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Effects of citicholine and dimethylsulfoxide on transepithelial transport of passively diffused drugs in the Caco-2 cell culture model

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Abstract

The objective of this study was to determine, using a Caco-2 cell monolayer model, the extent to which the paracellular and transcellular routes are altered by citicholine (CDP-Ch) and DMSO in the presence of human serum albumin (HSA). The apparent permeability (Papp) of mannitol in the presence of 4% (w/v) HSA was investigated using 0, 0.5, 1.0, 2.5, 5.0, and 10.0% (v/v)) of DMSO. The Papp for mannitol ranged from 0.56×10^{-6} to 0.89×10^{-6} cm/s (mean 0.77×10^{-6}). Increasing the concentration of DMSO does not appear to have an effect on the paracellular transport of mannitol and on the transepithelial resistance (TEER) of the monolayer, ($P > 0.05$). The effect of citicholine (CDP-Ch) was investigated in confluent Caco-2 cell monolayers incubated in the presence of 2, 4, 10, 40, 60, 100 and 200 mM CDP-Ch at 37 °C in an atmosphere of 7% CO_2 and 95% relative humidity. Papp of mannitol and diltiazem in the presence of CDP-Ch ranged from 0.53×10^{-6} to 8.52×10^{-6} cm/s and from 1.30×10^{-5} to 2.71×10^{-5} cm/s, respectively. CDP-Ch may have an effect on the stability of the tight junction complex resulting in an increase in the apparent permeability of mannitol.

Keywords: Caco-2 cells; DMSO; Citicholine; Paracellular; Transcellular; Albumin

1. Introduction

The intestinal epithelial cell lining forms a boundary between the intestinal tract and the systemic circulation. This boundary is the major barrier for the absorption of orally administered drugs, and is composed of a paracellular and a cellular component. The cellular component comprises the apical or luminal membrane, and the basolateral or albuminal membrane. The cellular component is usually referred to as the transcellular pathway. Although, the surface area is much larger for the transcellular route, the paracellular route is the preferred absorption pathway for many water–soluble and poorly lipid soluble drugs, ionized drugs, and high molecular weight compounds such as horseradish peroxidase that

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can diffuse within the subjunctional paracellular space, but are restricted by the tight junctions (Madara, 1989; Gumbiner, 1987; Powell, 1981). In addition, permeability of the intracellular junctions has been proven to be modulated with different compounds to enhance absorption (Madara, 1992). Transport across the paracellular pathway is controlled by the tight junctions, adherens or intermediate junctions, desmosomes, and gap junctions. However, the tight junctions are the most important in restricting drug transport. Thus, any effect on the tight junction complex or zonula occludens can significantly alter drug transport.

In this study, we have investigated the effects of increasing the concentration of dimethylsulfoxide (DMSO), a cryoprotective agent, in the presence of human serum albumin on the integrity of confluent Caco-2 cell monolayers. DMSO is often used to increase the solubility of highly lipophilic compounds with the purpose of facilitating transport studies through confluent Caco-2 cell monolayers. In vitro studies (Artursson, 1990) demonstrated that when Caco-2 cells monolayers are exposed to more than 1% DMSO, the integrity of the monolayer is lost due to a rapid osmolality increase in the media. To prevent this problem this study examines the effect of human serum albumin (HSA) as a cytoprotective agent. Caco-2 cell monolayers were exposed to several concentrations of DMSO up to 10% (v/v) in the presence of 4% (w/v) HSA in the basolateral side with the purpose of determining the maximum concentration of DMSO that could be used as a co-solvent to help in the solubilization of highly lipophilic compounds without causing cellular damage that may result in structural membrane changes affecting the paracellular and/or the transcellular pathways yielding fictitious permeability measurements. Reports in the literature (Krishna et al., 2001) support the use of 4% HSA and 5% DMSO to study the transport of poorly soluble drugs in the Caco-2 cell model. However, the results of this investigation do not support the increase in mannitol permeability reported by Krishna in the presence of 5% DMSO. In addition, the data in this investigation also supports the use of DMSO up to 10% (v/v) in the apical side in the presence of 4% BSA in the basolateral side.

Citicholine (Cytidine-5-diphosphocholine, CDP-Ch) (Fig. 1) is an essential intermediate in the biosynthetic pathway of structural phospholipids in the cell membrane. CDP-Ch is a mononucleotide composed of ribose, cytosine, pyrophosphate and choline. CDP-Ch has been used as a neuroprotective agent, specifically on the injury pathway that produces cellular membrane breakdown (Frontera and Secades, 1995). Thus, it was postulated that citicholine may have the potential of changing the effective permeability of permeants by changing the structural phospholipids of cell membranes. In summary, this investigation reports the effect of DMSO in the presence of albumin and also of CDP-Ch on the permeability of mannitol, a paracellular marker, and diltiazem, a transcellular marker across confluent Caco-2 cell monolayers.

2. Materials and methods

2.1. Chemicals

[^{14}C]Mannitol (specific activity 51.50 mCi/mmol) and [^{3}H]Diltiazem hydrochloride (specific activity 100 mCi/mmol) were obtained from New England Nuclear Research Products (Boston, MA). All labeled compounds used in the permeability experiments had radiochemical purity greater than 99%. DMSO (sterile, filtered 99% pure), albumin (human, fraction V, 96–99% pure) and cytidine-5′-diphosphocholine (CDP-Ch) (sodium salt from Yeast, 488.3 g/mol as a free acid, 98% pure), were purchased from Sigma, St. Louis, MO. Dulbecco's modified Eagle medium (DMEM, 10×, powder) with 4.5 g/l D-glucose and 584 mg/l L-glutamine was obtained from

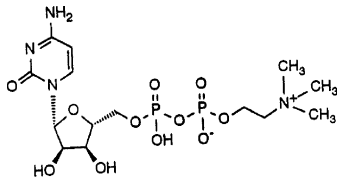

Fig. 1. Structure of citicholine (CDP-Ch).

Sigma. Phosphate buffered saline (PBS, 10 × solution) with calcium and magnesium, and PBS without calcium and magnesium (10 × solution) were obtained from Sigma. Non-essential amino acid solution (NEAA, 10 mM), sodium pyruvate solution, (100 mM), and hydroxyethylpiperazine ethane sulfonic acid (HEPES) were obtained from Gibco-BRL, Life Technologies (Gaithersburg, MD). Fetal bovine serum (FBS, certified), trypsin-EDTA (0.25% trypsin, 1 mM EDTA–4Na), and D-Glucose were obtained from Sigma. Transwell® cell culture chambers (24.5 mm diameter, 3.0 μm pore size, 4.71 cm^2 growth area), six well cluster plates, and cell culture flasks (T-25, T-75, and T162) were obtained from Corning Costar Corporation (Cambridge, MA). All other reagents were analytical grade.

2.2. Cell culture

The Caco-2 cell line originally harvested from a human carcinoma (Fogh et al., 1977) was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells from passage number from 25 to 35 were used for the transport experiment. The details for growing Confluent Caco-2 cell monolayers were previously described (Conradi et al., 1990; Pade and Stavchansky, 1997). Briefly cells were grown in normal tissue culture flasks (Costar, Corporation) in culture medium consisting of DMEM with 1% NEAA and 10% FBS. Before reaching confluency, cells were trypsinized with trypsin-EDTA solution and plated at a density of 63 000 cells/cm^2 in a culture medium on Transwell polycarbonate membranes (3.0 μm pore size, 4.71 cm^2 surface area). The culture medium was replaced (1.5 ml for apical, 2.5 ml for basal side) every other day for the first week and daily thereafter. Cells were maintained at 37 °C in an atmosphere of 7% CO_2 and 95% relative humidity.

2.3. TEER measurements

The complexity and the number of tight-junction strands generally correlate with the electrical resistance of the barrier. Thus, the integrity of the monolayer was evaluated by measuring the transepithelial electrical resistance (TEER) (expressed as Ω cm^2) using a Millicel-ERS Resistance System and silver/silver chloride electrodes (Millipore, Badford, MA). The TEER of the Caco-2 cell monolayers was measured 30 min before the transport studies. The TEER values were in the range of 300–430 Ω cm^2. These values take in to account the TEER measurements of the Transwell® cup without the monolayer, but in the presence of the transport buffer.

2.4. Transport studies

All transport experiments were conducted at 37 °C in an atmosphere of 7% CO_2 and 95% relative humidity conditions. The transport buffer consisted of a phosphate buffer saline at pH 7.2 (PBS), 1 g/l D-glucose and 15 mM HEPES. Due to the high glucose concentrations the transport medium was prepared daily. Prior to the experiments the culture medium of the Transwell®-grown Caco-2 cell monolayers were replaced with the transport medium by a mild washing step, and cell monolayers subsequently equilibrated for 30 min at 37 °C before undertaking the transport studies.

In the apical to basolateral (A → B) transport studies all wells in six well clusters received 2.5 ml of transport medium that had been previously equilibrated at 37 °C. While evaluating the effects of DMSO on the cell monolayer, 4% (w/v) human serum albumin was added to the transport medium. Transport experiments were conducted from apical to basolateral direction at pH 7.2, with 30 min sampling time interval. For the donor chamber, 1.5 ml [^{14}C]Mannitol (paracellular marker) solution (at the concentration of 1.604×10^{-4} mg/ml = 1.55×10^5 dpm/ml) was prepared with 0, 0.5, 1.0, 2.5, 5.0, and 10.0% (v/v) DMSO in transport medium at pH 7.2. For the receiver chamber, 2.5 ml of transport buffer was mixed with 4% (w/v) human serum albumin (fraction V).

The effect of CDP-Ch on the transepithelial transport was studied using mannitol and diltiazem hydrochloride (diltiazem-HCl) as paracellular and transcellular markers, respectively. The transport solution was prepared in the transport medium at pH 7.2 and contained [^{14}C]Mannitol

(4.14×10^{-5} mg/ml) and [^{3}H]Diltiazem-HCl (3.73×10^{-6} mg/ml). Prior to the transport experiments, the cell monolayers were washed with freshly prepared marker-free transport medium (pH 7.2) at 37 °C for 15 min. After this mild washing process, cell monolayers were incubated with different CDP-Ch concentrations: of 0, 2, 4, 10, 40, 60, 100, and 200 mM, for 1 h at 37 °C, 95% relative humidity, and 7% CO_2. After the incubation, transport experiments were conducted from the apical to the basolateral (A → B) direction at pH 7.2, with a 20-min sampling time interval.

Samples obtained during the transport studies were analyzed using a Beckman LS5000 model liquid scintillation counter. The amount of drug transported to the receiver side was normalized to account for loss in the apical concentration during each time interval. The effective permeability coefficients were calculated using Eq. (1).

$$\mathrm{Pe} = \left(\frac{V_\mathrm{d}}{A}\frac{\Delta\%}{\Delta t}\right) \quad (1)$$

where, Pe is the effective permeability coefficient in cm/s, V_d is the volume (cm^3) in the donor compartment, A is the surface area of the monolayers, 4.71 cm^2, and $\Delta\%/\Delta t$ is the percent mass transported per time interval. Finally mass balance calculations were determined to explore metabolism or adsorption to surfaces of the apparatus. The average recoveries from the apical to basolateral direction with and without the monolayer were found to be approximately 95.3 and 96.1%, respectively.

3. Results and discussion

Fig. 2 shows the apparent permeability of mannitol at five different concentrations of DMSO in the presence of 4% (w/v) human serum albumin in the basolateral side. No significant changes were observed on TEER values measured before and after the transport experiments. The apparent permeability coefficients of mannitol in the presence of 0, 0.5, 1.0, 2.5, 5.0, and 10.0% DMSO and 4% albumin ranged between 0.56×10^{-6} and 0.89×10^{-6} cm/s (mean $0.77 \times 10^{-6} \pm 0.11 \times 10^{-6}$ cm/s). No statistically significant differences were observed among the apparent permeability coefficients of mannitol in the presence and absence of DMSO ($P > 0.05$).

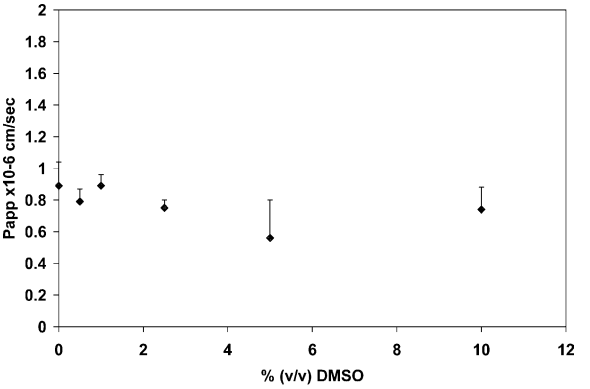

Fig. 2. Effect of DMSO on the apparent permeability of (Papp ± S.D.) of mannitol.

Fig. 3 shows the apparent permeability of mannitol, and diltiazem after a one hour incubation of 21-day-old Caco-2 cell monolayers with increasing concentrations of CDP-Ch (2, 4, 10, 40, 60, 100 and 200 mM). CDP-Ch has no effect on the permeability of the transcellular marker, diltiazem, while increasing the permeability of the paracellular marker, mannitol. The apparent permeability of the paracellular marker, mannitol, ranged between 0.53×10^{-6} and 8.52×10^{-6} cm/s, while that of the transcellular marker, diltiazem-HCl, ranged between 1.30×10^{-5} and 2.71×10^{-5} cm/s. There is a statistically significant difference in the apparent permeability coefficients of man-

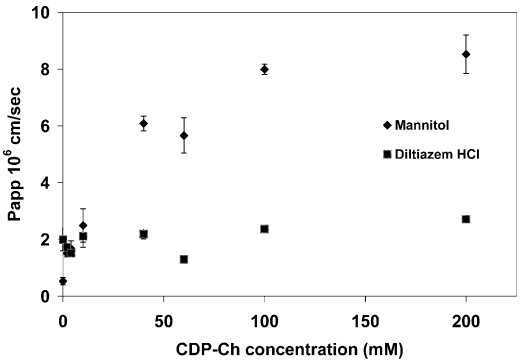

Fig. 3. Effect of CDP-Ch on the transport of mannitol and of diltiazem HCl (Papp ± S.D.).

nitol between the control and the CDP-Ch treated cell monolayers ($P < 0.05$, $P = 0.00412$). On the other hand, no statistically significant differences were observed among the apparent permeability coefficients of diltiazem hydrochloride for treated and untreated cell monolayers ($P > 0.05$).

The permeation enhancing effect of CDP-Ch may be explained in terms of membrane changes that may influence the tight junction complex disrupting its stability. This would increase tight junction permeability resulting in an increase in the apparent permeability of mannitol. Proteins associated with the tight junctions, for example, proteins in the ZO-1 multiprotein complex, could undergo conformational changes resulting in changes in their interaction with occludin (Furuse et al., 1993), a transcellular component of the tight junction. This could also result in changes in the apparent permeability of mannitol.

The Caco-2 cell model has been shown to be a valuable in vitro model of the intestinal mucosa (Artursson, 1990; Conradi et al., 1990; Wilson et al., 1990). When grown onto polycarbonate membranes, Caco-2 cells undergo differentiation ultimately exhibiting morphological and biochemical characterization similar to those observed in villus epithelial cells (Raub et al., 1989). The results of this investigation expand the use of the Caco-2 cell model to study transepithelial transport of highly lipophilic drugs solubilized in DMSO. The cytoprotective action of human serum albumin 4% (w/v) from the permeation altering effects of DMSO were successfully investigated using this model. Previous studies conducted with $>1\%$ (v/v) DMSO in the absence of albumin reported that DMSO rapidly penetrates cells causing rapid and dramatic changes in osmolality. Therefore, cells shrink almost instantaneously in effort to adapt to osmolality changes (McGann, 1988; Wolfinbarger and Hu, 1994). The results of the present transport studies with DMSO in the presence of 4% (w/v) HSA suggest that increasing the concentration of DMSO (0, 0.5, 1, 2.5, 5, and 10%; v/v) did not have a statistically significant ($P > 0.05$) effect on the paracellular transport of mannitol through the Caco-2 cell monolayers. In addition, no significant change was observed on TEER values measured before and after the transport experiments. The observed protective action of albumin can be explained in terms of forming a glycocalyx type coating on the basolateral cell membrane, and/or counteracting the osmotic pressure changes. In addition, Krishna et al. (2001) clearly demonstrates that inclusion of bovine serum albumin provides the necessary absorptive driving force reflecting in vivo sink conditions improving both recovery and the Papp of lipophilic and highly protein bound compounds. However, our results do not agree with the slight increase in permeability of mannitol in the presence of 5% DMSO in the apical side and 4% BSA in the basolateral side reported by Krishna. In fact, the results obtained with 10% DMSO do not support a significant change in the permeability of mannitol.

In conclusion, these studies and the work by Krishna clearly demonstrate the cytoprotective action of 4% human serum albumin from DMSO. This may facilitate the use of the Caco-2 cell model when using DMSO as a co-solvent to solubilize highly lipophilic molecules. Finally, the observed increases in paracellular permeability of mannitol in the presence of CDP-Ch suggest the use of CDP-Ch as a membrane modifier to increase the transport of macromolecules through the paracellular pathway.

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