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ORIGINAL ARTICLE

Study of the cholesterol extraction capacity of β-cyclodextrin and its derivatives, relationships with their effects on endothelial cell viability and on membrane models

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Abstract Endothelial cells (HUVEC) were treated with β-cyclodextrin and hydroxypropylated or methylated derivatives solutions in order to quantify their cholesterol extraction capacity. Non-toxic concentrations of cyclodextrins (CDs) were determined following methyl thiazol tetrazolium (MTT) assays, total protein measurements, morphological observations and trypan blue assays. The residual cholesterol content of cells was measured and the extraction power of CDs compared to results obtained by phase solubility diagrams. Cholesterol was extracted with a dose-response relationship, the lowest residual cholesterol content being obtained with β-CD at 10 mM. Low substituted derivatives (Crysmeb® and hydroxypropyl-β-CD) maintained liposomes integrity (as shown before), were the less cytotoxic and presented the lowest affinity for cholesterol contrary to methylated derivatives with degrees of substitution around 2.

Keywords Cyclodextrins · Cholesterol · Endothelial cells · Cytotoxicity · Viability · Membrane models

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Introduction

The effect of cyclodextrins (CDs) on red blood cells hemolysis has been extensively studied and used to predict irritation at the site of intravenous injection [1] and to explain some aspects of their parenteral toxicity. It is well documented that the dimethyl derivative shows substantial hemolysis, more than the parent β-CD. The hydroxypropyl derivatives on the contrary induce much less hemolysis [2]. In order to explain local irritation of the venous wall, endothelial cell cultures as used here are relevant models.

Lipid vesicles (liposomes) have long been considered as simple cell membrane models [3]. This is especially the case for small unilamellar vesicles (SUVs) which can be prepared with lipidic components similar to those present in the cell membrane. In previous studies, we have investigated the interaction of CDs with SUVs containing or not cholesterol [4–6]. The leakage of calcein, a fluorescent dye, was used to investigate the integrity of liposomes after interaction with CDs. The strongest effects were measured with β-methylated derivatives (dimethyl-β-cyclodextrin (Dimeb) > randomly methylated β-cyclodextrin (Rameb) > trimethyl-β-cyclodextrin (Trimeb)) except for Crysmeb®, a derivative with a low degree of substitution (DS). These effects were correlated with their affinity for lipidic components. It was also observed that membranes containing cholesterol are more sensitive to methylated cyclodextrins. These results are in good correlation with those obtained by Hatzi et al. [7] who drew the same conclusion with methyl-β-CD on SUVs. This can be explained if we accept that the interaction between CD and cholesterol is faster and of higher extent as compared to that between CD and saturated lipids [7]. Many authors agree that β-derivatives interact with cell cholesterol [8, 9] and the cytotoxicity of

some of the methylated CDs could be correlated with their ability to form inclusion complexes with cholesterol.

The aim of the present work is to assess whether the observations made on membrane models can be transposed to the cell level as the behaviour of CDs towards biological membranes, and especially their hemolytic and cytotoxic effects, is widely accepted to be related to lipid complexation and depletion [10] despite the lack of systematic quantitative study of their extraction capacity. Endothelial cell culture models have been chosen for our studies to simulate blood vessel endothelium.

Previous viability studies have been performed on the LT2 cell line originating from human umbilical vein endothelial cells (HUVEC) immortalized by large T SV40 antigen [11]. After 1 h of treatment with CD concentrations not higher than 10 mM, the number of cells was quantified by measuring DNA content as previously described [12]. From the experimental study, it has been concluded that Dimeb has the most damaging effect on the integrity of cell membranes. However, in the tested conditions, CDs were dissolved in a medium containing serum. As serum contains the same lipids as cell membrane and maybe numerous molecules able to be included in the CD cavity [10], lower cytotoxic effect could have been measured. Further studies have to be performed in serum-free medium in order to better understand CD-membrane interactions.

As explained above, the effect of CDs on model membranes and most probably on cell membranes should be essentially attributed to their interaction with cholesterol. Furthermore, CDs are widely described in the literature to modulate cell membrane cholesterol levels [13–16]. Very short incubation periods with CDs are sufficient to induce cholesterol depletion [14, 17, 18]. The mechanisms of cholesterol extraction from membranes by CDs is not yet fully understood [19]. As reminded by the latter author, neither significant binding to nor inserting of CDs into the membranes was observed in cholesterol extraction experiments. It was hypothesized that cholesterol efflux from the membranes occurs primarily by an aqueous diffusion mechanism in which cholesterol molecules desorb from the cell or vesicular membrane and are incorporated in the CD molecule after diffusion across aqueous layer [15].

The present work will try to bridge the gap between previous studies performed on membrane models and cytotoxic data about CDs by studying quantitatively their impact on cholesterol depletion and viability of endothelial cells. It is expected that direct measurements of residual cell cholesterol contents will be more appropriate to draw conclusions about the implication of cholesterol extraction in cytotoxicity than theoretical data obtained from in vitro studies.

Experimental

Materials

β-Cyclodextrin (β-CD) and dimethyl-β-cyclodextrin (Dimeb; DS 2.0) were kindly donated by Cyclolab (Budapest, Hungary). Hydroxypropylated β-cyclodextrin (HP-β-CD; DS 0.61) and Kleptose® Crysmeb (Crysmeb; DS 0.5) were a gift from Roquette Frères (Lestrem, France). Randomly methylated β-cyclodextrin (Rameb; DS 1.8) was offered by Wacker-Chemie GmbH (Munich, Germany). Methyl-β-CD (Mβ-CD; DS 1.9) and trimethyl-β-cyclodextrin (Trimeb, from Fluka; DS 3.0) were purchased from Sigma-Aldrich (Bornem, Belgium).

For the MTT experiments, human umbilical vein endothelial cells (HUVEC) were a kind gift from the Metastasis Research Laboratory of the University of Liège. For the further experiments, the cells were obtained from Lonza (Verviers, Belgium) as well as foetal calf serum (FCS) and phosphate buffer saline (PBS). MCBD 131 culture medium, L-glutamine, penicillin-streptomycin and trypan blue solutions were from Gibco®-Invitrogen. The endothelial cell growth supplement (ECGS) was purchased from Becton Dickinson Biosciences. Gentamicin (Geomycine®) and amphotericin B (Fungizone®) were respectively from Schering-Plough and Bristol-Myers Squibb. Sodium dodecylsulfate was from Pierce and dimethylformamide and methanol were from Merck. All other chemicals and cell culture reagents were purchased from Sigma Aldrich (Bornem, Belgium).

Cell culture

HUVEC were grown on 2‰ pork skin gelatin-coated culture dishes in MCBD 131 medium supplemented with 20% FCS, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL gentamicin, 0.5 µg/mL amphotericin B, 50 µg/mL ECGS and 7 U/mL heparin. The cells were maintained in a humidified 5% CO_2 and 95% air atmosphere at 37 °C and supplied with fresh medium every 2 or 3 days. Cells were used at passages between 2 and 6.

Preparation of cyclodextrin solutions

CD test solutions were prepared in a serum- and ECGS-free medium. CD mass was corrected for water content when calculating the molarity. After water content determination using a Mettler DL 35 Karl-Fischer titrator, appropriate amounts of CDs were dissolved in MCBD 131 medium containing 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The solutions were sterilized by filtration in aseptic conditions.

Osmolalities of 10 mM CD solutions were measured by freezing point depression in a Knauer semi micro osmometer (Germany). They were in the range 270–320 mOsmol/kg considered as isotonic.

Cell viability study (MTT assays)

The cell viability in the presence of CD solutions was evaluated in a preliminary study using the methyl thiazol tetrazolium (MTT) assay. Cells were seeded at an initial density of 10,000 cells/well in 96-well gelatin-coated culture plates and allowed to attach overnight. After washing, they were incubated for 1 h with 200 µL of CD test solutions (0.0, 1.0, 5.0, 10.0, and 2.5 mM for Dimeb and Trimeb). The cells were then washed and incubated for four additional hours with 200 µL of the serum free medium supplemented with 20 µL of MTT solution (5 mg/mL in PBS). After washing, the resulting intracellular purple formazan deposit was solubilized by 200 µL solution of sodium dodecylsulfate (10% m/V in dimethylformamide/water 50:50). The plates were then incubated overnight at 37 °C before spectrophotometric quantification using a Labsystems Multiskan MS microplate reader at 560 nm [20, 21]. The results were expressed in percentages of untreated controls (100% viability).

Cholesterol measurements

Regarding cholesterol measurements, the first step was the selection of the appropriate method to extract cholesterol from cells. Different procedures for lipid extraction from cells have been compared [22] and it was concluded that the Folch's method, using a chloroform/methanol at a 2:1 volume ratio [23], gave acceptable results for cholesterol extraction. In this study the Folch's method will be used according to the protocol of Fillet et al. [24]. Cholesterol measurements were performed using two 10 cm dishes of cultured cells grown to 80% confluence. After washing, the dishes were incubated for 1 h with 10 mL of CD solutions ranging from 0 to 10.0 mM. The cells were rinsed twice with ice-cold PBS, scraped in the same buffer, centrifuged at 1,000 rpm and the resulting pellet was homogenized in 300 µL of distilled water by sonication. An aliquot of the cell homogenate was taken for protein determination using the conventional Lowry technique according to the manufacturer's instructions (*DC* Protein Assay Kit®, BioRad) [18]. Cholesterol was extracted with the organic solvent mixture from a volume of cell lysate corresponding to 500 µg of proteins. After centrifugation at 4,000 rpm the organic phase was collected and evaporated to dryness [22–24]. The cholesterol content was determined by a colorimetric assay using the Elitech Cholesterol SL Kit® (Sopachem, Eke, Belgium). The principle of this enzymatic assay is as follows: cholesterol oxidase catalyses the oxidation of cholesterol with production of hydrogen peroxide. The hydrogen peroxide, which is produced stoichiometrically, reacts with 4-aminoantipyrine and phenol generating a chromogen, quinoneimine. This assay was successfully used for microplate reading using the microplate reader at 492 nm.

Cell morphology by phase contrast microscopy

To visualize cell morphology [1] after 1 h incubation with CDs, photographs of cells from the 10 cm dishes were taken at 40× magnification with a phase contrast microscope (Axiovert 25, Zeiss) equipped with a camera.

Trypan blue exclusion assay

The cells were washed after 1 h CD treatment. The adherent cells were detached using trypsin/EDTA and resuspended in 10 mL of fresh culture medium. They were centrifuged at 1,000 rpm and the resulting pellet was resuspended in 1 mL PBS. A 1:1 dilution was performed using the 0.4% trypan blue solution. Thoma chambers were filled with the resulting suspensions and visualized under light microscope using ×10 magnification. Counting of viable (seen as bright cells) and non-viable cells (stained blue) was performed. Cell viability was calculated in a population of approximately 150 cells as the number of viable cells in percents of total (viable + dead) cells.

Results

Cell viability study (MTT assays)

The effect of a 1 h exposure to different concentrations of CDs on HUVEC metabolic activity was evaluated using MTT assays (data not shown). In these assays, only the viable cells are able to reduce the MTT into formazan crystals by the mitochondrial succinate dehydrogenase. In our experiments, for most of the CDs and concentrations tested, more than 93% of MTT conversion was measured. Only Dimeb and Trimeb at 10 mM showed a significant cytotoxicity (53 and 68% viability respectively). In the following studies, these CDs will be used at a maximum of 5 mM.

Total protein amount

The total protein content of each sample was determined to evaluate cell number after CD treatment (Table 1). The principal aim of this step was to estimate the effectiveness of cholesterol extraction on an equivalent quantity of cell

Table 1 Determination of the total protein contents

CD concentration	1 mM	2.5 mM	5 mM	10 mM
β-CD	110.2 ± 9.4	103.9 ± 13.3	82.9 ± 10.6	71.8 ± 8.0
HP-β-CD	110.0 ± 9.2	104.7 ± 8.1	98.1 ± 16.0	83.7 ± 21.3
Crysmeb	116.3 ± 13.4	115.4 ± 15.3	96.6 ± 17.0	62.0 ± 6.4
Rameb	107.7 ± 10.2	90.2 ± 8.5	79.7 ± 12.5	60.6 ± 11.6
Mβ-CD	96.6 ± 10.9	86.8 ± 8.8	64.8 ± 11.4	52.2 ± 3.6
Dimeb	94.3 ± 7.9	80.0 ± 10.1	53.3 ± 8.1	–
Trimeb	102.4 ± 10.9	99.0 ± 15.9	85.1 ± 18.9	–

Data are expressed in percentages of untreated controls taken as 100% ± standard deviation to the mean (SD) ($n \geq 3$)

lysate. As the protein amount can be correlated with the cell number, this assay has been used to evaluate cell viability [1, 25, 26]. For each tested CD the lowest values were obtained at 10 mM. Values lower than 100% were obtained at 10 mM for Crysmeb and HP-β-CD and at concentrations $\geq$5 mM for β-CD and Trimeb, concentrations considered as cytotoxic. Only concentrations $\leq$1 mM were not toxic for Rameb, Mβ-CD and Dimeb.

Cell morphology by phase contrast microscopy

Phase contrast microscopy pictures were performed to observe morphological changes which could be induced by CD cytotoxic concentrations evaluated here above. Figure 1 shows a representative picture of changes observed with CD concentrations resulting in protein contents significantly lower than in untreated controls. Loss of cell-cell contact, decrease in the number of remaining attached cells as well as morphological changes (rouding cells) were observed confirming the cytotoxicity [1, 27].

Trypan blue exclusion assay

A trypan blue exclusion assay was performed at the maximal non-toxic CD concentrations, on the basis of total protein amounts as detailed in Table 1, to confirm their lack of cytotoxicity. All the values obtained were around 100% showing that cells are viable at the tested concentrations (Table 2). The maximum non-toxic CD concentrations will be used to interpret the CD effects in future studies.

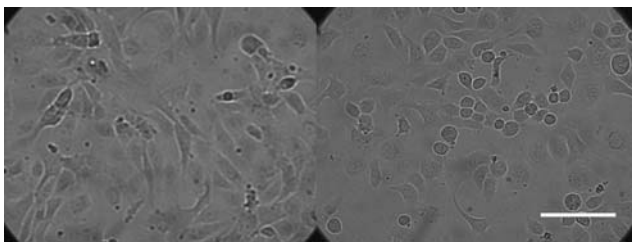

Fig. 1 Representative phase contrast microphotographs of cells incubated for 1 hour without (left side) and with 5 mM Dimeb solution (right side) that induces 50% of protein loss (scale bar = 100 µm)

Cholesterol measurements

Preliminary assays were performed on increasing cell number in order to evaluate the efficiency of the cholesterol extraction protocol. A linear response was obtained with the colorimetric assay confirming that cholesterol is completely extracted by the solvent mixture (Fig. 2).

Table 2 Cell viability evaluated by the trypan blue exclusion assay for individual minimum CD concentrations that induce no protein loss

CD	Concentration (mM)	% Viability
β-CD	2.5	99.0 ± 1.4
HP-β-CD	5	98.4 ± 2.1
Crysmeb	5	98.8 ± 1.4
Rameb	1	96.5 ± 2.4
Mβ-CD	1	99.0 ± 1.2
Dimeb	1	97.4 ± 2.9
Trimeb	2.5	97.0 ± 2.7

Data are expressed in percentages of untreated controls taken as 100% ± SD ($n \geq 9$)

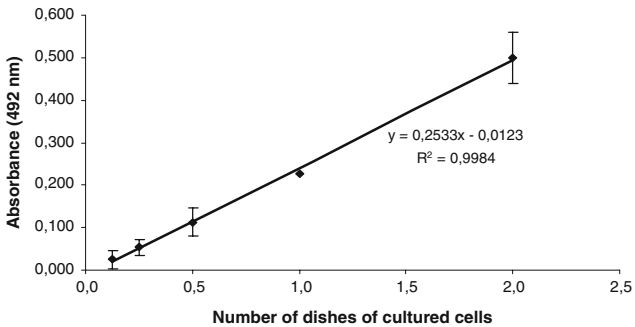

Fig. 2 Cholesterol assay after extraction by the Folch's method on increasing cell number ($n \leq 2$)

Table 3 Residual cholesterol content of HUVEC after treatment with CDs (measurements performed on equivalent cell lysates)

CD concentration	1 mM	2.5 mM	5 mM	10 mM
β-CD	84.0 ± 6.5	65.0 ± 4.2	53.1 ± 7.3	39.0 ± 5.3
HP-β-CD	90.1 ± 9.4	74.0 ± 5.3	67.5 ± 7.4	60.2 ± 10.3
Crysmeb	83.4 ± 7.6	77.0 ± 8.5	61.7 ± 9.6	54.9 ± 5.1
Rameb	71.9 ± 2.9	65.4 ± 5.1	57.0 ± 8.5	51.7 ± 7.5
Mβ-CD	76.0 ± 7.7	65.2 ± 6.7	57.9 ± 9.5	53.7 ± 2.7
Dimeb	76.0 ± 5.8	69.5 ± 6.4	65.2 ± 5.9	–
Trimeb	96.9 ± 11.4	91.0 ± 8.8	81.9 ± 15.9	–

Data are expressed in percentages of untreated controls taken as 100% ± SD ($n \geq 3$)

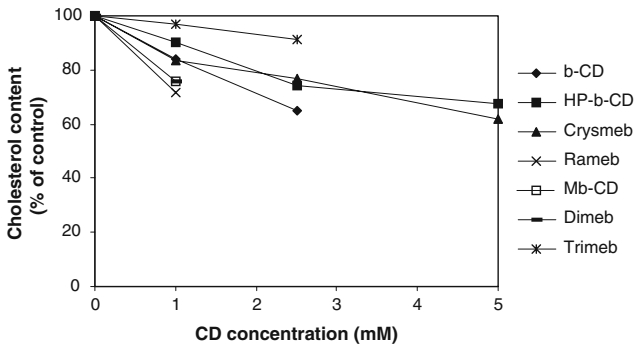

Fig. 3 Residual cholesterol content of HUVEC after treatment with CD concentrations leading to total protein amounts around 100% (measurements performed on equivalent cell lysates). Data are expressed in percentages of untreated controls ± SD ($n \geq 6$)

To evaluate the efficiency of cholesterol extraction by CDs, an equal amount of cells corresponding to 500 µg proteins was used. For most of the conditions tested, decrease in the HUVEC cholesterol content was observed, with a dose-response relationship (Table 3). Depending on their nature, CDs were able to decrease the cholesterol content of remaining attached cells up to 40%. Trimeb was less effective, especially at low concentrations. These results have to be taken with care especially when considering total protein amounts lower than 100%. On Fig. 3, results obtained with concentrations for which no decrease in the total protein amount was measured are presented (for clarity reasons, SD are not represented on the figure but they can be found in Table 3).

In order to compare the extraction power of CDs, the 1 mM concentration which is non-toxic for each CD has been chosen. Methylated derivatives with DS around 2 are the most effective while Trimeb has almost no effect. The low substituted derivative Crysmeb and β-CD give intermediate results while HP-β-CD is less effective.

Discussion

Conversion of MTT by the mitochondria of living cells is one of the assay currently used to evaluate the cell viability. HUVEC have been submitted for 1 h to CD solutions ranging from 0.0 to 10.0 mM in order to determine their cytotoxicity and also to select the ideal concentrations for further studies. A significantly decreased MTT metabolizing activity was observed for Dimeb and Trimeb at 10 mM suggesting that these high concentrations were cytotoxic and will not be used anymore in further studies.

The number of cells remaining attached after CD treatment was also evaluated from total protein content. Significant reductions of total protein content were obtained at 10 mM for all CDs. At 5 mM, the lowest value was obtained with Dimeb which confirms its most cell damaging effect. From these results the safety of CDs can be classified as follows: HP-β-CD > Crysmeb > β-CD > Trimeb > Rameb > Mβ-CD > Dimeb. This classification can be partially compared to those obtained on other cell lines although the authors did not evaluate this wide range of CDs [21, 28–30].

The cell viability was confirmed by morphological observations and trypan blue assays.

Decrease in the cholesterol content of remaining attached cells was measured for most of the CDs with a dose-response relationship, the lowest residual cholesterol value being obtained at the highest CD concentration. A maximum of 60% cholesterol was extracted with 10 mM β-CD. However, high concentrations of some CDs appeared to result into cell loss perhaps by detachment due to a perturbation of the integrins and focal adhesions known to be associated in lipid rafts [31–33]. To make relevant comparisons on the cholesterol extracting efficiency of the CDs, we chose the 1 mM concentration that is non-toxic for any of the CDs. Based on mean values, results show that their interaction with cellular cholesterol seems to increase in the order Trimeb < HP-β-CD < β-CD = Crysmeb < Dimeb = Mβ-CD < Rameb. Except for Trimeb and β-CD, these results show good correlation with phase solubility diagrams previously published [4] as the affinity of CDs for cholesterol was classified as follows: βCD < HP-β-CD < Crysmeb < Dimeb < Rameb = Trimeb. The observed effects are comparable with cholesterol extraction from other biological membranes [15, 34]. Results obtained with β-CD can be explained by the formation of cholesterol-β-CD insoluble complexes not detectable with phase solubility diagrams [35]. However, this complex formation exists and is confirmed by cholesterol extraction from cells. It is more difficult to explain the unexpected behaviour of Trimeb as soluble complexes are formed in aqueous solution [36]. However, when

considering the complexity of the cell membrane, we can envisage the possibility of higher affinity with other components explaining the lower extraction of cholesterol. As an example, Beseničar et al. [19] recently showed on lipid membranes that the amount of extracted cholesterol is highly dependent on their compositions, i.e. the presence of sphingomyelin (constitutive of cell membranes) drastically reduced and slowed down cholesterol extraction by methyl-β-CD.

Concerning the extrapolation of results obtained on liposomes to the cell level, we can conclude that except for Trimeb (and β-CD which was not studied on SUV's), similar conclusions can be drawn if we consider two groups of CDs: on the one hand the three methylated derivatives with DS around 2 and on the other hand low substituted derivatives. Indeed, HP-β-CD and Crysmeb maintained liposomes integrity, were the less cytotoxic ones and presented lower affinity for cholesterol unlike the other derivatives for which inverse observations have been done.

As a conclusion, findings obtained on lipid vesicles as model membranes have been partially confirmed when using biological membranes. However, liposomes are certainly very simplified models compared to the complicated biologic ones (lipid composition and asymmetric distribution, organisation in rafts and caveolae, number and nature of membrane proteins, ion channels, transporters, …) [3]. Future studies will be conducted with the safe concentrations selected here in order to evaluate the effect of CD interactions on cellular functions and to correlate it with cholesterol depletion.

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