Original Article

Evaluation of the potential toxicity of unmodified and modified cyclodextrins on murine blood-brain barrier endothelial cells

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ABSTRACT — In this study, we investigated the cytotoxic effects of unmodified α -cyclodextrin (α -CD) and modified cyclodextrins, including trimethyl- β -cyclodextrin (TRIMEB) and hydroxypropyl- β -cyclodextrin (HP β CD), on immortalized murine microvascular endothelial (cEND) cells of the blood-brain barrier (BBB). A CellTiter-Glo® viability test, performed on the cEND cells showed significant differences among the different cyclodextrins. After 24 hr of incubation, TRIMEB was the most cytotoxic, and HP β CD was non-toxic. α -CD and TRIMEB exhibited greater cytotoxicity in the Dulbecco's modified Eagle's medium than in heat-inactivated human serum indicating protective properties of the human serum. The predicted dynamic toxicity profiles (T_d) for α -CD and TRIMEB indicated higher cytotoxicity for these cyclodextrins compared to the reference compound (dimethylsulfoxide). Molecular dynamics simulation of cholesterol binding to the CDs suggested that not just cholesterol but phospholipids extraction might be involved in the cytotoxicity. Overall, the results demonstrate that HP β CD has the potential to be used as a candidate for drug delivery vector development and signify a correlation between the *in vitro* cytotoxic effect and cholesterol binding of cyclodextrins.

Key words: Cyclodextrins, Cytotoxicity, Blood-brain barrier, Endothelial cells, Molecular dynamics, Cholesterol binding

INTRODUCTION

Cyclodextrins (CDs) have a wide range of biomedical usage in the fields such as pharmacy, chemistry, biotechnology and medicine. They are oligosaccharides produced through the enzymatic degradation of starch and typically exist as hexameric α -, heptameric β - and octameric γ -CDs (Zidovetzki and Levitan, 2007). CDs are also known to enhance the solubility of lipophilic biologically active compounds and thus have the potential to integrate into sophisticated drug-delivery systems to enhance the permeation of substances through different barriers, such as the blood-brain barrier (BBB) (Shityakov *et al.*, 2014; Shityakov *et al.*, 2015). The permeation of CDs through the BBB could be an especially important issue in the case of some neurological diseases, such as Niemann-Pick type C, which is currently cured by some modified CDs

administered intrathecally to avoid the BBB (Vecsernyés *et al.*, 2014). Moreover, water-soluble CD derivatives with low toxicities are required to improve drug bioavailability, reduce drug immunogenicity, modify drug metabolism, and increase biological half-life of drugs after their systemic administration (De Jong and Borm, 2008; Sanvicens and Marco, 2008; Jansook *et al.*, 2010).

Considered as cage molecules, CDs have a core consisting of a hydrophobic cavity which traps or encapsulates other molecules, thus enabling its complexation with other substances (Crini, 2014). CDs do not readily permeate biological membranes due to their low lipophilicity. In fact, it has been shown that only negligible amounts of hydrophilic/amphiphilic CDs and drug-CD complexes are able to penetrate through lipophilic membranes like the gastrointestinal (GI) mucosa and skin (Irie and Uekama, 1997; Matsuda and Arima, 1999; Loftsson *et al.*, 2005).

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Due to the minimal absorption of CDs by the GI tract, they are proven to be non-toxic when administered orally (Irie and Uekama, 1997). Nonetheless, methylated CDs that are more lipophilic can be absorbed more readily (~10%) by the GI tract. However, owing to the toxic potential of CDs in this case, only limited amounts are incorporated in pharmaceutical formulations for oral administration (Irie and Uekama, 1997; Loftsson *et al.*, 1999, 2005). Also, in some animal experiments, certain CDs are proven to be non-toxic when administered intravenously (Munro *et al.*, 2004). In particular, 2-hydroxypropyl- β -cyclodextrin (HP β CD) is well tolerated in rats, mice, and dogs when orally administered and shows only limited toxicity (Gould and Scott, 2005).

On the other hand, the cytotoxicity of CD molecules is often tested *in vitro* through their hemolytic activity. Their ability to induce hemolysis differs depending on the type of CD ring present. Additionally, methylation of the cyclodextrin rings in β -CD also imparts cytotoxic activity observed in *in vitro* experiments (Kiss *et al.*, 2007, 2010).

It has been shown that exposure of tissue culture cells to CDs results in rapid cholesterol depletion (Christian *et al.*, 1997). Due to the relatively hydrophobic cavity of CDs, they are capable of extracting cholesterol from cell membranes. For instance, β -CDs are being used to modify the cholesterol content of cell membranes (Zidovetzki and Levitan, 2007). A study using bovine pulmonary endothelial cells showed that treatment with methylated β -CD resulted in a significant loss of membrane cholesterol, cytotoxicity, and decreased trans-endothelial electrical resistance (Hinze *et al.*, 2012). In another study, the cytotoxicity of novel β -CD derivatives was evaluated in relation to cholesterol depletion using human embryonic kidney 293A cells (Kiss *et al.*, 2010). This investigation showed that cholesterol depletion may be used as a biomarker to evaluate cytotoxicity.

In this context, the biological and toxic effects of cyclodextrins, their interaction with cellular membranes, and the extraction of different lipid components are highly relevant at the BBB level. Therefore, in the present investigation, we analyzed the cytotoxicity of the α -cyclodextrin (α -CD), trimethyl- β -cyclodextrin (TRIMEB) and HP β CD molecules on immortalized murine microvascular endothelial (cEND) cells and the mechanisms which are involved in the cytotoxic process using *in silico* and *in vitro* methods.

MATERIALS AND METHODS

Materials

The α -cyclodextrin (Fig. 1 [A]) compound was

Fig. 1. Schematic representations of α -CD (A), TRIMEB (B; R = CH₃) and HP β CD (B; R = (CH₂)₃OH) molecules. In the case of HP β CD model, the primary hydroxyl groups are only substituted.

purchased from Sigma-Aldrich Productions GmbH (Steinheim am Albuch, Germany). The pure forms (98% purity) of trimethyl- β -cyclodextrin and hydroxypropyl- β -cyclodextrin substances (Fig. 1 [B]) were the products of Cyclolab Cyclodextrin R&D Laboratory Ltd. (Budapest, Hungary).

Cell culture

Immortalized murine microvascular endothelial cells (Förster *et al.*, 2005; Silwedel and Förster, 2006; Golenhofen *et al.*, 2002) were seeded on collagen type 4-coated Transwell® filters (Greiner Bio-One, GmbH, Frickenhausen, Germany) and cultured using Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), and a penicillin/streptomycin mixture. The cells were grown for 5 days and differentiated with DMEM containing 1% charcoal-stripped fetal calf serum (sFCS) to build up tight junctions for 2 days.

Cell viability assay

cEND cells were seeded in a 96-well plate and grown to reach 90% of confluence in DMEM containing 10% FCS, 50 U·mL⁻¹ penicillin/streptomycin, and 1% L-glutamine. Once confluent, cells were forced to differentiate in 1% sFCS for two days. Next, cells were incubated with the α -CD, TRIMEB and HP β CD substances for 24 hr at 37°C in a concentration ranging from μ g·mL⁻¹ to mg·mL⁻¹ in DMEM or heat-inactivated (56°C for 30 min) human serum (Institute of Transfusion Medicine and Haemotherapy, Würzburg, Germany). Afterwards, cell viability was measured using the CellTiter-Glo® luminescent cell viability assay (Promega, Madison, WI, USA) kit, and used according to the manufacturer's instructions.

Briefly, the test compounds and controls were added to the cEND cells and after the intended incubation period, a 30-min incubation at room temperature (RT) followed. CellTiter-Glo® solution was then added. Following this, cell lysis was induced for 2 min with shaking followed by a 10-min equilibration at RT. Luminescence was read using the Tecan GENios Microplate Reader (MTX Lab Systems, Inc., Vienna, VA, USA). 10% solution of dimethyl sulfoxide (DMSO) was used as a positive control.

Light microscopy and image analysis

The morphology of cEND monolayers cultivated onto the cell culture flasks and Transwell® membranes with 0.4 µm pore size were examined by using light microscopy. 8-bit greyscale images of the analyzed micrographs were prepared using the ImageJ software (National Institute of Health, Bethesda, MD, USA) to calculate the percentage of pixels (P_p) for cytotoxic cyclodextrins.

Analysis of dynamic cytotoxicity

The MATLAB R2012a software (MathWorks, Natick, MA, USA) was used to predict the dynamic toxicity (T_d) profiles for cEND cells treated with α -CD, TRIMEB and DMSO substances in DMEM, or heat-inactivated human serum. Sigmoid logistic function ($f(x)$) was implemented in the MATLAB script using the following equation:

$$f(x) = \frac{a}{b + e^{-cx}} \quad (1)$$

where x is any independent variable described by *linspace* function; a and b are a parameterization factor; e is an exponential constant ($e = 2.718$); and c integer is a Hill slope. This equation was further parameterized to predict the dynamic toxicity profiles from the cEND static toxicity (T_s), namely:

$$T_s = 100\% - P_p \quad (2)$$

$$T_s = 100\% - P_L \quad (3)$$

$$T_d = \frac{T_s}{(b + e^{-cx}) \times 100} \quad (4)$$

where P_L is a percentage of luminescence; and P_p is a percentage of pixels.

The *linspace* function ($x = \text{linspace}(0, 24)$) was used to generate linearly spaced vectors for a 24-hr interval curve. The parameterization ($b = 0.01$) and Hill slope ($c = 0.5$) integers were adjusted to the T_d logistic function to establish 100% toxicity rate as a reference curve reaching the upper plateau level with maximal effect after 24 hr.

In silico methods to evaluate cholesterol binding to cyclodextrins

The β -CD structure (ID: 1BFN) was retrieved from the Protein Data Bank (Adachi *et al.*, 1998) to be used as a template for the construction of α -CD molecule via a deletion of one α -D-glucopyranose unit. Finally, methyl and 2-hydroxypropyl groups were added to the hydroxyl groups of β -CD to build TRIMEB and HP β CD. A cholesterol molecule was obtained from the Protein Data Bank (Ligand ID : CLR) and optimized at a neutral pH using Monte Carlo methodology with an OPLS 2005 force-field (Damm *et al.*, 1997). The ANTECHAMBER module was implemented to calculate the atomic partial charges for all molecules. Molecular geometries were evaluated with the Gaussian 09 program (Frisch *et al.*, 2009) implementing the DFT (Density Functional Theory) approach with the B3LYP/6-31G* level of theory (Kim *et al.*, 1994). The Rosetta docking protocol (Das and Baker, 2008) was used to create the cholesterol-CD complex as an initial structure suitable for a molecular dynamics (MD) simulation. All classical MD simulations were performed by the Amber 12 package (Case *et al.*, 2005) using the GAFF (General Amber Force Field) and GLYCAM_06j-1 force-fields for the cholesterol and CD molecules. The systems were solvated with TIP3P water models using the tLEaP input script available from the AmberTools. Long-range electrostatic interactions were applied via the PME (Particle-Mesh Ewald) method (Essmann *et al.*, 1995). The SHAKE algorithm (Miyamoto *et al.*, 1992) was used to constrain the length of covalent bonds, including hydrogen atoms. The Langevin thermostat was used to equilibrate the temperature (T) of the systems ($T = 300$ K). All simulations were run to minimize the system for 20,000 steps and equilibrate it for 2-ns time period with 2.0 fs time step. Finally, 50-ns classical MD simulation with no constraints was performed for each of the CD-cholesterol complexes, and all trajectory frames were recorded for the post-processing analysis. The MM-PBSA (Molecular Mechanics Poisson-Boltzmann Surface Area) method (Miller *et al.*, 2012) was implemented to estimate the free energies of binding (ΔG_{bind}) for the CD-cholesterol complexes. The python script (MMPBSA.py) from the AMBER 12 package was executed in the python environment to calculate the energetic profiles of analyzed complexes. The ΔG_{bind} values were determined by subtracting the sum of cyclodextrin (ΔG_{CD}) and cholesterol (ΔG_{CH}) free energies from the complex free energy ($\Delta G_{\text{complex}}$) as depicted according to following equation:

$$\Delta G_{\text{bind}} = \Delta G_{\text{complex}} - (\Delta G_{\text{CD}} + \Delta G_{\text{CH}}) \quad (5)$$

The free energy of binding can be represented as a composition of enthalpy (ΔH) and entropy ($T\Delta S$) as follows:

$$\Delta G_{\text{bind}} = \Delta H - T\Delta S \quad (6)$$

The interaction energy (ΔE_{MM}) was calculated by the molecular mechanics method as the energy between the CD and cholesterol, namely:

$$\Delta E_{\text{MM}} = \Delta E_{\text{elec}} + \Delta E_{\text{vdW}} \quad (7)$$

, where each of the ΔE_{elec} and ΔE_{vdW} terms defines either electrostatic or van der Waals (vdW) interaction energies, respectively. Finally, the ΔH and ΔG_{sol} (free energy of solvation) parameters can be rewritten as:

$$\Delta H = \Delta E_{\text{MM}} + \Delta G_{\text{sol}} \quad (8)$$

$$\Delta G_{\text{sol}} = \Delta G_{\text{PB}} + \Delta G_{\text{NP}} \quad (9)$$

, where ΔG_{PB} defines the polar solvation energy calculated by Poisson-Boltzmann method; and ΔG_{NP} describes non-polar solvation energy, which is predicted through the estimation of the repulsive (ΔG_{rep}) and attractive (ΔG_{att}) vdW interaction energies between solute and solvent:

$$\Delta G_{\text{NP}} = \Delta G_{\text{rep}} + \Delta G_{\text{att}} \quad (10)$$

The ΔG_{NP} and $T\Delta S$ values were calculated with the LCPO (Linear Combinations of Pairwise Overlaps) and RRHO (Rigid Rotor Harmonic Oscillator) methods, respectively. For the ΔG_{NP} calculation, a probe with the radius of 1.4 Å has been assigned to the solvent molecules.

RESULTS AND DISCUSSION

In vitro cytotoxicity study for unmodified and modified cyclodextrins on endothelial cells

Taking into consideration the previously published concentrations of either some cyclodextrin-formulated drugs, such as sevoflurane in the concentration of 20–40 $\mu\text{g}\cdot\text{mL}^{-1}$ (Nitzschke *et al.*, 2013) complexed with α -CD (relative inclusion content [w%] = 94%) and midazolam with TRIMEB in the concentration of 40 $\text{mg}\cdot\text{L}^{-1}$ (Shityakov *et al.*, 2014), or HP β CD, functionalized with calcium carbonate microparticles in the concentration of 50 $\mu\text{g}\cdot\text{mL}^{-1}$ (Zhang *et al.*, 2015), the CellTiter-Glo® luminescent cell viability assay was used to determine the CD cytotoxicity effect on endothelial cells in DMEM and heat-inactivated human serum. Although the high purification of TRIMEB and HP β CD was achieved with a significantly improved aqueous solubility (31 g and > 50 g in 100 mL at 25°C), in comparison to α -CD (15 g in 100 mL at 25°C), the TRI-

MEB and HP β CD powder might incorporate some traces (< 0.25%) of undermethylated and unpropylated products (unpublished data). cEND cell viability was assessed by the amount of adenosine triphosphate (ATP) produced by the metabolically active cells. The released ATP converts the luciferin substrate to luciferin oxide, and the released luminescence signals were recorded.

As the growth of the cEND cells is moderately sensitive to the presence of toxic compounds, the applied methodology allows to discriminate between the effects of highly toxic vs. moderately toxic vs. non-toxic samples. The results of this assay showed the absence of a toxic effect of HP β CD on cEND cells in either DMEM or in human serum after 24 hr of incubation. It was determined that even at the highest HP β CD concentration, this compound is well tolerated by cEND cells, confirming the results described previously in the literature (Gould and Scott, 2005).

Overall, with increased time, a significant difference from the actual cell viability for α -CD and TRIMEB was revealed concerning the median toxic concentration (TC_{50}) calculated as the concentration of substance at which toxicity occurs in 50% of cases. Their luminescence levels remained mainly above the TC_{50} threshold when tested in the $\mu\text{g}\cdot\text{mL}^{-1}$ range, but those levels were reduced dramatically, especially in DMEM at higher concentrations in the $\text{mg}\cdot\text{mL}^{-1}$ range. Moreover, TRIMEB was detected to be highly toxic even at 4.0 $\text{mg}\cdot\text{mL}^{-1}$ in DMEM judging by the lowest P_L value (0.4%). On the other hand, α -CD showed a mild cytotoxic effect at 6.3 $\text{mg}\cdot\text{mL}^{-1}$ in DMEM with the P_L value of 70%. The cytotoxicity effects of α -CD and TRIMEB were started at lesser concentrations in DMEM and only observed at the highest concentrations in the serum, thereby indicating the protective properties of the heat-inactivated human serum. It is possible that some serum proteins, such as albumin, interact with CDs decreasing their bioavailability and toxic effects.

A significant reduction in luminescence activity was also discovered after the treatment with 10% DMSO, which served as a positive control, thereby indicating massive cell death (Fig. 2 [A–C]). Similar results have already been published for the hemolytic effect and Caco-2 cell viability of methylated cyclodextrin derivatives, thus concluding that the incorporation of negatively charged hydroxypropyl groups in the molecule inhibits the cytotoxic effect while the methyl substituents promotes it (Hirayama *et al.*, 1999; Kiss *et al.*, 2010).

Evaluation of cytotoxicity of unmodified and modified cyclodextrins using light microscopy

To confirm the cytotoxic properties of cyclodextrins,

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Fig. 2. cEND cell viability profiles after 24 hr of incubation with α -CD (A), TRIMEB (B) and HP β CD (C) compounds at different concentrations in Dulbecco's modified Eagle's medium (DMEM) and heat-inactivated human serum. The total percentage of luminescence (P_L) is measured to a control group contained untreated cells. 10% solution of dimethyl sulfoxide (DMSO) was used as a positive control. The median toxic concentration is calculated as the concentration of substance at which toxicity occurs in 50% of cases and abbreviated as TC_{50} . The TC_{50} threshold is depicted as dashed line. The data represent as means $\pm$ S.D. of three independent experiments.

the morphological alterations in the cEND cells were investigated. These cells grow in monolayer and have a characteristic morphology that changes substantially following exposure to cytotoxic cyclodextrins. Under light microscopy, it was observed that the untreated cEND cells grew in an unrestricted manner forming confluent monolayers with tight junctions between neighboring cells. After being treated with cyclodextrins at the highest concentration for 24 hr in DMEM, the gaps between the cells became larger and a number of the cells diminished with toxicity levels below the TC_{50} threshold. When the cells were exposed to the α -CD compound at 63 mg $\cdot$ mL $^{-1}$, clear cytotoxic effect was observed in numerous cells probably due to their necrotic and apoptotic changes, and the majority of the cells became lean and thin. When treated with TRIMEB at 40 mg $\cdot$ mL $^{-1}$, the cell morpho-

gy changed significantly followed by massive cell death (Fig. 3 [A]). Similarly, the number of viable cEND cells was evaluated by measuring the P_p values, thereby demonstrating a significant toxic potential of the analyzed cyclodextrins especially for TRIMEB (Fig. 3 [B]).

Prediction of cEND dynamic toxicity profiles for unmodified and modified cyclodextrins

To assess the cEND dynamic toxicity profiles after 24 hr of incubation with the α -CD and TRIMEB substances at highest concentrations in DMEM or heat-inactivated human serum, the logistic dose-response function was transformed according to equations (3) and (4) in order to model the experimentally-determined toxicity profiles as a total percentage of luminescence or pixels. The same toxicity pattern curve has already been observed in a

Fig. 3. Unprocessed and ImageJ-processed light microscopy images taken at different magnifications (A) to measure the total percentage (P_p) of pixels (B) and dynamic toxicity (T_d) profiles (C) for the cEND monolayers. The cells were treated with α -CD and TRIMEB at the highest concentrations for 24 hr in Dulbecco's modified Eagle's medium (DMEM) in comparison to untreated control. The HeLa cytotoxicity data for 9.5 mM doxorubicin (Doxo) is used as experimentally determined reference T_d curve. The median toxic concentration is calculated as the concentration of substance at which toxicity occurs in 50% of cases and abbreviated as TC_{50} . The total percentage of pixels are abbreviated as P_p . The TC_{50} threshold is depicted as dashed line. The data represent as means $\pm$ S.D. of three independent experiments.

wet experiment during the time-lapse recording of HeLa cells with ‘round phenotype’ corresponding to cytotoxicity (Kühn *et al.*, 2013; Rappaz *et al.*, 2014). The percentage of apoptotic HeLa cells was determined after 24 hr of treatment with 9.5 mM of doxorubicin (Doxo), to be implemented as an experimentally-determined reference T_d curve. The T_d parameters demonstrated a strong correlation with the aforementioned data, as shown in Fig. 2, indicating that TRIMEB exhibited greater cytotoxicity than α -CD in DMEM under visual inspection with light microscopy (Fig. 3 [C]). Moreover, it should be noted that, in the case of the methylated CDs (TRIMEB), cell toxicity mainly depends upon the number and position of the methyl groups in the CD molecule (Kiss *et al.*, 2010). From the CellTiter-Glo® viability test analysis results, both cytotoxic cyclodextrins (α -CD/TRIMEB) were found to be more toxic in comparison to the 10% solution of DMSO, as they started to differ from the positive control after 12 hr of incubation in DMEM and after 6 hr in heat-inactivated human serum (Fig. 4 [A, B]). Finally, the maximal cEND toxic levels plateaued after 18 hr of incubation with CDs and after 9 hr of incubation with Doxo.

In silico evaluation of binding affinity to cholesterol for unmodified and modified cyclodextrins

The cholesterol depletion from lipid membranes via its binding to cyclodextrins associated with cytotoxicity is a well-known phenomenon leading to cell lysis (Arima *et al.*, 2004; Ohtani *et al.*, 1989; Fenyvesi *et al.*, 2008). Therefore, we tried to correlate our *in vitro* toxicity data to the CD-cholesterol binding affinity profiles

based on the evaluation of the free energy of binding.

The CD-cholesterol complex can be formed by desolvation of cholesterol into an aqueous environment (‘solvent mediated’) or by its direct desorption from the cellular membrane (‘surface mediated’) into the cyclodextrin cavity (López *et al.*, 2011). To model the first situation, we docked the cholesterol molecule with its hydrophobic tail into the CD amphiphilic interior through the smaller opening where the primary hydroxyl groups were located. In reality, the HP β CD molecule is usually randomly substituted having hydroxypropyl substituents at both the primary and secondary sides at C-2 and C-6 position. To simplify the molecular dynamics simulation, only the primary hydroxyl groups were substituted in the case of HP β CD model.

Assuming implicitly a 1:1 CD-cholesterol stoichiometry ratio, the CD-cholesterol complexes, as starting conformations, were subjected to 50-ns MD simulation with the MM-PBSA approach (Supplementary material). During the first 20-25 ns, the ‘flip-flop’ motion of cholesterol within the CD hydrophobic channel was observed, thus indicating the prolonged ‘equilibration’ phase in the MD simulation. After that, cholesterol was either embedded within the binding cavity of α -CD and TRIMEB with the dimethyl end of the lipophilic tail or it remained unbound to HP β CD floating in the vicinity of the larger opening of cyclodextrin (Fig. 5).

The estimated cholesterol binding energy profiles for unmodified (α -CD) and modified (TRIMEB and HP β CD) cyclodextrins are depicted in Table 1. During the free energy calculation, we observed that the highest affinity to bind cholesterol belongs to TRIMEB ($\Delta G_{\text{bind}} = -7.37$ kcal $\cdot$ mol $^{-1}$), which is in line with our cytotoxicity results.

Fig. 4. cEND dynamic toxicity (T_d) profiles after 24 hr of incubation with α -CD, TRIMEB and DMSO substances at highest concentrations in Dulbecco's modified Eagle's medium (DMEM) (A) or heat-inactivated human serum (B). The HeLa cytotoxicity data for 9.5 mM doxorubicin (Doxo) is used as experimentally determined reference T_d curve. The median toxic concentration is calculated as the concentration of substance at which toxicity occurs in 50% of cases and abbreviated as TC_{50} . The TC_{50} threshold is depicted as dashed line. The data represent as means $\pm$ S.D. of three independent experiments.

Fig. 5. Clustering analysis of cholesterol interaction with unmodified (α -CD) and modified (TRIMEB, HP β CD) cyclodextrins over the last 25 ns of the trajectories. All CD molecules are depicted using the solid surface representation method with a probe radius of 0.4 Å, density isovalue of 0.7 and grid spacing of 0.5 to visualize the binding cavity. CDs are colored according to their atom types. All hydrogen atoms are omitted to enhance clarity.

The HP β CD and α -CD represented no affinity to interact with cholesterol due to the positive free energy of binding values especially for the latter cyclodextrin molecule ($\Delta G_{\text{bind}} = 9.50 \text{ kcal}\cdot\text{mol}^{-1}$).

According to the literature, α -CD does not interact with cholesterol because of the narrow binding cavity size (Shvartsman *et al.*, 2006). Moreover, the β -CD derivatives enhance the solubility of cholesterol while α -CD derivatives do not, concerning the cholesterol-solubilizing effect for the three CDs obtained in the order of TRIMEB $\gg$ HP β CD $\gg \gg$ α -CD (Rajnavölgyi *et al.*, 2014). Therefore, it should be noted that not exclusively cholesterol but also the phospholipids have to be considered in the case of the α -CD complexation as these lipids might stand in the background of observed α -CD toxicity.

Cholesterol clustering analysis revealed different conformational patterns for unmodified and modified cyclodextrins. In particular, numerous cholesterol conformations, deviating greatly from each other, were obtained from the cholesterol/ α -CD inclusion complex with root-mean-square deviation (RMSD) of 4.6 Å because of the narrow α -CD binding cavity. Whereas no significant cholesterol deviations were detected for TRIMEB (RMSD

Table 1. Summary of energetic terms for cholesterol bound into the binding cavity of α -CD, TRIMEB and HP β CD using the MM-PBSA method.

Energy*	α -CD	TRIMEB	HP β CD
ΔG_{bind}	9.50	-7.37	6.47
ΔH	-7.08	-24.9	-12.51
$T\Delta S$	-16.58	-17.53	-18.98
ΔG_{solv}	19.76	3.98	4.09
ΔE_{elec}	-0.05	-0.23	-0.08
ΔE_{vdW}	-26.79	-28.65	-16.52
ΔG_{PB}	8.21	5.23	9.54
$\Delta G_{\text{elec}}^{\text{tot} **}$	8.16	5.0	9.46
ΔG_{NP}	11.55	-1.25	-5.45

*- $\text{kcal}\cdot\text{mol}^{-1}$; **- $\Delta G_{\text{elec}}^{\text{tot}} = \Delta E_{\text{elec}} + \Delta G_{\text{PB}}$

= 1.4 Å), indicating the convergence of the cholesterol structure towards an equilibrium state. The conformational alterations of lipophilic cholesterol in the binding cavity of different cyclodextrins might be explained by very low octanol-water partition coefficients ($\log P$) of α -CD ($\log P = -12.7$) and HP β CD ($\log P = -14.57$) in comparison to the TRIMEB ($\log P = -2.67$) compound (Hazai *et al.*, 2010). Furthermore, the cavity size of the latter molecule is large enough to allow the guest molecule to enter by holding it inside via a low thermodynamic equilibrium constant ($K_c = 3.79 \mu\text{M}$). The K_c parameters for all of the complexes were calculated from the ΔG_{bind} values as follows: $K_c = \exp([\Delta G_{\text{bind}} \cdot 1000]/[R \cdot T])$, where R (gas constant) is $1.98 \text{ cal}\cdot(\text{mol}\cdot\text{K})^{-1}$ and T (room temperature) is 298.15 Kelvin (Shityakov *et al.*, 2012).

On the other hand, enthalpy-entropy compensation might also play an important role in the CD-cholesterol interaction resulting in a net-free energy change. Finally, the negative ΔH and $T\Delta S$ values (Table 1) suggested that the CD-cholesterol interaction is mainly an enthalpy-driven mechanism with vdW interaction as a dominating force. Similar results have been reported for the cholesterol/methyl- β -CD dissociation by Tsamaloukas and coauthors with the standard free energy gain of $\sim -(2.88 \pm 0.24) \text{ kcal}\cdot\text{mol}^{-1}$ of an enthalpic nature and a negligible entropy change ($T\Delta S = -(0.24 \pm 0.72) \text{ kcal}\cdot\text{mol}^{-1}$). The same authors also defined the cholesterol binding to CDs as a phenomenon governed by the hydrophobic effect through the cholesterol occupancy of the CD hydrophobic surface area avoiding its exposure to water (Tsamaloukas *et al.*, 2005).

The role of electrostatic interactions in the cholesterol binding to α -CD, TRIMEB and HP β CD was also eval-

uated via the electrostatic contribution to the free energy of binding and solvation. In particular, the ΔE_{elec} and ΔG_{PB} electrostatic components were only found to play a minor role, which can be neglected. Moreover, all of the analyzed complexes were found to have positive total electrostatic energy ($\Delta G_{\text{elec}}^{\text{tot}}$), so the electrostatic contribution is not favored toward the binding. In agreement with our experimental data, indicating the best aqueous solubility for HP β CD, our calculations demonstrate that this compound has also been found to be highly water-soluble due to its low non-polar solvation energy ($\Delta G_{\text{NP}} = -5.45 \text{ kcal} \cdot \text{mol}^{-1}$). The future perspectives will include the synthesis of drug/CD complexes as subjects for further *in vitro* and *in vivo* pharmacological delivery testing. Additionally, the results of this study will be useful in eliminating CD cytotoxicity in those experiments and pointing towards viable passage through the BBB using primary endothelial cell lines and murine model.

In conclusions, in the current study, we analyzed the cytotoxic effect of the unmodified (α -CD) and modified (TRIMEB and HP β CD) cyclodextrin molecules on immortalized murine microvascular endothelial cells of the BBB. A CellTiter-Glo® viability test performed on the cEND cells revealed significant changes in cytotoxicity for analyzed CD compounds. The cell toxicity levels of α -CD and TRIMEB were higher than the more water-soluble HP β CD, which was detected to be non-toxic. The data also demonstrated that toxic CDs represented greater cytotoxicity in the Dulbecco's modified Eagle's medium than in heat-inactivated human serum indicating protective properties of the latter substance. Overall, the *in vitro* cell toxicity decreases in the following order: TRIMEB (highly toxic), α -CD (intermediate toxicity), HP β CD (nontoxic). Predicted dynamic toxicity profiles for α -CD and TRIMEB confirmed their more prominent cytotoxic effects in comparison to the 10% DMSO reference compound. Atomistic molecular dynamics simulation analysis of cholesterol inclusion by CDs suggested the major role of cholesterol extraction involved in the TRIMEB cytotoxicity. According to the results of this study, HP β CD might be a potential candidate for drug delivery vector development, and a strong correlation exists between the *in vitro* cytotoxic effect and cholesterol complexation of cyclodextrins.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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