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The *in vitro* investigaiton of the effects of the new-generation β-cyclodextrins on the cell membrane and on the transport of the BCS IV. class taxol

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DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

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The Examination takes place at Department of Pharmacology, Medical and Health Science Center, University of Debrecen, 2011.10.25. 11:00.

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INTRODUCTION

Drug administration can be performed in different ways. The most preferred one is the oral administration, when the active agent is absorbed from the gastrointestinal tract. For the total absorption numerous physicochemical properties of the active agent and dosage form should be proper. The solubility and the permeability of the drug across the biological membranes will determine the dosage form and the auxiliary materials which we should apply.

A biopharmaceutic classification system (BCS) for correlating *in vitro* drug product dissolution and *in vivo* bioavailability is based on recognizing that drug dissolution and gastrointestinal permeability are the fundamental parameters controlling rate and extent of drug absorption. These Biopharmaceutic Drug Classes are defined as:

- Class I High solubility-high permeability drugs: They can be easily dissolved in gut medium, and absorbed by epithelial cells into the blood circulation.
- Class II Low solubility-high permeability drugs: Although the drugs have good permeability across the membrane, they possess low bioavailability because of their low solubility.
- Class III High solubility-low permeability drugs: These active agents' bioavailability mostly depends on their transport speed across the cell membrane.
- Class IV Low solubility-low permeability drugs: Oral application of these drugs can be carried out with special auxiliary materials.

The bioavailability of Class II., III. and IV. drugs increases by amendment of solubility and permeability properties. In the interest of bioavailability enhancement various chemical and physico-chemical changes take place. In addition, special auxiliary materials can significantly modulate biopharmaceutical properties with such as absorption-enhancing materials,

efflux inhibitors larger quantities of active substances can penetrate the membrane of intestinal epithelial cells, the permeability increases.

Oral formulation of Class IV. drugs is enormous challenge. The taxol, an anticancer drug belongs to Class IV., and the oral formulation of taxol is in the focus of scientific interest.

One possibility of solubility enhancement is the molecular complexation. The cyclodextrins (CD) as ideal inclusion complex forming materials are able to hold small molecules with low water solubility in solution by, forming molecular complexes. However, we suppose that they also have membrane modulating effects, which can explain the increased permeability of the active agents.

Cyclodextrins

The product of photosynthesis is the starch. The building units of the two main components of starch (amylose and amylopectin) are D-glycopyranoside units. Several thousand glucopyranose units are linked to form these macrmolecules. There are specific enzymes as cyclodextrin-glycosyl-transferase, which not only degrade the macromolecules to smaller units, dextrins, but simultaneously have the two ends of dextrin molecules reacted forming cyclic dextrins, called cyclodextrins (CD). Three basic CDs are formed in significant amounts: α-CD, β-CD, γ-CD. Due to their steric structures these are torus-shaped and resemble conical cylinders, with internal axial cavity. The outer surface of the molecules is hydrophilic, while the internal cavity is lipophilic. The CDs can be considered as empty cylindrical (open both ends) molecular size capsules. In an aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecules which are energetically unfavoured (polar-apolar interaction), and therefore can be readily substituted by appropriate “guest” molecules, which are less polar than water. One, two or three CD molecules contain one or more entrapped “guest” molecules. The most frequent

host:guest ratio is 1:1. This is the essence of the molecular encapsulation. The active agent complexes formed with CDs occur in several dosage forms, for example oral, parenteral, nasal, transdermal, ophthalmological, rectal, pulmonary dosage forms. Besides CDs are widely used, their toxicological properties should be mentioned. The most important toxic effect is the hemolytic activity that shows diversity. The difference between the hemolytic activities is caused by the different solubilizing effect on the membrane components. The CDs remove mainly cholesterol and phospholipids, especially phosphatidylcholine and sphingomyelin, from the outer half of the membrane bilayer, leading to imbalance of the bilayer. In aspect of pharmaceutical technology β-CD derivatives have got the greatest importance. Various β-CD derivatives have been synthesized and tested in order to improve their physicochemical properties, inclusion capacities, as well as to decrease their toxicity.

Taxol and CDs

Taxol (paclitaxel) has a significant importance in the treatment of various solid tumors. Oral administration is hampered because:

- Water solubility of taxol, which belongs to Class IV of the BCS, is very low.
- Taxol is a well-known P-glycoprotein (Pgp) substrate. Network of MDR-ABC transporters (multi drug resistance – ATP binding casette) form a "chemoimmunity" system, which protects the body against the accumulation of foreign chemical agents. These transporters selectively modulate their substrates' entry and exit through the cell membrane. One of the best known MDR-ABC transporters is Pgp /MDR1 (ABCB1).
- Taxol has a good affinity for intestinal and liver cytochrom P450 (CYP 450) metabolic enzymes. Pre-systemic metabolism causes inactive hydroxy-, and dihydroxypaclitaxel metabolites.

In this view the improvement of oral bioavailability of taxol (1-2%) can be worked out in three ways. An ideal auxiliary material or delivery system is able to increase the solubility, and inhibit Pgp and possibly CYP 450. Moreover we can take account of the modulation of tight junctions (TJ) and the lipid content of the membrane. TJ modulation may cause paracellullar and lipid content modulation of the membrane may cause transcellular transport increase.

CDs can also be useful tools to enhance solubility and efficacy of poorly soluble drugs, such as taxol. The highest level of complex formation with paclitaxel in phosphate-buffered saline (PBS) was found using methylated-β-cyclodextrins, including 2,6-di-O-methyl-β-cyclodextrin (Dimeb) and randomly methylated β-cyclodextrin (Rameb), with approximately 90% inclusion efficiency. Besides the solubility enchancer effect, growing body of evidences shows that appreciably interact with the cell membrane and the Pgp. By these information, it has been suggested, that β-CDs could be useful in the oral formulation of taxol. But not enough information has been known so far about the toxicity of new β-CDs and their effect on the cell membrane.

Cell culture models, Caco-2

Testing the potential drugs on cells, like Hela (human cervix carcinoma cell line) or Caco 2 (human colon carcinoma cell line) is a very important part of the early pharmaceutical development process. By these tissue culture systems the pharmacokinetic properties and toxicity of drugs and auxiliary materials can be predicted. The Caco-2 *in vitro* transport model is currently the best characterized and most commonly used cell system for gastrointestinal absorption studies. Caco-2 cells possess all of the criterias that are required to model the intestinal drug absorption. Polarized enterocytes form confluent monolayer with tight junctions between the cells, and the monolayer can be used to study drug transport by all routes. The apical surface of the differentiated cells is covered

by intact microvilli, and in their membrane there are active transporters and enzymes as same as in human gut epithelium.

AIMS OF THE STUDY

The increase of drug permeability especially in case of Class IV. drugs is a really important task. By the *in vitro* investigation of pharmacon absorption those factors can be determined, of which modulation the biopharmateutical properties can be positively changed. It is crucial to know and understand those processes, by which a larger amount of drug can penetrate through the membrane of the gastrointestinal epithel cells, meaning the fact that the drug has increased permeability through the gut epithelium. As well the properties of the applied excipients are tremendously affecting the entrance way of the drug and the execution of the dosage form. Therefore it is important to know the effect of excipients on the absorption and their possible toxic properties.

While planning our experiments these aspects were considered and the following aims were settled:

1. Installation of the Caco-2 cell *in vitro* transport model and its characterization with TEER measurements and also with known permeability drugs.
2. Citotoxicity and hemolititic activity investigation of the new-generation methylated-β-CDs.
3. Exploring the coherences between the structure, the cholesterol solubilizing capacity, the citotoxicity and the hemolytic activity of the investigated CDs.
4. Investigating the effects of best-choice CDs based on citotoxicity and cholesterol solubilizing capacity on the transport of taxol in the Caco-2 transport model.
5. Investigating the effects of chosen CDs on the active efflux of taxol and on the barrier functions.

MATERIALS AND METHODS

Cyclodextrins

Rameb and Crysmeb (methylated-β-cyclodextrin) were purchased from Wacker Chemie (Germany) and Roquette Fréres (France), respectively. The other CD derivatives, cholesterol/Dimeb and cholesterol/Rameb complexes with 4.63(m/m)% and 4.82(m/m)% cholesterol content, respectively, were the products of Cyclolab Cyclodextrin R&D Laboratory Ltd. (Hungary)

Cell culture

Hela cell line

Hela cells were obtained from the European Collection of Cell Cultures (ECACC) and maintained in Dulbecco's Modified Eagel's medium (DMEM Sigma-Aldrich, Hungary), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, Hungary),2 mM L-glutamine (Sigma–Aldrich, Hungary) and 100 mg/l gentamycin (Sigma-Aldrich, Hungary) in 5% CO_2 at 37 °C.

Caco-2 cell line

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, UK). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich Ltd., Hungary) supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich Ltd., Hungary), 1% non-essential amino acid (Sigma–Aldrich Ltd., Hungary) and 100 mg/l gentamycin at 37 °C in an incubator containing 5% CO_2. The passage number of the cells was between 25 and 42.

Cholesterol solubilization by CDs

CD derivatives at a concentration of 0.04 M were dissolved in distilled water and excess amount of cholesterol was added. The suspensions were mixed at room temperature for 12 h. After the incubation, the suspensions were filtered,

and cholesterol contents of the filtrates were determined by HPLC using Nucleosil 120-5, C18 4 mm×100 mm (Macherey Nagel) column at 40 °C, UV detection at 210 nm, and acetonitrile:2-propanol (3:1) as eluent. The experimental error of the measurements did not exceed 10%.

MTT assay

Hela cells

The cytotoxic effect of CDs was evaluated by a colorimetric cytotoxicity method i.e. the MTT test. The test was performed as follows: Hela cells in complete medium were seeded to 24-well plate at a final density of 8×10^4 cells/well. After 2–3 days the medium was removed, the cells were washed with isotonic phosphate buffered saline (PBS) and the CD test solution was added. The cells were then incubated for 30 min at 37 °C in a 5% CO_2-air incubator. After incubation, the samples were removed, and the cells were washed twice with 1 ml PBS. At the end, 0.9 ml medium and 100 ml MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] solution (5 mg/ml) (Sigma–Aldrich, Hungary) were added to each well. The plates were further incubated for 4 h, the MTT solution was removed and 2 ml of DMSO were added to dissolve the formed formazan crystals. The absorbance of each sample was recorded at 570 nm by a Shimadzu UV-1601 spectrophotometer. Data were expressed as the percentage of viable control cells calculated from the absorbance at 570 nm, corrected for background absorbance

Caco-2 cells

MTT method was used to determine the viability of Caco-2 cells. Cells were exposed to increasing concentrations of β-CDs dissolved in isotonic phosphate buffered saline (PBS) at 37 °C for 30 min. Control groups were processed equally and incubated without CDs simultaneously. After 30 min incubation cells were washed with PBS and the MTT solution was added to each well at a

final concentration of 0.5 mg/ml. After 3 h of incubation at 37 ◦C the derived formazan crystals were dissolved in 2-propanol/1N HCl (25:1) and the absorbance was measured at 570 nm with a FLUOstar OPTIMA microplate reader (BMG LABTECH, Germany). Absorbance values were corrected with background absorbance, measured at 690 nm. Cell viability was expressed as the percentage of the untreated control. From the cytotoxicity results a dose–response curve was determined, and the concentration that induced 50% inactivation of mitochondrial dehydrogenase enzyme (IC_{50}) was subsequently calculated.

Testing the hemolytic activity

Hemolysis measurements were performed by a self-developed method for setting complete dose–response curves for each CD and computing their HC_{50} values. Human blood was collected from healthy donors. Erythrocytes were separated from citrated blood by centrifugation at 2500×g for 10 min, washed three times with PBS and resuspended in the same solution. Aliquots of the cell suspension with the respective red blood cell number of 5×10^7 were added to the buffer solution (PBS pH 7.2) containing increasing concentrations of the CDs investigated in the study. After gently mixing each mixture was incubated at 37 °C for 10 min and followed by a rapid centrifugation at 5000×g. Finally, the absorbance of the hemoglobin released into the supernatant was measured at 540 nm with the use of a Hitachi 220A spectrophotometer and a 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. From the hemolysis results, a dose–response curve was determined, and the concentration that induces hemolysis 50% of the erythrocytes (HC_{50}) was subsequently calculated.

Caco-2 transport model characterization by transepithelial electrical resistance

For transport experiments, Caco-2 cells were seeded at density of 200,000 cells/well on Transwell (Corning Costar, USA) polycarbonate filters (pore size 0.4 μm, surface area 1.12 cm^2). Culture medium was replaced with fresh medium every 2 days in the inserts. Monolayers were used for the experiments between 20 and 30 days after seeding. The formation of functional epithelial layers was monitored by the development of transepithelial electrical resistance (TEER) and measured with a Millicell–ERS voltohmmeter (Millipore, Hungary). The measured TEER values should be corrected by the following equation:

$$TEER(Ohm\times cm^2)=[Measured\ value(Ohm)–value\ of\ empty\ insert(Ohm)]\times effective\ area(cm^2)$$

Inserts were used for experiments when TEER values were more than 1000 $Ohm\times cm^2$

Caco-2 transport model characterization by drugs with well known human absorption

For characterization we investigated permeability of drugs with well known human absorption through Caco-2 monolayer. The permeability studies were carried out as follows:

TEER values were measured before and after the experiments to check the integrity of monolayers. Before the experiments, monolayers were washed and incubated in HBSS (Hanks' balanced salt solution at 37°C for 30 min. The basal chamber contained the acceptor solution (HBSS). The donor solution containing 0.5 μCi/ml drug in HBSS was added into the apical chambers. Samples were taken from the basolateral side of the filters at 10. 30, 60, 120. min. The volume was replenished with HBSS, which was corrected during further calculations. In

the case of drugs transported with high speed, the samles were taken at 5., 10., 15., 90. minutes. The radioactivity of the samples was measured by liquid scintillation counter (Tri-Carb, PerkinElmer, USA). The apparent permeability coefficients (P_{app}) were calculated as

$$P_{app} = dQ/dt \times 1/(C_0\ A)$$

where dQ/dt is the permeability rate of taxol (dpm/s),
C_0 is the initial conc. of radiolabeled chemicals in the donor chamber (dpm/ml),
A is the surface area of the membrane (cm^2).

Transepithelial taxol transport experiments

Before the experiments, monolayers were washed and incubated in HBSS at 37 °C for 30 min. TEER values were measured before and after the experiments to check the integrity of monolayers. The permeability studies were carried out as follows.

Pretreatment with CDs, Protocol 1.

Monolayers were incubated apically with 20mM Rameb, 20mM MaRameb (methylated-6-monodeoxy-6-monoamino-β-cyclodextrin), and 20mM SuRameb (Succinylated methyl-β-cyclodextrin) at 37°C for 30 min, then the cells were washed twice with HBSS. After CD pretreatment, permeability of taxol was determined in apical to basolateral and basolateral to apical direction in different experiments. In apical to basolateral (A-B) permeability measurements, the donor solution containing 0.5 μCi/ml [^{3}H]taxol (Moravek Biochemicals USA) and 0.5 μCi/ml [^{14}C]mannitol (Amersham USA) in HBSS was added into the apical chambers. The specific activity of taxol was 36.0 Ci/mmol, and the concentration in the donor compartment was 11.9 ng/ml. In this experimental setting, the basolateral chambers contained only HBSS. Samples were taken

from the basolateral side of the filters at intervals of 15–30 min till the 90th min. The volume was replenished with HBSS, which was corrected during further calculations. In some experiments, [^{14}C]PEG-4000 (ARC, USA) was used as paracellular marker instead of [^{14}C]mannitol. In basolateral to apical (B-A) transport permeability measurements, the donor solution containing 0.5 μCi/ml [^{3}H]taxol and 0.5 μCi/ml [^{14}C]mannitol in HBSS was added into the basolateral chambers. The apical chambers contained the acceptor medium, HBSS. Samples were taken from the apical side of the filters at intervals of 15–30 min till the 90th minute. The volume was replenished with HBSS, which was corrected during further calculations.

Co-treatment with CDs

0.5 μCi/ml [^{3}H]taxol and 0.5 μCi/ml [^{14}C]mannitol were mixed in 20 mM Rameb, 20mM MaRameb, and 20mM SuRameb solutions and preincubated at 37°C for 30 min. In A-B permeability measurements, Caco-2 monolayers were treated apically with taxol–CD solutions for 90 min and samples were collected from the basolateral side at intervals of 15–30 min, thus CD treatment continued until the end of the experiments. In B–A permeability studies, 20 mM Rameb, 20mM MaRameb, and 20mM SuRameb solutions were added to the apical chambers, whereas 0.5 μCi/ml [^{3}H]taxol and 0.5 μCi/ml [^{14}C]mannitol were administered basolaterally. Samples were taken from the apical side and the volume was replenished with CD solutions. For Pgp inhibition, 10 μM cyclosporine A (CSA) was added to the apical chamber both in A-B and B-A studies. For all experiments, three inserts were used for each treatment group. The radioactivity of the samples was measured by liquid scintillation counter (Tri-Carb, PerkinElmer, USA). The apparent permeability coefficients (P_{app}) were calculated as in the previous experiment.

Immunohistochemistry

The CD treated and control Caco-2 monolayers on the membrane of the culture inserts were washed in PBS (pH 7.3) and fixed with 3% paraformaldehyde in PBS for 30 min. Cells were blocked with 3% bovine serum albumin and incubated with 5 μg/ml concentration of primary antibodies against ZO-1 (Invitrogen, Hungary), claudin-1 (Invitrogen, Hungary), and β-catenin (Sigma–Aldrich Hungary) in blocking buffer for 1 h. Incubation with secondary antibody Cy3-labeled anti-rabbit IgG (2 μg/ml) and bis-benzimide (10 μM) to stain cell nuclei (both from Sigma–Aldrich, Hungary) lasted for 1 h. Between incubations, cells were washed three times with PBS. Filter membranes with the stained cells were mounted in Gel Mount (Biomeda, USA) to glass slides and examined by a NikonEclipse TE2000 fluorescent microscope (Nikon, Japan), and photographed by a Spot RT digital camera (Diagnostic Instruments, USA).

Statistical analysis

Data have been analyzed using SigmaStat (version 3.1, SPSS Inc., USA) and are presented as means ±SD. Comparison of groups was performed by one-way analysis of variance, and followed by Holm-Sidak post-hoc test. Differences were considered significant at $p < 0.05$. Experiments were performed in triplicate and repeated at least twice.

RESULTS AND DISCUSSION

Solubilization of cholesterol

As the high affinity for cholesterol of methylated-β-CD molecules is well known, we studied the effect of the methyl substituents number on the cholesterol-solubilizing ability. By increasing the number of methyl groups in the CD molecule the cholesterol concentration in the aqueous CD solutions was enhanced reaching the maximum at around 14 methyl groups corresponding to Dimeb. The isomeric purity had hardly any effect on the cholesterol solubilization: 4.2, 3.57 and 3.65 mg/ml cholesterol concentration was measured in the 0.04 M solutions of Dimeb with 50, 80 and 95% isomeric purity, respectively. The values scatter within a 10% range around the average of 3.8 mg/ml. The studied non-methylated-CD derivatives, such as HPBCD (Hydroxypropylated-β-cyclodextrin), QABCD (Quaternary amino-β-cyclodextrin) and CMBCD (Carboxymethylated-β-cyclodextrin) having hydroxypropyl, quaternary amino or carboxymethyl groups on the CD molecule showed much lower solubilizing potential towards cholesterol: only 0.32, 0.05 and 0.055 mg/ml cholesterol concentrations were achieved, respectively. In order to decrease the high affinity of Rameb and Dimeb towards cholesterol some ionic groups were introduced into their structure. The obtained compounds, called “second generation β-CD derivatives”, contain either cationic (amino) or anionic (carboxymethyl, succinyl, sulfopropyl, etc.) groups in addition to the methyl substituents. Modification of Rameb with 1 or 2 ionic groups in a CD molecule resulted in reduced cholesterolsolubilizing effect compared to Rameb: 2.4 and 0.5 mg/ml cholesterol were dissolved in 0.04 M SuRameb and MaRameb solutions, respectively. On the other hand, modification of Dimeb with 2 or 3 ionic groups destroyed its high cholesterol-solubilizing potential: <0.1 mg/ml cholesterol could be dissolved in the solutions of SPDimeb (Sulfopropylated heptakis(2,6-di-O-methyl)-β-cyclodextrin), CMDimeb (Carboxymethylated-heptakis(2,6-di-O-methyl)-β-cyclodextrin

sodium) and CMHEDimeb (Carboxymethyl-hydroxyethylated-heptakis(2,6-di-O-methyl)-β-cyclodextrin).

Correlation among the structure, cholesterol complexation ability and cytotoxic attributes of β-CD molecules

First we tested the cytotoxicity of several β-CDs on Hela cells with using MTT assay. The resulting viability diagrams showed that there are significant differences between the cytotoxicity of the β-CD derivatives. The cytotoxicity was dependent on the number and the position of the methyl groups and ionic substituents could decrease the toxic effects as compared to the electroneutral methylated molecules.

In further study the toxic properties of β-CDs were investigated *in vitro*, on Caco-2 human colon carcinoma cells and in parallel on human erythrocytes. It is well known that β-CDs interact with cell membrane cholesterol; however this mechanism has not been fully investigated in the context of structure, cholesterol solubilization capacity and cytotoxicity. These properties are influenced by the number and position of methyl groups and introducing further ionic and non-ionic substituents the fine tuning of cyclodextrin ring can be achieved. To reveal the mechanism of CD's acute cytotoxic effects on epithelial cells we used a short experimental protocol. During the short CD cell treatment we should not consider the cell proliferation, the observed effects originate from the interaction between CDs and cell membrane, and thus the concentration-dependent cytotoxicity can be correlated with the cholesterol solubilization. Regarding to our observations major differences have been demonstrated among cytotoxic properties of various CD derivatives and some structure–activity relationship have been revealed. Increasing the number of methyl groups on the molecule enhanced its toxic effect: Crysmeb with three to four methyl groups/CD are practically non-toxic, while Trimeb Rameb and Dimeb are highly toxic on Caco-2 cells. Similar trend was observed on the hemolytic effect of

these derivatives. It can be concluded, that in the case of methylated-β-CD compounds the cell toxicity depends highly on the number and slightly on the position of the methyl groups. Ionic groups in the CD molecule can significantly decrease the cytotoxic effect, compared to the electroneutral methylated molecules. Regarding to the ionic substituents the introduction of negatively (carboxymethylated) or positively (quaternary amino) charged groups into the molecule hinder the development of the cytotoxicity in the concentration range examined in our study. A strong relationship between the *in vitro* hemolytic activity and the cholesterol-solubilizing ability of the CD derivatives was found by others.

The solubilization effect of the CDs is different from detergents, since CD ring form a new compartment in the aqueous phase and particularly the lipid components of cell membrane are reversibly extracted. The removal of the lipid components from the membrane (mainly cholesterol) can lead to the lysis of the cell. This hypothesis is strongly supported by our following experimental observations: (i) the cytotoxic properties of cholesterol complexes of the most "toxic" derivatives are significantly lower than the cytotoxic profile of the native compounds and (ii) significant correlation can be observed between the cytotoxicity, hemolytic activity and the cholesterol complexation capacity of various CD compounds.

Our data confirm the view that the cytotoxicity of various CD derivatives depends on their cholesterol extraction capacity from the cell membrane. Based on the above presented correlations we propose that cholesterol-solubilizing properties can be a predictive factor for β-CD cell toxicity which depends on the structure of the CD derivative, e.g. on the number and position of the methyl groups and the presence of ionic groups.

Caco-2 transport model and permeability studies

Another aim of our study was to investigate the effect of randomly methylated-β-CDs on taxol permeability, which has not been investigated yet. For modeling the human intestinal absorption we applied Caco-2 transport model which is an appropriate model for studying the different ways of absorption. During the characterization of the transport model, we followed up the development of Caco-2 monolayer with TEER measurements and permeability measurements of drugs, of which human absorption is well-known. Our results harmonized with the official P_{app} values of the investigated materials.

The Caco-2 intestinal absorption models widely used to characterize transcellular permeability of drugs, because both passive and active mechanisms can be investigated, Pgp substrates can be identified and the reasons of the insufficient absorption of active substances can be revealed. The failed absorption and inadequate distribution of several drugs, such as anticancer agents, are often due to the function of active efflux transporters such as Pgp expressed in intestinal epithelial cells and brain capillaries. The high barrier function reflected by the TEER values and low permeability coefficients for paracellular markers, and the very high B–A/A–B ratio of Pgp substrate taxol permeability indicates that the Caco-2 model is suitable to study the effect of randomly methylated β-CDs on taxol permeability.

Effective oral formulation of the Pgp substrate, anticancer agent taxol, is widely investigated but still not resolved. Oral bioavailability of taxol depends on two major factors, solubility and efflux by ABC transporters. Methylated β-CDs are effective to increase taxol solubility but their use as excipients is limited by their toxicity

Effect of Rameb derivatives on taxol permeability

According to t results of cholesterol solubilization and toxicity experiments, we selected three β-CD derivatives: Rameb, SuRameb and MaRameb. Rameb is a conventionally used methylated β-CD, whereas MaRameb and SuRameb are second-generation Rameb derivatives containing cationic and anionic groups, respectively. Introduction of ionic groups into the molecule decreases cholesterol solubilizing capacity and cytotoxicity, as our previous results show.

The concept behind the pretreatment experiment was that CDs remove cholesterol from the membranes changing their structure, permeability, and potentially the Pgp activity in the apical membrane in accordance with findings with Dimeb, resulting in higher permeability for taxol. This hypothesis, however, was not proved in case of Rameb, MaRameb, and SuRameb because pretreatment of the Caco-2 monolayer did not cause any significant changes in taxol permeability compared with the untreated control.

In Protocol 2, when taxol was co-administrated with CDs in the apical compartment the A–B taxol permeability increased more effectively than the well-known Pgp inhibitor CSA. From these results, we could have deduced that CD treatment inhibited Pgp function effectively in this system but this assumption was not confirmed by B–A permeability experiments. B–A taxol flux was not decreased in the presence of Rameb and its derivatives, as expected, whereas CSA induced an effective decrease in B–A taxol permeability by Pgp inhibition. These results clearly show that the presence of these CD derivatives is necessary for the enhanced A–B taxol permeability but not Pgp inhibition is responsible for the increased A–B flux.

Kinetic calculations of taxol transport show that the investigated CDs not only could increase the transport speed of taxol, but the quantity also.

The asymmetry ratio of B–A/A–B taxol flux was similar in the presence of CDs and CSA. As taxol crosses biological barriers by lipid-mediated free diffusion, only efflux pumps ABCB1 and ABCG2 in the apical membrane modify its

transport. On the basis of these assumptions, the balance between apical influx and efflux processes will determine the direction of the transport, and the B–A/A–B ratio. In control cells, a significant taxol efflux was measured, demonstrated by a B–A/A–B ratio of 12, indicatingthat the net efflux is 12 times bigger than the passive influx. In case of CSA, no significant change was seen in the permeability coefficient values of taxol in A–B direction, but a significant drop in B–A direction to one-third of the control explaining the drop of the B–A/A–B ratio to 2. In the presence of Rameb, MaRameb, and SuRameb (co-treatments), the permeability coefficient values of taxol in A–B direction were increased significantly by 5.9, 4.0, and 3.9 times, respectively, compared with control, in contrast to CSA treatment. The drop of the B–A/A–B ratio to 3 can be only explained if the net efflux is unchanged compared with control, indicating no change in efflux pump activity. If CDs would have induced efflux pump inhibition, this ratio should have fallen much below the calculated B–A/A–B ratio of 3. These data indicate that the effect of these CDs and CSA on taxol permeability is very different.

The possible mechanism of action of Rameb, MaRameb, and SuRameb on taxol permeability

There are four possible mechanisms to explain the taxol permeability enhancing effect of CDs:

1. The enhancement of taxol solubility
2. Permeabilization of cell membrane by altering the lipid composition
3. Changes in the function of TJs, and
4. The possible inhibition of efflux pumps

The solubility increasing property of Rameb was already reported for taxol. As there were no available data for Rameb derivatives, Cyclolab also determined taxol solubility in water at room temperature and found that they

could improve it by three magnitude of order in aqueous solution. Taxol solubility in 20 mM solutions of Rameb, SuRameb, and MaRameb are 30, 25, and 26 mg/ml, respectively. Rameb, Dimeb, and HPBCD were reported to form complex with taxol yielding 100% inclusion efficiency at 1/20 (taxol mol/CD mol) ratio. The molar ratios of [^{3}H]taxol (15 nM) and CDs (20 mM) in the present experiment were 1:1000000, resulting in a great excess of CDs ensuring 100% complex formation. These data indicate that Rameb and its derivatives can keep taxol in solution effectively in aqueous condition. The excess amount of CDs might also exert other effects. Cyclodextrins change membrane structure; physicochemical properties, fluidity, and can cause membrane permeabilization too. CDs are cell membrane cholesterol depleting agents; therefore, they are able to alter the overall membrane lipid packing.

Changes in membrane composition by CDs can lead to altered partition of substrates into the lipid bilayer. In addition, CDs as permeation enhancers overcome the aqueous barrier and carry the drug to the membrane surface. On the contrary, recent results demonstrate that β-CDs are able to overcome the barrier of cell membrane and enter the cells probably via endocytosis. This pathway can be utilized also for drug delivery, thus CD–taxol complexes may penetrate into the cells and circumvent efflux proteins.

On the other hand, at higher concentration (more than 10 mM) methylated β-CDs can inhibit the CYP 450 enzymes including CYP 2C, which converts taxol into its inactive, water soluble metabolite

β-CDs extract cholesterol from cell membrane. The cholesterol depletion decreases ATPase activity. Pgp utilizes the energy of ATP hydrolysis and if the activity of ATPase is decreased, the Pgp activity should be decreased. Hereby β-CDs should increase the transport of Pgp substrates through the cell membrane. But in our experiments we didn't detect Pgp function inhibition by Rameb, SuRameb and MaRameb at the applied concentration.

There are data indicating that CDs can change epithelial barrier function too. TJs are associated with cholesterol-rich lipid rafts, and depletion of cholesterol in Caco-2 cells led to alteration and reorganization of TJ architecture and distribution of specific TJ proteins.To test the effect of Rameb derivatives on barrier function, we measured TEER, paracellular permeability, and visualized monolayer integrity by TJ immunostaining. TEER values showed significant decrease after apical Rameb treatment but remained above 1000 Ohm × cm^2. This alteration was not reflected in paracellular permeability changes, but redistribution of TJ proteins could be visualized. Rameb and SuRameb treatment caused weaker and uneven staining of junctional proteins at the cell borders, whereas punctated cytosolic staining could be detected, indicating the redistribution of TJ proteins. The effect of RAMEB on the reorganization of TJ proteins is also reflected in the significant decrease of TEER, and could contribute to the increase in taxol permeabilities in both A–B and B–A directions.

The detected increase in taxol permeability in A–B direction, in the presence of MaRameb might not originate from the loss of barrier function. This conclusion is supported by the fact that MaRameb had similar permeability enhancing effect even if it was nontoxic and had no impact on TEER and TJ proteins.

All things considered, in oral formulation of taxol MaRameb proved to be the most suitable excipient as solubility, permeability enhancer excipient.

SUMMARY

1. A Caco-2 transport model was installed and characterized at the Department of Pharmaceutical Technology, University of Debrecen. The Caco-2 intestinal model is widely used for *in vitro* simulation of drug absorption, and is an appropriate model for studying the different ways of absorption.
2. The new generation CD derivatives showed lower cytotoxicity than the parent methylated compounds. The complexation of cholesterol into the cyclodextrin ring dramatically decreased the cytotoxicity of CDs on Caco-2 cells. The cytotoxicity of various CD derivatives depends on their cholesterol extraction capacity from the cell membrane. Hemolytic activity test results were in accordance with MTT test results.
3. From structure-activity analysis it can be concluded that in the case of methylated-β-CD compounds the cytotoxicity depends highly on the number and slightly on the position of methyl groups. Significant correlation can be observed between the cytotoxicity, hemolytic activity and the cholesterol complexing capacity of various CD compounds. I propose that cholesterol-solubilizing properties can be a predictive factor for β-CD cell toxicity which depends on the structure of the CD derivative.
4. Based on their cytotoxicity profile, three CD derivatives were chosen: Rameb, MaRameb, and SuRameb. I have demonstrated that Rameb and its less toxic ionic derivatives MaRameb and SuRameb effectively increase taxol permeability in Caco-2 monolayers, but the continuous presence of these CD derivatives is necessary for the enhanced taxol permeability in taxol-CD complex form. This effect could primary be mediated by increased complexation and solubility of taxol and also possibly by the interaction of CDs with cell membrane.

5. In contrast to the well-known active efflux pump inhibitor CSA, the examined CD derivatives at 20 mM concentration did not influence the activity of active efflux pumps. In the case of Rameb, the increased paracellular permeability can indicate attenuated barrier integrity. However the ionic derivatives of Rameb did not change the barrier function of the Caco-2 monolayer and did not induce a drop in TEER or internalization of specific tight junction proteins, they are able to enhance the taxol influx in Caco-2 cell monolayer. MaRameb proved to be the most suitable β-CD in oral formulation of taxol.

PUBLICATIONS

Candidate: Tímea Kiss

Neptun ID: LZME2Y

Doctoral School: Doctoral School of Pharmaceutical Sciences

List of publications related to the dissertation

1. Fenyvesi, F., **Kiss, T.**, Fenyvesi, É., Szente, L., Veszelka, S., Deli, M.A., Váradi, J., Fehér, P., Ujhelyi, Z., Tósaki, Á., Vecsernyés, M., Bácskay, I.: Randomly Methylated beta-Cyclodextrin Derivatives Enhance Taxol Permeability Through Human Intestinal Epithelial Caco-2 Cell Monolayer.
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