Drug Delivery Studies in Caco-2 Monolayers. IV. Absorption Enhancer Effects of Cyclodextrins

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Purpose. The purpose of the present study was to use the human colorectal carcinoma cell line, Caco-2, as a human intestinal epithelial model for studying the effects of cyclodextrins as absorption enhancers. ***Methods.*** Cyclodextrins of varying sizes and physicochemical characters were investigated. The effects of the cyclodextrins were evaluated by means of staining of the cytoplasma and determination of the mitochondrial dehydrogenase activity as well as by transport enhancement of the macromolecular pore marker polyethylene glycol 4000 (PEG-4000) across the Caco-2 monolayers. ***Results.*** The transport enhancing properties of the cyclodextrins were found to follow the lipophilicity of the core in their cyclic structure. Dimethyl-β-cyclodextrin was the most powerful in all aspects and caused an increase in the permeability of the cytoplasma membrane in a concentration dependent manner. It was possible to increase the overall transport of PEG-4000 10-fold by the use of dimethyl-β-cyclodextrin in low concentrations where the toxic effects on the monolayers were insignificant. It was further observed that the basolateral membrane was significantly more sensitive to cyclodextrins than the apical membrane. ***Conclusions.*** Since dimethyl-β-cyclodextrin was able to produce an absorption enhancing effect on PEG-4000 in concentrations where the toxic effects on Caco-2 monolayers were low it is worth to pursue the compound as an absorption enhancer.

KEY WORDS: cyclodextrin; oral drug delivery; absorption enhancer; Caco-2; cell culture; peptide.

INTRODUCTION

The cyclodextrins (CDs) constitute a family of cyclic oligosaccharides. The repetitive unit in the molecules is glucose and owing to steric reasons, only 6-, 7-, and 8-membered rings (α-, β- and γ-CD) are formed naturally (1). The natural occurring species are quite different in cavity size, ranging from 174 Å^3 to 427 Å^3. Therefore, they can act as hosts for a wide variety of lipophilic compounds (1). The CDs can be derivatized on the hydroxy groups. The most common derivatives applied in pharmaceutics are the dimethylated- and the hydroxypropylated β-cyclodextrin, DM-β-CD, and HP-β-CD. These CDs have a higher solubility as well as a more hydrophobic cavity compared to the parent compound. CDs have been studied as absorption enhancers for mucosal transport of poorly absorbed drug molecules such as peptides. Significant rectal absorption enhancement of insulin by CD was reported from hollow-type suppositories in rabbits (2). An important conclusion from that work was that the barrier function of the rectal membrane restored itself within 24 h after the CD treatment. The CDs have also been studied extensively for nasal delivery of peptides (2–7). All reports showed considerable absorption enhancement of *e.g.*, insulin and human granulocyte colony-stimulating factor. Also, the oral administration of insulin with CD has been explored in rats (8). Insulin was found to be absorbed better in the presence of CD, however, at the same time a proposed dissociation of the hexamer of insulin to monomers resulted in a higher degradability by α-chymotrypsin.

Some CDs are approved as food additives and are widely used in the food industry (1). However, the pharmaceutical use is only limited as of now (9). Few studies deal with the toxicity of CDs in pharmaceutics. The effects on the nasal membrane has been shown to be largely reversible (7) and in combination with fatty acids, bile salts, and a phospholipid, the overall cytotoxicity has been lowered (10). It has been suggested that the toxicity of CDs depend on the relative proportion of cellular to extracellular molecules likely to complex with and, thereby, occupy the CDs' hydrophobic core (11). Elsewhere, it was proposed that the undesired effects of large doses of CDs after long term administration could be explained by the absorption of otherwise not absorbed food contaminants (12).

In the present work, CDs of varying sizes and derivatizations were tested for their ability to enhance the transport of macromolecules across Caco-2 monolayers, which was previously shown to be a valuable model for evaluation of absorption enhancers (13–15). Cytoplasmic stainings and enzyme activity tests were employed to elaborate upon the relation between cytotoxicity and absorption enhancement as well as to propose a possible mechanism of action.

MATERIALS AND METHODS

Various cyclodextrins, α-, β-, γ-, and hydroxypropyl-β-cyclodextrin (degree of substitution = 4.9), were generously donated by Amaizo Co. (Hammond, IN). Dimethyl-β-cyclodextrin, trypan blue, thiazolyl blue (MTT), and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). Radiolabeled ^{14}C-polyethylene glycol 4000 (PEG-4000) was obtained from New England Nuclear (Boston, MA). The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagles medium (DMEM), fetal calf serum (FCS), non-essential amino acids (NEAA), L-glutamine as well as benzylpenicillin, streptomycin and Hank's balanced salt solution (HBSS) were obtained from Biological Industries (Israel). Polycarbonate filters (Transwell®) in clusters of 12 wells with a pore size of 0.4 μm and an area of 1 cm^2 used for transport experiments and transparent filters (Transwell-Col®) with the same specifications used for microscopic studies were obtained from Costar (Cambridge, MA). ELISA plates (Microwells®) were obtained from Nunk (Denmark). Culturing flasks and other disposables were obtained from Greiner (Austria). Buffer substances and all other chemicals were analytical grade and were used as received without further purification.

Caco-2 Cell Culturing

The Caco-2 cells were grown at 37°C, 90% relative hu-

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midity, and 10% CO_2/air in a standard medium consisting of DMEM, 9% FCS, 1% NEAA, 1% L-glutamine, 100u/ml benzylpenicillin, and 10u/ml streptomycin. The cells were seeded onto Transwell® polycarbonate filters and used for studies between passage 27 and 35. Mycoplasma tests were performed by The National Veterinary Institute (Uppsala, Sweden). No infections were found. The confluence of the monolayers was followed by measurements of the transepithelial electrical resistance (TEER) using Millicell® ERS (Millipore, USA). This varied from 500 to 900 $\Omega*cm^2$ for Transwell-Col®- and Transwell® filters, respectively.

Osmolarity

Prior to all experiments the osmolarities of test solutions were measured by freezing point depression in a Knauer Semi-micro osmometer (Germany). The cyclodextrins were dissolved in HBSS in concentrations ranging from 0.25% to 5%, except β-CD which has a maximal solubility of 1.8%.

Mitochondrial Dehydrogenase Activity (MTT-test)

Thiazolyl blue (MTT) is a complexing agent for dehydrogenases in the mitochondria of cells (16). This was used as a cell culture test for the cytoxicity of DM-β-CD (DM-β-CD was the only CD with transport enhancing effects in the concentrations tested). The test was performed as previously described (15). In brief, the cells were plated at a density of 40,000/ml into 96 ELISA wells. The ELISA plates were kept under standard cell culturing conditions for 18 to 22 h before tests. The culture medium was removed and replaced by 100 µl test solution in HBSS. After 10 min, 20 µl of a 5 mg/ml MTT solution in HBSS was added and the cells were further incubated for 90 min. In order to dissolve precipitated complex bound MTT, 100 µl of a solvent containing SDS, butanol, and hydrochloric acid was added. The crystals were allowed to dissolve and the visible absorption was measured in a Thermomax microplate reader (Molecular Devices, USA) at 570 nm.

Cytoplasmic Staining

The Caco-2 cells were grown on transparent Transwell-Col® filters as described above. Trypan blue is a large molecule with a molecular weight of 961 g/mol which is not able to penetrate into cells through an unperturbed cytoplasmic membrane. The cell monolayers were treated apically for 15 and 60 min with solutions of CD in HBSS. CD solutions were removed and replaced by a solution of 0.4% trypan blue in order to stain the cytoplasma. After 60 sec the cells were washed twice with HBSS and observed by light microscopy. Staining of the cells were quantified by counting the staining frequency in a population of approximately 250 cells as described previously (15).

Transport Studies

The profiles of ^{14}C-PEG-4000 transport across Caco-2 monolayers were in all cases used as the measure of transport enhancing ability of the CDs. Three experimental protocols were followed. The first protocol was a simple transport of PEG-4000 across the monolayers in the presence of various CDs. Therefore, the CD under investigation and PEG-4000 dissolved in 0.6 ml HBSS were added simultaneously to the apical side of the cell monolayers at time zero. The basolateral compartment contained 1.5 ml HBSS. Samples of 100 µl were taken from the receptor side and replaced with fresh HBSS as previously described (15). In the second protocol, the apical side of the monolayers were pretreated with DM-β-CD solutions for 45 or 90 min prior to start. The DM-β-CD solutions were removed prior to the start of the actual experiment. The third protocol described the transport of PEG in apical to basolateral or basolateral to apical direction in presence of DM-β-CD on the apical, basolateral or bilateral sides of the cells. All experiments were performed as triplicates.

Analytical Procedure

The quantitative determination of ^{14}C-PEG-4000 was performed by scintillation counting using a Minaxi Tri-Carb® 4000 from United Tech. Packard (IL). Each 100 µl sample was added 400 µl destilled water and 4.5 ml pico-aqua scintillation cocktail for counting.

RESULTS AND DISCUSSION

Osmolarity and Transepithelial Electric Resistance

The osmolarity of all CD solutions were in the range from 300 to 360 mOsm. The TEER of the Caco-2 monolayers were not affected by treatments with CDs other than DM-β-CD. Figure 1 illustrates the time course of TEER with DM-β-CD. The control resistance did not change throughout a period of 3 h. This was representative for α-, γ-, and HP-β-CD in 5% solutions and β-CD in a 1.8% solution. A solution of 2.5% DM-β-CD reduced the TEER to the background levels after 3 h, whereas a 5% solution did this within 1 h. This might suggest that the tight junctional complexes were interrupted during the treatment. This result will be discussed later.

Mitochondrial Dehydrogenase Activity (MTT-test)

From the MTT-test the inhibitory concentrations, IC50,

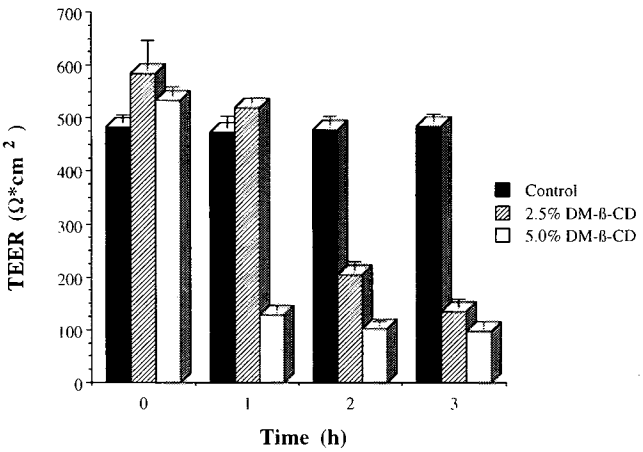

Fig. 1. Changes in the transepithelial electrical resistance of Caco-2 monolayers with time as a function of the concentration of DM-β-CD. Error bars indicate standard deviations.

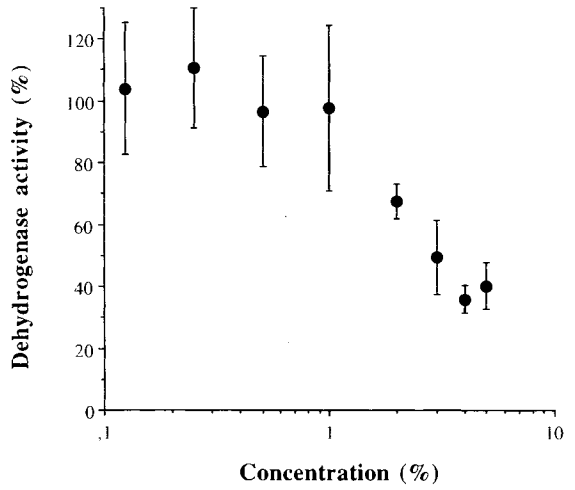

Fig. 2. Mean ± SD dehydrogenase activity of the mitochondria in Caco-2 cells following exposure to DM-β-CD in varying concentrations.

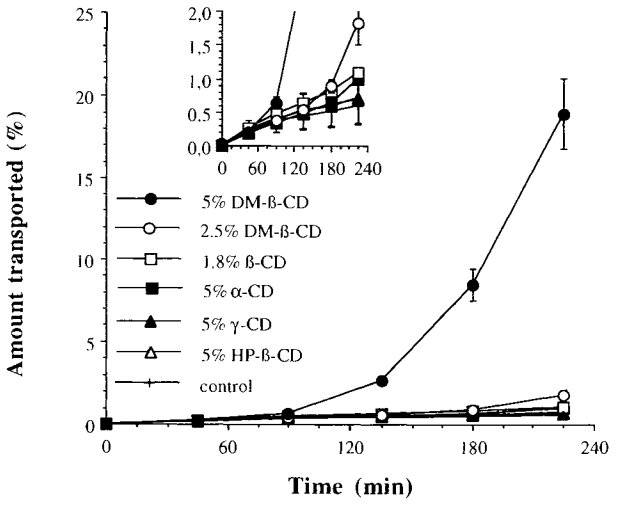

Fig. 3. Effects of various cyclodextrins on the mean ± SD amount of PEG-4000 transported across Caco-2 monolayers.

given as the concentration resulting in a 50% decrease in the dehydrogenase activity, was determined by exposure of the monolayers to DM-β-CD solutions from 0.125% and up to 5% (Figure 2). DM-β-CD showed an IC50 of 3%. The core of DM-β-CD is the most hydrophobic in the family of CDs and the enzyme inhibitory effect is most likely due to its strong complexation and extraction of cholesterol and other components from the cytoplasma membrane (1).

Cytoplasmic Staining

Vital staining methods have been applied to pharmaceutical enhancer studies (14,15). In Table I the stainings of monolayers treated with DM-β-CD are reported. It is evident that both the time of exposure and the concentration are important factors for the viability of the cells. As the time increased from 1 h to 3 h, the fraction of stained cells increased from 1 to 5%, respectively, for the lower concentration of DM-β-CD. In the same time-span stainings of 4 to 18%, respectively, of the cells were observed for the higher concentration of DM-β-CD. No control treatments caused any staining. It is important to notice that no treatments, except 5% DM-β-CD for 3 h, caused stainings of more than 6% of the cells. Thus, in those cases the culture was considered fully viable (15).

Transport Studies

Although DM-β-CD appeared to be most promising for absorption enhancement, all CDs were tested for absorption enhancing effects. Figure 3 illustrates the effects on the transport of PEG-4000 across Caco-2 monolayers. At the bottom of the figure coinciding profiles for PEG transport in the presence of α-, β-, γ-, and HP-β-CD is found. No significant beneficial effects on transport was seen for these CDs relative to the control. However, DM-β-CD showed remarkable impact on the transport rates for PEG-4000. At a concentration of 2.5% it produced a small increase in transport, but at 5% a tremendous effect was found, the flux reached approximately 10%/h*cm^2. The effects of DM-β-CD were not evident until 90 min after the application. Therefore, a pretreatment with 2.5% and 5% DM-β-CD for 45 and 90 min was investigated (Figure 4). After the specified time of pretreatment the DM-β-CD solutions were removed and replaced by PEG-4000 in HBSS. The fluxes found were not as high as that found in Figure 3. This probably owing to the progressing membrane effect on the cell monolayers when DM-β-CD was present through out the full time span of the experiment. This is supported by the increased staining in-

Table I. Effects of DM-β-CD on the degree of trypan blue staining in Caco-2 monolayers.*

	Time (h)		
Concentration	1	2	3
2.5%	1	2	5
5.0%	4	6	18

* All data are presented as percentages of stained cells in a population of approximately 250 cells.

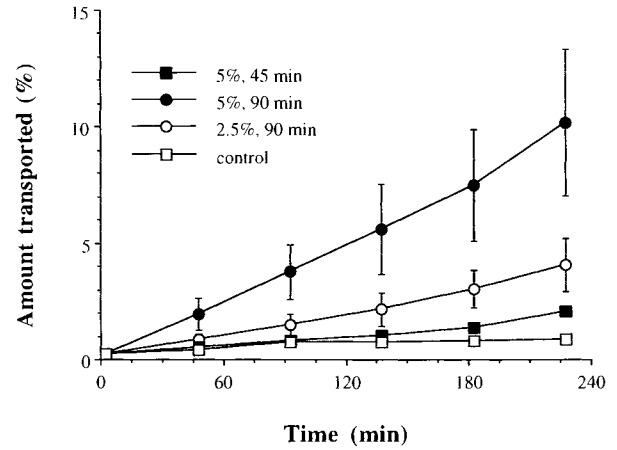

Fig. 4. Effects of the time of pretreatment and the concentration of DM-β-CD on the mean ± SD amount of PEG-4000 transported across Caco-2 monolayers.

tensity of the cells by trypan blue with time (Table I). It was evident that the time and concentration factors were important for the degree of enhancement. The 5% treatment for 45 min just barely raised the transport flux above that of the control, whereas 2.5% for 90 min significantly increased the flux to about 1.5%/h*cm^2. The CD effects on the cell monolayers were found not to be symmetrical. In Figure 5 the apical to basolateral transport of PEG during apical, basolateral and bilateral exposure to DM-β-CD is presented. It is evident from the profiles that the treatment of the basolateral membrane was most effective. In the case of 5% treatment basolaterally, the effect occurred about 1h earlier with a flux close to 20%/h*cm^2, compared to apical treatment where the flux was 10%/h*cm^2. Even the low concentration applied basolaterally produced a tremendous effect. A concentration of 2.5% applied basolaterally to the cells resulted in a flux of 20%/h*cm^2, whereas the same concentration applied apically only had low effect. Figure 6 illustrates some of the similar treatments to those in Figure 5, but the transport of PEG-4000 was followed from basolateral to apical side. Again the basolateral treatment was more effective. The flux in the direction basolateral to apical was generally not as high as in the other direction. A factor contributing to this observation is the different volumes of the apical and basolateral compartments.

When the Caco-2 monolayers were treated with DM-β-CD the TEER decreased dramatically. This was an indication that the tight junctional complexes were disrupted. However, this cannot be the only effect of DM-β-CD due to the fact that the cytoplasma was stained and enzyme activities were inhibited. Transport rates for PEG-4000 in untreated cells was independent on side of application. Therefore, the intercellular diffusional areas of the two sides are identical. Since basolateral enhancement was more effective than apical enhancement, the basolateral membrane must be more sensitive. Preliminary studies in our laboratories have shown that cholesterol is important for the recovery of the monolayers after a given DM-β-CD treatment. Thus, part of the mechanism may be explained by an extraction of cholesterol or other lipids (1). We believe this indicates that DM-β-CD has multiple effects on the epithelium and that the

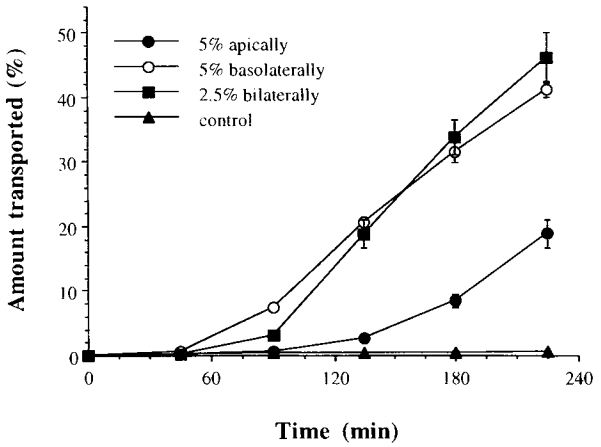

Fig. 5. Effects of the way of treatment and the concentration of DM-β-CD on the mean ± SD amount of PEG-4000 transported across Caco-2 monolayers from the apical to basolateral side.

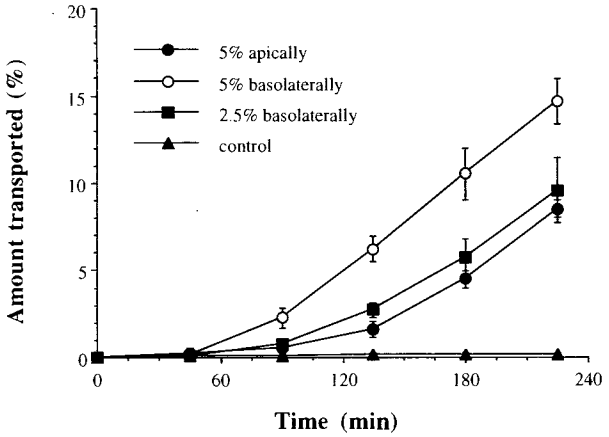

Fig. 6. Effects of the way of treatment and the concentration of DM-β-CD on the mean ± SD amount of PEG-4000 transported across Caco-2 monolayers from the basolateral to apical side.

extraction of membrane components is the primary effect by the CD. Secondary to this as a result of a membrane contraction or intracellular mediators, the tight junctional complex opens. This hypothesis must be proven in the future.

In conclusion, CDs have been shown to impair the cytoplasmic membrane of Caco-2 cells in monolayers. The action of the different CDs varies widely. It has been shown that the most effective CD is DM-β-CD and that it can enhance the transport of a macromolecule greatly under conditions where the cytotoxic effects are minimal. CDs, therefore (especially DM-β-CD), appear to be worth pursuing as enhancers for absorption of poorly absorbed drug molecules.

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