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Biocompatibility evaluation and synthesis of macrocyclic compounds

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1. Abbreviations

AcACD	acetylated-α-cyclodextrin
ACD	α-cyclodextrin
API	active pharmaceutical ingredient
BCS	biopharmaceutical classification system
C4S	*para*-sulphonato-calix[4]arene
C6S	*para*-sulphonato-calix[6]arene
C8S	*para*-sulphonato-calix[8]arene
CD	cyclodextrin
CMBCD	carboxymethylated-β-cyclodextrin
CnS	*para*-sulphonato-calix[n]arene
DIMEA	hexakis (2,3-di-O-methyl)-α-cyclodextrin
DIMEB	heptakis (2,3-di-O-methyl)-β-cyclodextrin
DS	degree of substitution
HBSS	Hank's balanced salt solution
HC_{50}	concentration which causes 50% hemolysis
HPACD	(2-hydroxypropyl)-α-cyclodextrin
HPBCD	(2-hydroxypropyl)-β-cyclodextrin
i.v.	intravenous
IC_{50}	half maximal inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PARMEA	partially methylated α-cyclodextrin
PARMEB	partially methylated β-cyclodextrin
PBS	phosphate-buffered saline
RAMEA	random methylated α-cyclodextrin
RAMEB	random methylated β-cyclodextrin
RBC	red blood cell
RT-CES	real time cell electronic sensing
SBEBCD	sulfobutylether-β-cyclodextrin
SuACD	succinylated-α-cyclodextrin
TEER	transepithelial electrical resistance
THF	tetrahydrofuran
TRIMEA	hexakis (2,3,6-tri-O-methyl)-α-cyclodextrin
TRIMEB	heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin

2. Introduction and objectives

Regarding the up-to-date databases of novel drug candidates, the tendency of increased lipophilic nature and molecular size is conspicuous. However, the biological activity properties and solubility are inversely proportional. The Biopharmaceutical Classification System classifies the APIs based on their aqueous solubility and gastrointestinal permeability, as fundamental characteristics. An orally administered drug has to be dissolved in the aqueous environment along the gastrointestinal tract, however a somewhat lipophilic characteristic is also indispensable for permeating through the membrane barriers.

Macrocyclic compounds consist of repeating units with no terminal ends. Owing to the cyclic structure each possess a cavity, which is suitable for accommodating guest molecules. Highly water soluble macrocyclic rings are able to form inclusion complexes with BCS II and IV APIs, facilitating their solubility, thus bioavailability enhancement.

The major classes of macrocyclic organic host compounds are the cyclodextrins, calixarenes, crown ethers, and cucurbiturils. Cyclodextrins had been known first, and the subsequent extensive investigations resulted their current application not only as pharmaceutical excipients, but as orphan drug as well.

Despite of the practically unlimited possibilities of CD modification, just a few derivatives have been applied in medical formulations, and mainly β-CDs. The biocompatibility of several other β-CDs has been evaluated for more than a decade in the Department of Pharmaceutical Technology, University of Debrecen. The small dimension of the α-CD cavity limits the range of guest molecules, which explains their less frequent pharmaceutical application. Our aim was to determine the biocompatibility of those α-CD derivatives, which bear similar structural modifications than the already studied β-CDs. The biocompatibility evaluation included *in vitro* methodologies, such as cell viability assays and hemolysis test. The apprehension of the correlations between the structural modifications and the toxic effects in each system, helps to ascertain the connection between the chemical structure and the safety profile.

Our commitment towards α-CDs had been reinforced by the research group of Applied Supramolecular Chemistry (CSAp), University of Lyon 1. They have demonstrated an advantage of α-CD application, namely that they form stable nanoparticles, which facilitates sustained drug release. The goal of our cooperation with the CSAp group was the synthesis of alkyl ether α-CDs, with well-defined structure. Modifications at the primary, secondary and

both faces aimed to clarify how the reducing free hydroxyl group number or the increasing length of the alkyl substituent effects the cytotoxic or hemolytic activity.

In correspondence with the proportion of experimental work performed, the main part of the dissertation deals with cyclodextrins. The second part of our work was focused on *para*-sulphonato-calix[n]arenes, which have several similarities to CDs. *Para*-sulphonato-calix[n]arenes have been widely studied and have a great possibility to future pharmaceutical application. However, their biocompatibility evaluations are still incomplete. Our aim was to complement the missing data on their effects on Caco-2 cells, more precisely their impact on paracellular absorption from the small intestine.

3. Materials and methods

3.1 Materials

The commercially available α-CD derivatives and the RAMEA:phosphatidylcholine complex were generously offered by Cyclolab Ltd. (Budapest, Hungary). The other α-CDs were synthetized in the University of Lyon 1, as described in the dissertation. *Para*-sulphonato-calix[4]arene (C4S), *para*-sulphonato-calix[6]arene (C6S) and *para*-sulphonato-calix[8]arene (C8S) were synthetized by Florent Perret and Anthony W. Coleman as described in the literature.

Reagents and methanol were purchased from Sigma Aldrich (France) the other solvents from VWR (France). The use of anhydrous solvents was provided by PureSolv solvent purification system by Innovative Technology. Anhydrous THF was freshly distilled in the presence of Na and benzophenone. All reactions were carried out in N_2 atmosphere with the exception of hydrogenation reactions where H_2 was used.

3.2 Caco-2 cell line

Caco-2 cell line was obtained from the European Collection of Cell Cultures. Cells were grown in plastic cell culture flasks in Dulbecco's Modified Eagle's Medium, supplemented with 3.7g/L $NaHCO_3$, 10% (v/v) heat-inactivated fetal bovine serum, 1% (v/v) non-essential amino acids solution, 1% (v/v) L-glutamine, 100 IU/mL penicillin, and 100µg/mL streptomycin at 37ºC in an atmosphere of 5% CO_2. The cells were routinely maintained by regular passaging. For cytotoxic experiments, cells were used between passage numbers 20 and 40. The culture media was replaced with fresh media in every 72 hours.

3.3 Hemolysis test

Hemolysis test was performed on fresh human blood, collected from healthy donors. Erythrocytes were separated from citrated blood by centrifugation at 2500 × g for 10min, washed three times with PBS and re-suspended in the same solution. Aliquots of the cell suspension with the respective RBC number of 5×10^7 were added to the buffer solution (PBS, pH 7.2) containing increasing concentrations of the samples investigated in the study. After mixing them gently, each solution was incubated at 37°C for 10 minutes and then centrifuged at 5000 × g. Finally, the absorbance of the hemoglobin released into the supernatant was measured at 540nm with FLUOstar OPTIMA Microplate Reader (BMG Labtech, Germany). The percentage of hemolysis was expressed as the ratio of hemoglobin in the supernatant of

the sample solutions, related to the hemoglobin concentration after the complete hemolysis of erythrocytes in water. The dose–response curve was determined, and the HC_{50} concentrations were subsequently calculated.

3.4 MTT cell viability assay

The cells were seeded in 96-well plate until the cell monolayer become confluent. The medium was changed once. After one week the medium was removed, the cells were washed with PBS and exposed to increasing concentrations of the certain substance dissolved in PBS. Cells were incubated for 30 minutes on 37 ºC. Control groups were processed equally and incubated only with PBS. After treatment, MTT dye (3-(4,5-dimethylthiazol-2-yl))-2,5-diphenyltetrazolium bromide, 5mg/mL) was applied to each well for 3 hours. MTT solution was removed and isopropanol:hydrochloride acid (25:1) was added to dissolve the formed formazan crystals. The absorbance was measured at 570nm against a 690nm reference with FLUOstar OPTIMA Microplate Reader. Cell viability was expressed as the percentage of untreated control.

3.5 Real-Time Cell Electronic Sensing

The E-plate was coated with 0.2% rat tail collagen–DW solution for 20 minutes at 37°C. Culture media (60µL) was added to each well for background readings than 100µL Caco-2 cell suspension was dispensed at the density of 1.5×10^4 cells/well. The cells were grown for 2 days. The medium was changed to excipients solutions and the cells in the E-plate were kept in an incubator at 37°C for 8 hours and monitored every 5min. The cell index at each time point was defined as $(R_n–R_b)/15$, where R_n is the cell–electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the media alone.

3.6 Statistical analysis

Raw data were compared with one-way ANOVA (using Geisser-Greenhouse correction) followed by Tukey post-testing (because all data passed the D'Agostino and Pearson omnibus normality test). Statistical significance for the difference of means was assigned into one of five categories: $p > 0.05$ (not significant), $p < 0.05$, $p < 0.01$, $p < 0.001$ or $p < 0.0001$. Statistical analysis was performed with GraphPad Prism 6.05, while other calculations were made by Microsoft Office Excel 2013.

3.7 Transport study

Caco-2 cells ($2x10^5$) were seeded in 12-well Transwell inserts at a density of 2×10^5 cells per cm^{-2} and grown for 30-35 days. TEER was measured when the medium was changed every third day (Millicell-ERS volthommeter, Millipore, USA). The inserts were used for experiments when the TEER values reached $1000\Omega \times cm^2$. The culture medium was removed and the apical and basolateral compartments of the Caco-2 cell monolayers were washed with Hank's Balanced Salt Solution. In the experiments involving pre-treatment with calixarenes, the cells were exposed to calixarene solution dissolved in HBSS and applied in the apical chamber. HBSS solution (1.5mL) was placed in the basal chamber. The pre-treatment was carried out for 30min at 37°C. After treatment cells were washed with HBSS solution and then Lucifer Yellow dye was applied at 40μg/mL concentration diluted with HBSS into the apical chamber and HBSS into the basal chamber. In each case, samples were regularly taken from the basal chamber and were replaced with fresh HBSS. Samples were collected in 96 well black plate and fluorescence was measured at an excitation wavelength of 450nm by FLUOstar OPTIMA Microplate Reader. The values of TEER was measured before and after the experiment.

3.8 Synthesis

Our aim was to investigate the effect of well characterized structural changes on the toxic behavior, we carried out individual primary and secondary face per-substitution, also complete per-substitution. The synthetized derivatives were alkyl ethers bar none. Regarding the substituent types methyl-, ethyl-, propyl- and butyl chains were introduced. Due to the low yield of per-6 and complete per-substitution, only the di-substituted derivatives were tested *in vitro*, although, the increasing length of the alkyl chains at the secondary phase caused decreasing aqueous solubility. Further modifications were performed on the free OH-groups at the C-6 position. Namely, negatively charged groups were substituted to increase the hydrophilicity. The presence of a sulfate or sulfopropyl group has significantly increased their solubility. Sulfopropylation had a main drawback, namely the outstandingly difficult purification procedure.

3.9 Contributions

RT-CES experiments were performed with the help of Dr. Alexandra Bocsik and were observed by Dr. Mária Deli. The comparison of the toxicity of RAMEA and RAMEA-phosphatidylcholine complex was done with Dr. Zoltán Ujhelyi. Dr. Rudolf Gesztelyi did the

statistical analysis. Dr. Ildikó Bácskay observed and helped in the evaluation of biocompatibility assays. Dr. Florent Perret monitored the evaluation of the NMR spectra. Prof. Anthony W. Coleman evaluated the results of transport study on calixarenes.

The rest of the experimental work including cell viability assays, hemolysis tests, transport study, and α-CD synthesis have been done by the author.

4. New scientific results of the thesis

4.1 α-cyclodextrins

Our curiosity was awakened by the growing interest in α-CD utilization, due to its favourable effect on blood lipids and weight loss. On the other hand, as similar chemical modifications have been done on α- and β-CDs, we presumed that the comparison of their *in vitro* behavior may provide indispensable information on structure-activity correlations. Our work provided information on three levels:

- effect of certain substituents on the biocompatibility, compared to the parent molecule,
- understanding the influence of the number of building units on the toxic profile,
- evaluation of each derivative's effects in three different systems.

In vitro tests were chosen by the endeavour to model the *per os* and intravenous administration routes. As well known, both orally or *i.v.* administered drugs must comply with the crucial criteria of good aqueous solubility. When it can only be provided in the presence of excipients (e.g. CDs), the throughout evaluation of their behavior in a biological system is essential. It has to be clarified, whether the CD has a disadvantageous effect on the endothelium, thus the enhanced absorption only results from the disruption of the barrier, or we face indeed an advantageous bioavailability enhancer effect. Furthermore, when they are administered intravenously, the possibility of hemolysis must be excluded. Our work targets the limitation of adverse effects in human, by a reliable guide for the determination of a safe concentration range for those α-CDs, which have a potential for future pharmaceutical applications.

Tests were performed on Caco-2 cells and on human erythrocytes. IC_{50} values were determined by both MTT assay and RT-CES method, and HC_{50} by hemolysis test. MTT assay is based on the enzymatic conversion of MTT dye in the mitochondria, and detects early cytotoxicity compared to the two other methods. Hemolysis test was a simple and rapid investigation process to range the CDs according to their hemolytic activity. Both methods are so called end-point tests, in the sense that they visualize if cell death/lysis befell, but do not provide kinetic data. RT-CES does not only support the determination of toxic concentrations, but as the assay is performed for the whole length of the experiment, it characterizes the kinetic factors of toxic processes. The resulting data are biologically relevant, because the elimination of labels brings the cells closer to the physiological conditions. RT-CES provides reliable and dynamic cytotoxic parameters. This method was the most sensitive technique in our study.

The influence of the α-CD derivatives on Caco-2 cell viability, and their hemolytic evaluation led us to four general conclusions, as follows:

1. **Chemical modification on the free hydroxyl groups have a definite impact on toxicity.** In each case, a structural modification resulted change in both the IC_{50} and HC_{50} values, compared to the parent molecule. Both methods performed on Caco-2 cells indicate, that the presence of three methyl groups per glucopyranose residue (TRIMEA), phosphate groups (phosphated ACD) or succinyl groups (SuACD) resulted in lower IC_{50} values, thus increased the toxicity. On the other hand, hydroxypropylation (HPACD) and acetylation (AcACD) caused such a reduction, that their IC_{50} values could have not been determined up to 100mM.

2. **Toxicity depends on the number of building units.** The IC_{50} and HC_{50} value of those α- and β-CD derivatives, which have undergone the same chemical modifications, were correlated to their parent molecules, and then compared. We have discovered that a certain substitution pattern may have contrary effect, meanwhile others shifted the toxic profile similarly. For example, hydroxypropylation decreases the toxicity: neither HPACD, nor HPBCD has a ponderable IC_{50} value up to 100mM and 200mM, respectively. Although, HPBCD is hemolytic from 57mM, but HPACD is safe to use up to 100mM. On the other hand, TRIMEA proved to have more severe cytotoxic and hemolytic effect than RAMEA, while RAMEB has a lower IC_{50} and HC_{50} concentrations, than TRIMEB.

3. **The rate of toxicity depends on the exposition time.** In general, it can be stated that longer exposition cause lower IC_{50} values, thus more severe toxic effect. Apart from HPACD and AcACD, which were non-toxic in all systems, lower IC_{50} concentrations were determined by RT-CES, than by MTT assay. The main difference is the prolonged exposition time, and the permanent contact between the cells and CDs along the RT-CES experiment. For instance, RAMEA showed toxic effect from 78mM with MTT test, but with RT-CES from 25mM. In case of sulfated ACD, no IC_{50} was defined by MTT, but by RT-CES it was determined from 10mM.

4. **The intensity of CD cytotoxicity varies on different cell types**. The rate of increase/decrease in the toxic effect of most derivatives, compared to the parent molecule, differs on Caco-2 cells and on RBCs. This difference is due to the distinct membrane composition of the two cell types. HPACD and AcACD are the exceptions, regarding their non-toxic behaviour in each case. However, while phosphated ACD proved to be toxic on

Caco-2 cells, it did not have effect on RBCs. RAMEA had relatively high IC_{50} value, but its hemolytic effect occurred already at 15mM. It is true in general, that HC_{50} values are lower than IC_{50} concentrations, which may indicate that α-CD derivatives have higher affinity to the erythrocyte membrane than to the intestinal cell membranes. Matilainen *et al.* have reported that hydroxypropylated CDs and native γ-CD seemed to be the safest on lung cells. Kiss *et al.* found the following order regarding the cytotoxicity of β-CDs on Caco-2 cells: RAMEB > TRIMEB > DIMEB > PARMEB > HPBCD ~ CMBCD. Interestingly, the cytotoxicity of α-CD towards human corneal epithelial cells was even greater than of DIMEB, to which a relatively low IC_{50} value belongs. These results all confirm that the intensity of cytotoxicity depends on the cell, thus membrane types. As every cell type has a unique membrane structure, a certain CD derivative disrupts their integrity in different rates.

Accurate structure-activity correlations were found when CD derivatives with slight structural differences were compared by MTT assay. Namely, those of the methylated CDs which possess a distinct number of substituents, showed significant differences in cytotoxicity. Increasing number of methyl groups resulted lower IC_{50} values, thus elevating toxicity. When the hydroxyl groups at each position were modified, thus no free hydroxyls were left at the CD ring (TRIMEA), IC_{50} was determined at a rather low concentration (1.8mM). DIMEA, RAMEA and PARMEA possessing 12, ~11 and ~9 methyl groups, respectively, proved to have toxic effect at elevating concentrations. These observations led to two main conclusions. Firstly, increasing number of methyl groups presented on the α-CD ring has no beneficial effect on CD toxicity. While DIMEA had similar effect to the parent molecule, only RAMEA and PARMEA reduced the cytotoxicity of the native α-CD. Thus, to improve the toxic profile of native α-CD, maximum 11 hydroxyl groups shall be modified, at least by methylation. This consideration drove to the second statement, which confirms the necessity of free hydroxyl groups for the amelioration of the cytotoxic profile.

Hexakis(2,3-di-O-methyl-6-O-sulfate)-α-CD (**12**) and hexakis(2,3-di-O-ethyl-6-O-sulfate)-α-CD (**13**) differ in the length of the alkyl group at positions C-2 and C-3. **12** have 12 methyl, **13** has 12 ethyl groups at the secondary face. The DS for the sulfate substituent is 6 in case of **12**, and slightly less, 5.5 for **13**. Compared to the parent molecule (DIMEA), the cytotoxicity of **12** is increased. It can be explained by the lack of free hydroxyl groups, also by the effect of sulfate groups. It has to be noted, that the parent molecule of **13** could not be tested due to the insolubility of hexakis(2,3-di-O-ethyl)-αCD in aqueous environment. Compound **13** had more

satisfactory toxic profile, as it has a higher IC_{50} value. This effect can be the result of the two excess methylene group per glucopyranose unit. However, as the DS for sulfate was not exactly 6 but lower, it implies the presence of a few free hydroxyl groups at the primary face; and as it was explained above, the presence of free hydroxyl groups has a highly beneficial vital effect *in vitro*.

Significant correlation was observed between the cytotoxicity determined by MTT on Caco-2 cells, the hemolytic activity and the cholesterol complexation capacity of various β-CDs. β-CDs can extract cholesterol from the lipid rafts and RAMEB can enter the intestinal epithelial cells by endocytosis. The mechanism of α-CD cytotoxicity is different, because its cavity is too small to include a cholesterol molecule. The acyl chain of phospholipids, however, fits well within the α-CD cavity. In our indirect verification, the RAMEA-phosphatidylcholine complex did not result in toxicity on Caco-2 cells, compared to the unloaded RAMEA. It proves, that toxic effect does not occur when the CD cavity is in complex. However, phosphatidylcholine extraction alone does not explain the toxic behavior. Phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin also shows affinity for α-CDs. Phospholipids are present in the external and internal layers of RBC membrane in different ratios. This explains the concentration dependent hemolytic effect of α-CDs. The most severe hemolytic activity was shown by TRIMEA and SuACD, in accordance with the MTT results. HPACD and AcACD had diminished hemolytic activity (HC_{50}>100mM). Our results are in correlation with the findings of Leroy-Lechat *et al.*, namely that hydroxypropylation lowers the hemolytic activity in case of α-, β- and also γ-CD. All authors, including us, agree with Irie *et al.* and Bost *et al.*: the complexation of the membrane components induces the lysis of the erythrocytes or the irreversible damage of Caco-2 cells.

Synthetic work

The fact has been accepted, that most of the commercially available CD derivatives are mixtures. The DS value gives useful information about the approximate structure, however one batch is composed of molecules with different substitution pattern. The importance of the number of substituents has been already shown by Irie and Uekama. They reported the different hemolytic activity of the methylated and hydroxypropylated β-CDs, which differ in the DS. Mosher and Thompson have published the dramatic difference found between the hemolytic effect of SBEBCDs, having 1, 4 or 7 substituents per CD ring. SBE1BCD already induced hemolysis at higher concentration than the parent molecule, SBE4BCD had significantly milder, while SBE7BCD practically had no hemolytic effect.

Our aim was to synthetize alkyl ether α-CD derivatives, which differ in the position and the length of the alkyl substituents. Modifications were made on the primary, secondary or both faces, solely focused on per-substitutions. The introduced alkyl chains consisted of one (methyl) to four (butyl) carbon atoms. In case of primary and secondary face per-substitutions, the 'long' reaction way was used, thus protective groups were used to avoid the over-substitution. Complete per-modification had been facilitated by using the 'sledgehammer' way, thus when reactants were added in high equivalents, and the substitution at each position were indiscriminate.

Secondary face substitutions resulted derivatives with twelve methyl, ethyl, propyl or butyl groups at the C-2 and C-3 positions. Despite of the presence of six free hydroxyls at the primary face, the aqueous solubility had been significantly decreased as the number of methylene groups increased within the side chains. Only the methyl substitution resulted a water soluble product. The other di-alkyl derivatives could have been dissolved only in the presence of an organic co-solvent, which hamstrung the *in vitro* evaluations. In order to overcome the problem of solubility, further structural changes were carried out. The primary hydroxyls were modified with hydrophile anionic substituents, namely sulfate or sulfopropyl groups. Both type of changes resulted in high aqueous solubility. The DS values related to the sulfate groups were significantly higher (6-5.1) than those of related to the sulfopropyl groups (4.6-2.9). Among sulfate derivatives, complete substitution could have been achieved on the methylated CD, but the DS of sulfates decreased as the alkyl chain lengthened. Sulfopropylation has put two obstacles in the way. Firstly, the DS values turned to be relatively low. Secondly, the purification procedure became painstaking, despite of the application of several techniques. The origin of this problem was the probable inclusion of sulfopropyl derived salts within the CD cavity.

Primary face alkylation has combined five reaction steps, which have the benefit of excluding alkylation at the secondary phase. Even though the individual steps can give a high yield, the main drawback of this synthesis was the really small overall yield, which hampered the further evaluations. Complete per-substitution provided similar challenges. The 'sledgehammer' way resulted in a mixture of derivatives bearing 1-18 alkyl groups. Due to the slight physicochemical differences between them, their chromatographic separation was particularly cumbersome, and the separated final product had often very small yield. Moreover, complete per-alkylation resulted products with poor water solubility, due to the lack of free hydroxyl groups.

4.2 Calixarenes

The seemingly beneficial sulfonation of CDs (regarding both solubility and toxicological profile) drove us to the similarly modified representatives of calixarenes. The focus of our attention was on the evaluation of their effects in Caco-2 cell systems.

It became evident quite soon, that the unique structure is not the only advantage of calix[n]arenes, but they deliver additional benefits regarding their versatile biological activity (e.g. antituberculous, antibiotic, antiviral, anti-thrombotic activity, etc.) Another improvement compared to CDs is their negligible hemolytic effect. For all CnS derivatives which were tested by *Coleman et al*., maximum 5% of hemolysis was observed up to 50mM. The toxicological profile of various calix[n]arenes in different *in vitro* systems has already been reported, and the results are unambiguously promising. Their mutagenic effect was excluded by Ames test, they did not affect the growth of human fibroblasts, neither activated neutrophil granulocytes.

However, to our best knowledge, *para*-sulphonato-calix[n]arenes have been scarcely investigated from the point of view of their effect on paracellular absorption from the small intestine. We found it crucial to establish these effects, as it represents an important step in their future application as transporter systems. However, calix[n]arenes are not yet approved by the FDA for pharmaceutical use, but their potential has already been demonstrated.

Our aim was to evaluate the effects of three calix[n]arene sulfonates (n=4, 6, 8) on paracellular absorption. In the first place, the IC_{50} concentration was defined for all derivatives on Caco-2 cells by MTT assay. Surprisingly, in each case, IC_{50} concentrations were quite low, thus their negative effect on cell viability had an early occurrence.

In the transport study, LY was used as the paracellular marker. The effect of *para*-sulphonato-calix[n]arenes were tested in two different experiments. First, the cell monolayer was pre-treated with the CnSs and the LY absorption was measured after. In the second case, CnS and LY were applied simultaneously. C6S did not enhance the paracellular transport of LY neither with, nor without pre-treatment. Interestingly, the behavior of C4S and C8S were similar: no passage was induced by pre-treatment, but when they were combined with LY, there was a detectable enhancement of LY transport.

In case of each pre-treatment, the TEER value remained above $1000\Omega xcm^2$ with no significant change compared to the starting value. Simultaneous application of the used materials manifested in serious TEER reduction in each case.

The difference between C4S and C8S on one hand and C6S on the other is of interest, and suggests that there may be a different mode of action with Caco-2 confluent monolayers, which is in agreement with a difference in the effect on TEER of C4S and C8S compared to the effect of C6S. Of particular interest is the requirement for the combined use of the *para*-sulphonato-calix[n]arenes and LY to induce transport. These differences have provided us a guidance for further evaluations, which aims the better understanding of the underlying mechanism.

4.3 Novelty and practical relevance of the work

Cyclodextrins

- Chemical modifications on the α-CD ring may reduce (e.g. hydroxypropylation, acetylation, methylation) or enhance (e.g. phosphorylation, succinylation) the toxic behaviour compared to the native parent molecule.
- Similar structural changes (e.g. methylation) may result in contrary effects on α- and β-CD rings.
- Methylation decreases the toxicity depending on the number of substituent groups, but complete per-methylation increases the cytotoxicity.
- The presence of free hydroxyl groups is possibly required for the elaboration of a safe toxic profile.
- α-CDs cause hemolysis on RBCs at lower concentrations, while their undesirable effect on Caco-2 cells arise only at higher concentrations.
- RT-CES showed that α-CD toxicity increases with the exposition time.
- Both hydroxypropylation and acetylation result α-CD derivatives with reduced toxicity, which could be possibly applied even in parenteral formulations.

Calixarenes

- Sulfonation of calix[n]arenes does not only result in better bioavailability, but seems to enhance paracellular absorption.
- *Para*-sulphonato-calix[n]arenes (n=4,6 or 8) have no effect on paracellular absorption when the cell monolayer is treated with calixarenes prior to the addition of the marker molecule.
- C4S and C8S increase the paracellular absorption of Lucifer Yellow when they simultaneously present with the marker molecule.
- C6S did not affect the paracellular absorption in neither case.
- The difference in paracellular absorption enhancement suggests distinct mechanism of calixarenes depending on the ring size.

5. Summary

The low solubility of drug candidates cause a major problem in pharmaceutical formulations, as the aqueous solubility is an indispensable criterion for appropriate bioavailability. Macrocyclic compounds possess a relatively hydrophobic cavity, which is suitable for guest molecule inclusion. Cyclodextrins and calixarenes are widely studied organic host-compounds, and CDs have already been used as pharmaceutical excipients for solubility enhancement. The macrocycles' chemical structure allows their versatile modification, which eventuates changes not only in physicochemical characteristics, but in their effects on living organisms, as well. Thus, the biocompatibility evaluation of the derivatives is fundamental.

Owing to the already performed assessment of numerous β-CD derivatives' biocompatibility, the aim of this research was to extend these experiments to commercially available α-CDs. They have been used less frequently, however several derivatives, which have not been tested yet *in vitro,* have the possibility of future pharmaceutical use. Their importance is also certified by their benefits in nanoparticle formation. We have been interested in concrete structure-toxicity correlations, thus alkyl ether α-CD derivatives were synthetized bearing increasing length alkyl chains, in different positions. *Para*-sulphonato-calix[n]-arenes have already been widely examined due to their efficient drug complexation and versatile biological activity, however, their effects on paracellular transport mechanism have not been evaluated until now.

The cell viability and hemolysis tests have allowed us to rank the α-CDs and to choose the safest derivatives, also to compare their toxic effects in different systems. The comparison of α- and β-CDs bearing the same chemical modifications highlighted the importance of the number of building units. Important information has been evaluated regarding the connection between the cytotoxic effect and the number of free hydroxyl groups. Derivatives with long alkyl chains possess low solubility, which led us towards further chemical modifications. Sulfonation seemed to have beneficial impact on the biocompatibility. Sulfonation also improved the solubility of calixarenes. C4S and C8S proved their positive effect on paracellular absorption in a non-toxic concentration range, however C6S had no similar effect, thus their behavior in *in vitro* absorption model system arose forward-looking questions.

Our research concludes, that the structural changes on the macrocyclic rings may have major impact on the biocompatibility. As the modification possibilities are practically unlimited, the evaluation of structure and activity cannot be avoided, facilitating the safest choice for further pharmaceutical use.

6. Résumé

La faible solubilité de certains médicaments cause des problèmes majeurs dans les formulations pharmaceutiques, puisque la solubilité dans l'eau est un critère indispensable pour la biodisponibilité. Les composés macrocycliques tels que les CDs et les calixarènes ont une cavité relativement hydrophobe, leur permettant ainsi d'encapsuler de nombreuses molécules. Les CDs ont déjà été utilisées comme excipients pharmaceutiques pour l'amélioration de la solubilité. La structure de ces macrocycles permet d'effectuer de nombreuses modifications, qui causent des changements tant au niveau de leurs caractéristiques physico-chimiques que sur leurs effets sur les organismes vivants. Ainsi, l'évaluation de la biocompatibilité de ces dérivés est primordiale en vue de leur utilisation en pharmacie. Puisque l'étude de la biocompatibilité de plusieurs dérivés de β-CD a déjà été étudiée, l'objectif de cette recherche était d'étendre ces expériences à des dérivés de l'α-CD qui sont disponibles dans le commerce. Nous nous sommes intéressés aux relations entre structure et toxicité. Ainsi les dérivés alkyl éther d'α-CD, avec des chaînes alkyle de longueur croissante et substitués sur différentes positions, ont été synthétisés et leur toxicité étudiée.

Les *para*-sulphonato-calix[n]-arènes quant à eux, ont souvent été étudiés et ont montré une forte capacité à complexer de nombreux médicaments. Ils ont aussi démontré une activité biologique polyvalente. Néanmoins, leurs effets sur le mécanisme de transport paracellulaire n'a jamais été évaluée.

Les tests de viabilité cellulaire et d'hémolyse nous ont permis d'une part de classer les α-CDs et de choisir les dérivés les plus sûrs, et d'autre part de comparer leur effets toxiques dans des systèmes différents. La comparaison des α- et ß-CDs portant les mêmes modifications chimiques nous a montré l'importance du nombre d'unités de construction. Le rapport entre l'effet cytotoxique et le nombre de groupes hydroxyles libres est également très important. Les dérivés portant de longues chaînes alkyles possèdent une faible solubilité, ce qui nous a conduits vers d'autres modifications chimiques : la sulfonation de ces derniers dérivés semble avoir un impact bénéfique sur la biocompatibilité de CDs. Elle a aussi amélioré la solubilité des calixarènes. Les calix[4] et [8]arène sulphonates ont prouvé leur effet positif sur l'absorption paracellulaire, tandis que le calix[6]arène sulphonate n'a pas eu d'effet similaire.

Notre recherche conclut que les changements structurels sur les anneaux macrocycliques peuvent avoir un impact majeur sur la biocompatibilité. Comme les possibilités de modification sont pratiquement illimitées, l'évaluation de la structure et de l'activité est indispensable pour faciliter les choix les plus sûrs dans les applications pharmaceutiques à venir.

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8. Publications

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Subject: PhD Publikációs Lista

Candidate: Eszter Róka
Neptun ID: VRH6VE
Doctoral School: Doctoral School of Pharmacy
MTMT ID: 10052077

List of publications related to the dissertation

1. **Róka, E.**, Ujhelyi, Z., Deli, M. A., Bocsik, A., Fenyvesi, É., Szente, L., Fenyvesi, F., Vecsernyés, M., Váradi, J., Fehér, P., Gesztelyi, R., Félix, C., Perret, F., Bácskay, I.: Evaluation of the Cytotoxicity of [alfa]-Cyclodextrin Derivatives on the Caco-2 Cell Line and Human Erythrocytes.
   Molecules. *20*, 20269-20285, 2015.
   DOI: http://dx.doi.org/10.3390/molecules201119694
   IF: 2.465
2. **Róka, E.**, Vecsernyés, M., Bácskay, I., Félix, C., Rhimi, M., Coleman, A. W., Perret, F.: para-Sulphonato-calix[n]arenes as selective activators for the passage of molecules across the Caco-2 model intestinal membrane.
   Chem. Commun. *51*, 9374-9376, 2015.
   DOI: http://dx.doi.org/10.1039/C5CC01777G
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List of other publications

3. Fehér, P., Ujhelyi, Z., Váradi, J., Fenyvesi, F., **Róka, E.**, Juhász, B., Varga, B., Bombicz, M., Priksz, D., Bácskay, I., Vecsernyés, M.: Efficacy of Pre- and Post-Treatment by Topical Formulations Containing Dissolved and Suspended Silybum marianum against UVB-Induced Oxidative Stress in Guinea Pig and on HaCaT Keratinocytes.
   Molecules. 21 (10), 1269, 2016.
   DOI: http://dx.doi.org/10.3390/molecules21101269
   IF: 2.465 (2015)

4. Réti-Nagy, K., Malanga, M., Fenyvesi, É., Szente, L., Vámosi, G., Váradi, J., Bácskay, I., Fehér, P., Ujhelyi, Z., **Róka, E.**, Vecsernyés, M., Balogh, G., Vasvári, G., Fenyvesi, F.: Endocytosis of fluorescent cyclodextrins by intestinal Caco-2 cells and its role in paclitaxel drug delivery.
   Int. J. Pharm. 496, 509-517, 2015.
   DOI: http://dx.doi.org/10.1016/j.ijpharm.2015.10.049
   IF: 3.994

5. Ujhelyi, Z., Kalantari, A., Vecsernyés, M., **Róka, E.**, Fenyvesi, F., Póka, R., Kozma, B., Bácskay, I.: The Enhanced Inhibitory Effect of Different Antitumor Agents in Self-Microemulsifying Drug Delivery Systems on Human Cervical Cancer HeLa Cells.
   Molecules. 20, 13226-13239, 2015.
   DOI: http://dx.doi.org/10.3390/molecules200713226
   IF: 2.465

6. Ujhelyi, Z., **Róka, E.**, Fenyvesi, F., Fehér, P., Váradi, J., Réti-Nagy, K., Vecsernyés, M., Bácskay, I.: Assessment of the hemolytic activity and cytotoxicity of different PEG-based solubilizing agents.
   Pharmazie. 68, 383-384, 2013.
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