrins are composed of eight glycopyranose units linked by α-1,4-glycosidic bonds. The inner cavity of these molecules is apolar, even the outer surface is polar, i.e. it is highly soluble in water. This unique property allows them to encapsulate other molecules, i.e. act as molecular capsules. There are number of free hydroxyl-groups on cyclodextrin molecules, which can be substituted with different ways, e.g. hydoxyalkylation (HPBCD), methylation (RAMEB), acylation or sulfobutylation. With the modification of free hydroxyl-groups and covalent bonds, we also have the opportunity to make larger structures. These molecules, which contains more than one cyclodextrin monomer within a molecule, called cyclodextrin polymer. The polymers offer an increased potential for the use of these excipients, as they increase the potential for interaction between the drug and the cyclodextrin cavity.

Cyclodextrins have also well-known biological effects, which largely determine their applicability and safety. Due to their complexing properties, they are also able to complex natural hydrophobic molecules of the cell membrane, which is associated with their cytotoxicity and the modulation of membrane protein functions such as P-glyoprotein. α-cyclodextrins can extract phospholipids, and even β-cyclodextrins can extract cholesterol from the cell membrane. The degree of lipid solubilization depends on the properties of the cyclodextrin molecule, such like ring size and substitution. **Kiss T. et al.** have shown a relationship between cytotoxicity, hemolytic activity and cholesterol solubilizing properties of β- cyclodextrins. (2- Hydoxypropyl)-beta-cyclodextrin (HPBCD) can enter the cells by endocytosis and it removes the abnormally accumulated cholesterol from the late endosomes/ lysosomes of Niemann-Pick type C (NPC) mutant cells. This discovery led to the clinical application of HPBCD in the treatment of Niemann-Pick disease type C. HPBCD also showed positive effects in Alzheimer's disease, in cardiovascular diseases and it may act as anticancer agent.

These positive effects revealed that cyclodextrins can be considered potential drugs and it is of growing importance to know more about the cellular effects of these cyclodextrin derivatives.

Thereto, cyclodextrins can exert their above-mentioned neuroprotective effects and increase the bioavailability of various drugs, cyclodextrins must cross the cell membrane. Due to their large molecular size and hydrophilic characteristics, they are unable to cross the cell membrane by passive diffusion. These excipients are able to enter the cells by endocytosis. This process form primary endocytic vesicles which deliver their content to early endosomes. During the endosomal maturation early endosomes are converted to late endosomes. Finally, these late endosomes can fuse with the lysosomes, where the relatively small fractions of internalized fluid, transported via the late endosomes were degraded. In mammalian cells several endocytic pathways can be differentiated depending on their dependence on proteins and lipids. The two major pathways are phagocytosis and pinocytosis. During pinocytosis the cells are able to take up fluids, and it can be divided into groups based on the size of the vesicles and the proteins involved in the process. According to these micropinocytosis, chlatrin-dependent and independent, and caveola-mediated endocytosis can be distinguished. **Hammoud et al.** are not found specific membrane receptor, which interacts with cyclodextrins, however the interaction of β-cyclodextrins with membrane cholesterol is well known and has several consequences. The extraction of membrane cholesterol may result in the alteration of signaling pathways and mechanisms.

Nuclear factor-κB (NF-κB) is a pleiotropic regulator of cell and viral genes, consisting of two subunits, the p50 and p65 subunits. Normally, they are found in the cytoplasm in an inactive form, but in certain cases, such as inflammatory stimuli the complex translocated into the nucleus and involved in the regulation of many biological processes. The possible stimulation of this major inflammatory pathway caused by the impairing effects of cyclodextrins on cell membrane and cellular components has not been studied yet. The effects of cyclodextrins on NF-κB activation was only investigated on macrophages. **Motoyoma et al.** found, that dimethyl-α-cyclodextrin antagonized the excess activation of LPS-stimulated macrophages.

Autophagy is a self-digestive process that is activated when the cell removes the pathologically active mitochondria, endoplasmic reticulum or peroxisomes, or the damaged, aggregated proteins. This process plays an important role in balancing energy sources. Autophagy has three main types: micro-, macro-, and chaperon-mediated autophagy. During this self-digestive process, the damaged proteins or injured cell compartments are removed from the cells via autophagosomes. In the case of macro-autophagy, dysfunctional cell organelles in the cytoplasm are sequestered by the expanding phagophore, and LC3 molecules are inserted into the phagophore membrane, which becomes autophagosomes. The final step of the process is the fusion with lysosomes. **Kameyama et al.** described, that folate-appended methyl-β-cyclodextrin induces the mitophagy, which means the selective elimination of mitochondria.

The small interfering ribonucleic acid (siRNA) is a double-stranded, non-coding DNA molecule, usually 20 to 27 base pairs, that plays an important role in gene silencing. The base of the RNA interference is the suppression of gene expression through translation repression by siRNA. Due to its large molecular size and polyanionic nature, it is difficult to transfect the siRNA into the cells and the naked siRNA easily degraded in the blood stream. A number of systems are available or under development to solve this problem. These systems can be polymer- (polyplex) or liposome-(lipoplex) based systems or conjugates. The negatively charged siRNA associates with the positively charged polymer molecule through electrostatic interactions or hydrogen bonding. The various delivery systems usually taken up by cells by receptor-mediated endocytosis. **Lee at al.** observed a dose- dependent decrease in the expression of P-glycoprotein (Pgp) on Caco-2 cells, transfected by siLentFect.

2. Aims

The aim of my doctoral research was to reveal the consequences of the cellular internalization of cyclodextrins and to investigate the siRNA carrying capacity of different cyclodextrin polymers.

In the first part we aimed to investigate the cellular internalization and the intracellular effects of β-cyclodextrin derivatives on Caco-2 intestinal epithelial cells and to examine and to compare the above-mentioned effects on HeLa human cervix epithelioid cells. Our purposes were:

- to investigate and to compare the cellular internalization of fluorescein (FITC) and rhodamine (Rho) labeled hydroxy-propyl-beta- (HPBCD) and random methyl-beta-cyclodextrin (RAMEB).
- to reveal the more precise mechanism of endocytosis
- to study the impact of the internalization of these cyclodextrin derivatives
- to examine the possible activation of nuclear factor- κB (NF- κB) pathway
- to investigate the autophagy, and the localization of these cyclodextrin derivatives in autophagosomes
- to study the localization of HPCD and RAMEB in lysosomes

In the second part we aimed to examine the siRNA carrying capacity of two cyclodextrin polymers. Our objectives were:

- to formulate polyplexes in different molar ratios and investigate their properties
- to examine the cellular internalization of these polyplexes

3. Materials and methods

The Caco-2 and HeLa cell lines were obtained from the European Collection of Cell Cultures (ECACC, UK) and were cultured with modified DMEM cell culture media. (2-hydroxypropyl)-β-cyclodextrin (HPBCD), random methyl-β-cyclodextrin (RAMEB) and their rhodamine (Rho) and fluorescein (FITC) labeled analogues were products of CycloLab Ltd. (Budapest, Hungary).

The cytotoxic effects of different concentrations (50 μM, 500 μM, 1 mM, 10 mM and 50 mM) of hydroxypropyl-beta (HPBCD) and random-methyl-beta-cyclodextrin (RAMEB) derivatives for 24 hours, and the effects on cell proliferation of different concentrations (50 nM and 100 nM) of cyclodextrin polymers were investigated for 2 days by RTCA method.

The cellular internalization of the fluorescently labeled cyclodextrin derivatives was investigated by fluorescence microscopy. During the experiments, Caco-2 and HeLa cells were incubated for 30 min at 37 °C with 50 μM fluorescently labeled cyclodextrin derivatives solutions, and after fixation and nuclear staining, the samples were examined by fluorescence microscopy. The type of the endocytosis mechanism was investigated by flow cytometry using different endocytosis inhibitors. The used inhibitors: wortmannin 50 nM, genistein 200 μM, filipin 1 μg/ml, nocodazole 10 μM, LY294002 50 μM and rottlerin 1 μg/ml. In these experiments, cells were preincubated with the different inhibitors for 40 min at 37 °C or 4 °C and then incubated with 50 μM fluorescently labeled cyclodextrin solutions for 30 min. Then the cells were washed, the dead cells were stained with propidium iodide, and the cellular fluorescence intensity was measured with a flow cytometer. In the case of Caco-2 cells the measurements were carried out by Béla Nagy Ph. D. and Zsolt Fejes Ph. D. at the Department of Laboratory Medicine.

The effect of cyclodextrins on NF-kB pathway was examined by fluorescence microscopy. During the experiments, cells were incubated for 30 minutes at 37 °C in 50 μM HPBCD or RAMEB, in the case of positive control in 50 ng/ml TNF-α solutions. Then cells were fixed, the nonspecific binding sites were blocked, and the p65 subunit was labeled with a primary and fluorescently labeled secondary antibody, and the nuclei were stained. The samples were examined by fluorescence microscopy, whereby the nuclear translocation of the NF-kB p65 subunit was monitored.

The effect of cyclodextrins on autophagy was investigated qualitatively and quantitavely. For the qualitative studies, cells were incubated for 24 h at 37 °C in 50 μM HBPCD or RAMEB, in the case of a positive control in 100 μM chloroquine solutions. Then cells were fixed, permeabilized, and the LC3B molecule in the membrane of the autophagosomes was labeled with primary and fluorescently labeled secondary antibodies (LC3B Antibody Kit for Autophagy, Thermo Fisher Scientific), and the nuclei were stained. The samples were then examined by fluorescence microscopy. For quantitative measurements, cells were incubated for 24 h at 37 °C in 50 μM HBPCD or RAMEB, in the case of a positive control in 100 μM chloroquine

solutions. Subsequently, the membrane of the autophagosomes was stained with a fluorescent dye (CYTO-ID ® Autophagy Detection Kit, Enzo Life Sciences) according to the kit, and the cellular fluorescence intensity was measured with a microplate reader.

Lysosomes presented in Caco-2 and HeLa cells were examined by fluorescent microscopy, whereby the cells were incubated for 30 min at 37 ˚C in 50 μM unlabeled or fluorescently labeled HPBCD or RAMEB solutions, then the membrane of lysosomes were stained by LysoTracker® (Thermo Fisher Scientific). After that nuclei were stained, and the samples were examined by fluorescence microscopy for qualitative studies and the cellular fluorescence intensity was measured with flow cytometer for quantitative studies

The properties of formulated polyplexes were examined by dinamic light scattering (DLS) and zeta potential measurements by ZetaSizer Nano (Malvern). In these experiments 100 μM siRNA and polypmer solutions were used. Detection was at an angle of 175° and the thermostat was set to 25 °C. The size distribution and polydispersity (PDI) of the different solutions were measured three times. The zeta potential was measured in a High Concentration Zeta Cell with a variable voltage current.

The properties of polyplexes were also investigated by gel retardation assay carried out by Alexandra Gyöngyösi Ph. D. and István Lekli Ph. D., at the Department of Pharmacology. In the experiments the 100 and 500 nM siRNA solutions were used to formulate different molar ratio polyplexes with QABCDP polymer. Electrophoresis was performed for 1 hour at 80 V and samples were detected on a ChemiDoc Touch (Bio-Rad Laboratories, Hercules, CA, USA).

The cellular internalization of the polyplexes was investigated by confocal microscopy and flow cytometry. The confocal microscopic measurements were carried out by György Vámosi Ph. D., at the Department of Biophysics and Cell Biology. In the experiments, cells were incubated for 30 min, 4 or 24 h in polyplex solutions with different molar ratios of 5 μM fluorescently labeled siRNA. Cells were then fixed, nuclei were stained, and samples were examined by confocal microscopy or cellular fluorescence intensity was measured with a flow cytometer.

4. Results

4.1 Investigation of the cellular effects of cyclodextrins

4.1.1. Investigation of the cytotoxicity

The cytotoxic effects of different cyclodextrin concentrations on Caco-2 and HeLa cells were investigated by RTCA method. In the case of HPBCD only the 50 mM concentration was cytotoxic, while in the case of RAMEB 10 mM concentration showed cytotoxicity. In the case of HeLa cells we found, that at certain cyclodextrin concentrations, cell index increased without any further cytotoxicity. We suppose that the possible explanation is the following: at certain non-toxic cyclodextrin concentrations, the cell membrane is reversibly compromised causing increased membrane permeability or morphological changes, which are reflected in the increased cell index values. RTCA cell index measurement is based on the attachment of cells on the surface of plate electrodes, and any alterations in cellular morphology, attachment or electrical resistance between cells and plate surface cause cell index changes. The RTCA results seem to support this theory, because there was a non-toxic 50 μM HPBCD concentration, which had no significant effect on cells, as well as non-toxic concentrations, which increased cell index between 500 μM–10 mM, and a toxic, 50 mM HPBCD concentration, which decreased cell index.

As we used, in our further experiments, 30 min of incubation with cyclodextrins, we analyzed the RTCA normalized cell index values at this time point. We found that only 50 mM RAMEB treatment showed significant toxic effects compared to the control cells after 30 min. We used 50 μM non-toxic concentration of HPBCD and RAMEB with 30 min of incubation in our further experiments.

4.1.2. Investigation of the cellular uptake of cyclodextrins

The cellular internalization and intracellular localization of the fluorescein (FITC) or rhodamine (Rho) labeled (2-hydroxypropyl)-beta-cyclodextrin (HPBCD) were visualized by fluorescent microscopy using Caco-2 and HeLa cells. Smaller and larger cyclodextrin-loaded vesicles were detected along the cell membrane and around the cell nucleus for both cell lines. The fluorescent labelling did not influence the endocytosis of these derivatives.

The results were confirmed by flow cytometry. For this, some samples were pretreated with different endocytosis inhibitors and treated with 50 μM FITC-HPBCD or FITC-RAMEB at 0 °C or 37 °C. At the end of this experiment dead cells were stained with propidium iodide. Fluorescence intensity was measured by flow cytometer. We compared the fluorescence intensities of the inhibitor-treated cells to the untreated samples (100%). In the case of both cell line, cooling significantly inhibited the cellular uptake of cyclodextrin derivatives. In the case of Caco-2 intestinal epithelial cells, rottlerin, which is the inhibitor of macropinocytosis inhibited significantly the uptake, while in the case of HeLa cells, remarkably increased FITC-RAMEB and slightly enhanced FITC-HPBCD uptake. In contrast with rottlerin, the wortmannin and

LY294002, which another inhibitors of macropinocytosis, had no significant effect on these cyclodextrin derivatives uptake. This can be explained by the different mechanisms of the actions of the two inhibitors. Rottlerin is a protein kinase C inhibitor, while wortmannin and LY294002 inhibit the PI3K. Chlorpromazine is a cationic amphiphilic drug, which is applied to inhibit clathrin-coated pit formation. The effect of chlorpromazine is highly cell-type dependent. In Caco-2 cells interestingly, significantly increased the uptake of both cyclodextrin derivatives, while in HeLa cells had no significant effects. Filipin, which inhibits the caveola-mediated endocytosis, did not decrease significantly the uptake of cyclodextrins in Caco-2 and HeLa cells. Nocodazole, which disrupts the microtubules, did not affect the uptake of the cyclodextrins either. According to these results, in Caco- 2 cells, macropinocytosis can be the main mechanism of the cellular internalization of the fluorescent HPBCD and RAMEB derivatives, while in HeLa cells is presumably several simultaneous processes.

4.1.3 Investigation of NF- κB pathway

In this experiment the effects of non-toxic concentration of HPBCD and RAMEB on the NF-κB inflammatory pathway were tested on Caco-2 and HeLa cells. The p65 subunit of NF-κB was labeled with anti-p65 antibody and the cell nuclei were stained with Hoechst 33,342. The possible activation of NF-κB pathway by the different cyclodextrin derivatives were visualized by fluorescence microscopy. Cells were pre-incubated with 50 μM cyclodextrin solutions and after that primer and secondary antibody solutions. In this case, a green signal was detected only in the cytoplasm. It means, that the p65 subunit was found only in the cytoplasm. Cyclodextrins did not activate the translocation of this subunit from the cytoplasm to the cell nucleus, so these derivatives did not activate this inflammatory pathway in the cells. In the case of positive control, cells were pre-incubated with TNF-α and a green signal was detected in the cell nuclei, indicating that TNF-α activated the NF-κB pathway.

4.1.4. Investigation of autophagy

The effects of (2- hydroxypropyl)-beta-cylodextrin (HPBCD) and random-methyl-beta-cyclodextrin (RAMEB) on the induction of autophagy was evaluated on Caco-2 intestinal epithelial and HeLa cervical epithelioid cells. First of all, we tested it qualitatively with fluorescence microscopy labeling the LC3B molecule in the autophagosome membranes with an anti-LC3B antibody while the cell nuclei were stained with Hoechst 33,342. After rhodamine labeled HPBCD and RAMEB treatments, the presence of autophagosomes was detectable, similar to the control sample. At the same time, chloroquine treatment, which was the positive control, caused more intensive autophagosome formation than in the case of cyclodextrin treatment. The autophagosome formation with Rho-cyclodextrin derivatives was investigated in order to determine the localization of the cyclodextrins in the cells. In

the case of both cell line, we could detect limited colocalization of the green signal of LC3B and red signal of Rho-cyclodextrins in some intracellular vesicles (yellow signal) indicating that cyclodextrins were only partially localized in the autophagosomes.

The obtained results were confirmed with quantitative experiments. The membrane of autophagosomes was stained with CYTO-ID® Green Detection Reagent and the cell nuclei were stained with Hoechst 33,342. The fluorescence intensity was measured with a microplate reader. Results were normalized to cell number and expressed as a relative fluorescence intensity. HPBCD and RAMEB treatments did not increase autophagosome formation compared to the control sample both on Caco-2 and HeLa cells. The difference between the chloroquine-treated sample and the control was significant, indicating that chloroquine used as a positive control induce autophagosome formation, autophagy.

4.1.5. Investigation of lysosomes

The effects of rhodamine (Rho) labeled HPBCD and RAMEB on the lysosomes were assessed on Caco-2 intestinal epithelial and HeLa cervical epithelioid cells. First, a qualitative test with fluorescence microscopy was performed. After a non-toxic concentration (50 μM) of cyclodextrin treatments, lysosomes were detectable in the cytoplasm similar to the control sample. The green and the red signals are colocalized in many intracellular vesicles (yellow pixels), indicating that significant amount of the cyclodextrins could be detected in the lysosomes after internalization. It can be noted that in Caco-2 cells, larger lysosomal vesicles could be identified with higher cyclodextrin content than in HeLa cells.

We confirmed our results by flow cytometry. After unlabeled HPBCD and RAMEB treatments, the intensity of Lysotracker lysosomal staining (red fluorescence) is similar to the sample stained only with Lysotracker. After fluorescein labeled HPBCD and RAMEB treatments, the green fluorescence intensity increased, and no red fluorescence was detected. This is indicating, that cyclodextrins were taken up by cells, and localized in the cytoplasm. Applying both FITC-cyclodextrins and Lysotracker, the lysosomal fluorescence did not exceed the value of only Lysotracker-stained samples. It means, that cyclodextrins did not increased the amount of lysosomes.

4.2. Investigation of the siRNA carrying capacity

4.2.1. Characterization of the polyplexes

The formation of the polyplexes was characterized by dynamic light scattering (DLS) and gel retardation assay. The hydrodynamic diameter was measured by DLS of the polyplexes. The siRNA was 336.26 ±3.29 nm with a polydispersity index (PDI) of 0.467 ± 0.008, QABCDP was 85.96 ± 0.89 nm with a PDI of 0.44 ± 0.006, NHBCDP was 19.58 ± 0.37 nm with a PDI of 0.37 ± 0.01 and PEI was 4693.8 ±731.88 nm with a PDI of 0.36 ± 0.05. The complexation was successful in every case, because the hydrodynamic diameter of the formulated polyplexes was between the polymer and siRNA diameter. The QABCDP: siRNA in molar ratio 1:1 was 145.82 ±6.94 nm with a PDI of 0.57 ± 0.11, in molar ratio 2:1 was 78.71 ± 0.97 nm with a PDI of 0.42 ± 0.01. The

NHBCDP: siRNA in molar ratio 1:1 was 31.9 ± 0.93 nm with a PDI of 0.62 ± 0,02, in molar ratio 2:1 was 15.43 ± 0.12 nm with a PDI of 0.30 ± 0. 02. The PEI: siRNA in molar ratio 1:1 was 1712.6 ± 276.3 nm with PDI of 0.94 ± 0.03, in molar ratio 2:1 was 7024,4 ±1404.4 nm with a PDI of 1.0.

The zeta-potential of siRNA was -18.96 ± 0.42 mV, the QABCDP was 26.53 ± 14.69 mV, the NHBCDP was 3.89 ± 0.98 mV and the PEI was 3.54 ± 1.82 mV. These results confirmed that the formulationwas successful, the polymers bound the siRNA, because the zeta-potential of the polyplexes was positive, but not larger than the polymer. The QABCDP: siRNA in molar ratio 1:1 was 2.99 ±0.18 mV, in molar ratio 2:1 was 1.69 ± 0.31 mV. The NHBCDP: siRNA in molar ratio 1:1 is 3.11 ±0.2 mV, in molar ratio 2:1 is 2.73 ±0.66 Mv. The PEI: siRNA in molar ratio 1:1 was1.94 ± 0.04 mV, in molar ratio 2:1 was 1.88 ± 0.05 mV.

The gel retardation assay was performed using QABCD polymer and siRNA at different molar ratios. Interestingly the bounding of the siRNA was shown concentration dependency. According to our results, in the case of 100 nM siRNA concentration it was completely bound at polymer: siRNA ≥ 128 ratio, while in the case of 500 nM siRNA concentration, the polymer ratio which completely bound the siRNA was ≥ 64.

4.2.2. Investigation of the cell proliferation

The effects of the GAPDH siRNA, the different polymers and polyplexes on the cell proliferation of Caco-2 cells were investigated by RTCA method. In the case of all three polymer the tested concentration, 50 or 100 nM, the formulated polyplexes and the siRNA in 50 nM concentration had no significant inhibitory effect on the cell proliferation. In contrast the 1% Triton X-100 inhibited significantly the intestinal epithelial cell proliferation.

4.2.3. Investigation of the cellular uptake of polyplexes

The cellular uptake and the intracellular localization of the Cy3-labelled siRNA polyplexes were investigated by confocal microscopy. In the case of NHBCDP the siRNA did not enter the cells, such like in the case of naked siRNA. In the case of PEI red signal was detected along the cell membrane. It indicates that larger polyplexes-loaded vesicles were along the cell membrane or in membrane ligations. In the case of QABCDP red signal (siRNA) and green signal (polymer) were detected in the cytoplasm. These two signals could be detected colocalized (yellow pixels), which indicated that smaller and larger polyplex-loaded vesicles were in the cytoplasm, around the nucleus.

The obtained results were confirmed by flow cytometry measurements. For this the cells were incubated with the Cy3labelled siRNA polyplexes for 30 min and the cellular fluorescence intensity was measured. We compared the fluorescence intensities to the sample treated with naked siRNA. In the case of QABCDP polyplexes the fluorescence intensity was higher, but in the case of NHBCDP polyplexes it was

the same as for the control sample. In the case of PEI no living cells were detected, presumably this polymer concentrations (5 and 10 μM) were cytotoxic to Caco-2 cells. After 4 and 24 h of incubation, the 2: 1 molar ratio of QABCDP polyplex shows a green and red signal in colocalization, from which it is concluded that the polyplex is still present in the cells after a long incubation period. In the case of a polyplex with a molar ratio of 32: 1, no red signal was detected after either 4 or 24 hours, from which it is concluded that this polyplex is not taken up by the cells.

5. Discussion

5.1. Cellular effects of cyclodextrins

This PhD research reveals important details about the cellular effects of cyclodextrins on Caco- 2 and HeLa cells, and this is the first systematic comparative study that tests the interactions of cyclodextrins with different cell lines. The data show that there are important differences between the cellular internalization of cyclodextrins (e.g., type of endocytosis, volume of endocytosis) in different cell lines, which can be used in further developments for cell type specific drug delivery. This work demonstrates the possible selective application of cyclodextrins for endocytosis-mediated cellular uptake. On the other hand, the cellular effects and the intracellular fate of cyclodextrins are poorly studied. More information is needed to reveal the effects of these special macrocycles on cellular processes, especially on the major inflammatory, or metabolic routes, which are connected with endocytosis. To enrich our knowledge in this field, this work focused on the investigation of NF-κB pathway activation and autophagosome and lysosome formation after the endocytosis of cyclodextrins.

By using fluorescent cyclodextrin derivatives, we showed previously that cyclodextrins are able to enter Caco-2 intestinal cells by endocytosis, but the importance of the different fluorescent labeling has not been compared yet on the same cyclodextrin derivative. Now we have confirmed our previous results, that cyclodextrins enter the cells. In the present work we compared cyclodextrin derivatives labeled with two different fluorescent moieties: the green fluorescenyl and red rhodaminyl groups attached to the cyclodextrin molecules. The type of the fluorescence labeling of HPBCD did not influence the endocytosis of these derivatives. Both fluorescein- and rhodamine-labeled derivatives entered the cells and were localized in smaller vesicles along the cell membrane and in larger granules around the nucleus. The similar chemical structure explains this: both fluorescent moieties are carboxyphenyl xanthene dyes.

The endocytosis of these cyclodextrins was found to be temperature dependent on both two cell lines: cooling significantly inhibited the endocytosis of both FITC-HPBCD and FITC-RAMEB. However, based on our results, we concluded that the mechanism of the endocytosis on Caco-2 intestinal epithelial cells is the macropinocytosis, while on HeLa cells we could not found main mechanism. On Caco-2 cells rottlerin pretreatment reduced the fluorescence intensity significantly compared to the untreated samples. Rottlerin is one of the inhibitors of macropinocytosis. In contrast, wortmannin, another inhibitor of macropinocytosis, did not inhibit the uptake of these derivatives. This can be explained by the different mechanisms of the actions of the two inhibitors. Interestingly, on HeLa cervical epithelioid cells the rottlerin significantly increased the uptake of FITC-RAMEB and slightly enhanced the uptake of FITC-HPBCD. Filipin, the inhibitor of the caveola-mediated endocytosis and nocodazole, which disrupts the microtubules, did not affect

the uptake of the cyclodextrins either on Caco-2 and HeLa cells. **Plazzo et. al.** found, that chlorpromazine blocked the internalization of methyl-βCD tagged with fluorescein, and the fluorescent intracellular vesicles remained in the vicinity of the plasma membrane in HeLa cells. In contrast, we found, that this endocytosis inhibitor significantly increased the uptake of cyclodextrins on Caco-2 cells and had no significant effects on HeLa cells. The possible explanation can be, that we used flow cytometry to measure the effect of inhibitors. This method measures the whole cellular fluorescence intensity, while **Plazzo et. al.** obtained their results by fluorescence microscopy, which exactly shows the intracellular localization of endosomes.

The NF-kappa B pathway is an inflammatory pathway in cells in which nuclear translocation of the p65 subunit is a key event in inflammatory responses. This process may contribute to the opening of epithelial barriers at the level of signaling pathways. In our experiments, the above-mentioned p65 subunit was labeled by immunofluorescence methods based on the method developed by our research group and its nuclear translocation was monitored. (2- Hydroxypropyl)-beta (HPBCD) and random-methyl-beta-cyclodextrin (RAMEB) at a non-toxic concentration (50 μM) were found not to activate this inflammatory pathway in either the Caco-2 or HeLa cell lines. These results support new information on the safety profiles of cyclodextrins.

Autophagy is a self-digesting process that plays an important role in the breakdown and recycling of dysfunctional and redundant cellular components. We found that autophagosomes could be detected in both Caco-2 intestinal epithelial cells and HeLa cells after treatment with (2-hydroxypropyl)-beta and random-methyl-beta-cyclodextrin, these derivatives did not increase the amount of autophagosomes in the cells, compared to the control sample. However, when examined by fluorescence microscopy, we found that rhodamine-labeled HPBCD and RAMEB enter in small amounts into the autophagosomes, as inferred from the colocalization of the cyclodextrin's red and autophagosome's green signals. From all these results, we conclude that HPBCD and RAMEB at non-toxic 50 μM concentrations did not cause any damage to cellular components that would lead to autophagy.

Lysosomes are cell compartments in which the pH is acidic, which acidity is required for the enzymes to function. Lysosomes also play an important role in the breakdown of excess, abnormally functioning cellular components. As a final step in autophagy, autophagosome fuses with the lysosome, where the ingested components are degraded. Interestingly, we found that the two cyclodextrin derivatives we studied - HPBCD and RAMEB - are only slightly absorbed into the autophagosomes, while significantly engaged into the lysosomes. Larger lysosomal vesicles with higher cyclodextrin content were detected in Caco-2 cells than in HeLa cells. This difference between the two cell lines may be suitable for the development of lysosomal targeted therapies in the treatment of cancer.

5.2. siRNA carrying capacity of cyclodextrin polymers

In the second part of my PhD research the characterization, cellular uptake of different cyclodextrin- based polyplexes were investigated. In the recent years the

siRNA based therapies have undergone great development. Many carrier system already in the concept and under development, however, transfection of siRNA into cells poses number of challenges. In parallel, cyclodextrins gaining more interests in the recent years.

First of all the effects of polymers and polyplexes on cell proliferation was investigated by RTCA method. Based on our results, the applied concentrations of 50 and 100 nM, neither the polymers, nor the siRNA, nor the formulated polyplexes had a negative effect on cell proliferation. In our further experiments, we incubated the cells with the polyplexes for 2 or 5 days, so we examined their effect on cell proliferation at these time points as well. According to our results, polyplexes and polymers at a non-toxic concentration of 100 nM did not have a negative effect on the proliferation of Caco-2 cells even after 5 days.

We compared the siRNA binding and carrying capacity of two cationic cyclodextrin polymer and the polyethilineimine (PEI), as a positive control. Each of the polymers can bind and condenses the siRNA, because the size of the siRNA decreased and its negative zeta potential increased after the complexation. According to our results, measuring by gel retardation assay the bounding of the siRNA was concentration dependent. According to our results, in the case of 100 nM siRNA concentration it wascompletely bound at polymer: siRNA ≥ 128, while in the case of 500 nM siRNA concentration, the polymer ratio which completely bound the siRNA was ≥ 64.

We tested the cellular uptake of the formulated polyplexes by fluorescence microscopy. We found that the polymers show different cellular internalization on Caco- 2 cells. The NHBCDP was not able to enter the cells, such like the naked siRNA. In the case of PEI, the polyplexes localized in larger granules or in membrane ligations, while the QABCDP polyplexes localized around the cell nuclei in larger granules (Figure 3). **Di'Silvio et. al.** found that polyplexes formulated with polyallilamin (PAH) can be detected in the cells after 24. We found the same, QABCDP polyplexes can be found in the cytoplasm in similar amounts after 24 h than after 30 min.

6. Summary

Cyclodextrins are widely used excipients to increase the bioavailability of lipophilic drugs, as well as hydroxypropyl beta-cyclodextrin applied as an orphan drug. However, the intracellular effects of cyclodextrins were first investigated for their safety..

Based on our results:

- the fluorescent labeling does not influence the cellular internalization of HPBCD and RAMEB. After endocytosis, they are localized in smaller vesicles along the cell membrane and in larger vesicles around the nucleus, on both cell lines.
- In the case of Caco-2 cells, the more precise mechanism of endocytosis is macropinocytosis, even in the case of HeLa cells it is presumably several simultaneous processes.
- These derivatives do not cause the translocation of the p65 subunit into the nucleus, so these do not induce the NF-kappa B inflammatory pathway.
- Compared to the control sample, they do not induce the formation of autophagosomes and are found in small amounts in these vesicles.
- Neither HPBCD nor RAMEB induce lysosome formation, however, they are detectable in the lysosomes. Lysosomes with larger size and higher cyclodextrin content were observed in Caco-2 cells than in HeLa cells.
- the cyclodextrin polymers are able to complex siRNA, based on size, zeta-potential measurements and gel retardation assay
- QABCDP-polyplexes entered the cells, while the NHBCDP- polyplexesdid not entere and the PEI-polyplexes were found in membrane ligation along the cell membrane

7. List of publication

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Candidate: Ágnes Rusznyák
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Rusznyák, Á.**, Palicskó, M., Malanga, M., Fenyvesi, É., Szente, L., Váradi, J., Bácskay, I., Vecsernyés, M., Réti-Nagy, K., Vasvári, G., Haimhoffer, Á., Fenyvesi, F.: Cellular Effects of Cyclodextrins: studies on HeLa Cells.
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3. Le Khanh, H. P., Nemes, D., **Rusznyák, Á.**, Ujhelyi, Z., Fehér, P., Fenyvesi, F., Váradi, J., Vecsernyés, M., Bácskay, I.: Comparative Investigation of Cellular Effects of Polyethylene Glycol (PEG) Derivates.
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