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Comparison of different intestinal epithelia as models for absorption enhancement studies

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Abstract

In this study we compared the effect of two surfactants (laureth-6 and sodium docusate) on the permeability of a model hydrophilic drug across three different epithelia (Caco-2 cells, stripped porcine jejunum and rat ileo-jejunum). Among the tested epithelia Caco-2 cells are the tightest with the trans-epithelial electrical resistance of $372 \pm 4\ \Omega\ cm^2$ followed by porcine jejunum ($124 \pm 8\ \Omega\ cm^2$) and rat ileo-jejunum ($33 \pm 2\ \Omega\ cm^2$). Both surfactants decreased the trans-epithelial electrical resistance and increased the permeability of a model drug across Caco-2 cells at concentrations as low as 0.02 mg/ml, with more pronounced effect observed for laureth-6. On the other hand, ten times higher concentrations (0.2 mg/ml) did not affect the permeability of the model drug across the porcine jejunum. Similarly, laureth-6 at this high concentration had no effect on the trans-epithelial electrical resistance of the rat ileo-jejunum and did not increase the permeability of the model drug across this tissue. On the basis of these results we concluded that Caco-2 cells are much more sensitive to the investigated surfactants, that act as permeation enhancers, than the native intestinal tissues. Therefore, the results obtained in the experiments with Caco-2 cells might exaggerate the effects of the surfactants on the permeability compared to in vivo situation.

Keywords: Absorption enhancement; Caco-2 cells; Rat ileo-jejunum; Porcine jejunum; Trans-epithelial electrical resistance; Surfactants

1. Introduction

Many hydrophilic drugs such as bisphosphonate drugs, proteins, peptides and peptide-like drugs are poorly absorbed from the gastrointestinal tract, due to low permeability across the intestinal epithelium. One approach to improve the permeability of these drugs is co-administration of absorption enhancers, including surfactants, bile salts, calcium chelating agents, fatty acids, cyclodextrins, chitosans and other mucoadhesive polymers (Junginger and Verhoef, 1998; Aungst, 2000; Zadravec et al., 2000). These substances promote the permeability of poorly permeable drugs mainly by opening the tight junctions, leading to the increased

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paracellular permeability (Ward et al., 2000). Besides that, it was shown that several surfactants often used as pharmaceutical excipients could improve the permeability in the absorptive direction by the inhibition of secretory transporters including P-glycoprotein and several multidrug resistance associated proteins (MRPs) (Lo, 2003).

The main problem of absorption enhancers is the narrow concentration range between their ability to increase the intestinal permeability and cytotoxicity (Ward et al., 2000). In general, a positive correlation between the toxic and the enhancement effects is established (Quan et al., 1998). Different models of intestinal epithelia such as Caco-2 cells and rat native intestine are used for studying the effectiveness and toxicity of various absorption enhancers (Aungst, 2000). The concentrations of absorption enhancers that is needed to improve the permeability of polar drugs across the native intestine are usually very high (>10 mM) (Yamamoto et al., 1996; Sugiyama et al., 1997; Lo and Huang, 2000), while the absorption enhancers promote the permeability of drugs across Caco-2 cells at much lower concentration (Quan et al., 1998). These observations suggest that Caco-2 cells are much more sensitive to the absorption enhancers than the native intestinal tissue. Therefore, it is possible that the conclusions drawn out from the experiments with Caco-2 cells might not adequately reflect the activity of certain absorption enhancers in the native intestinal tissue.

The purpose of the present study was to compare the effects of two surfactants (laureth-6 and sodium docusate) on the permeability of a model hydrophilic drug across the different models of intestinal epithelia. For that purpose Caco-2 cell monolayers, stripped porcine jejunum and rat ileo-jejunum were used. Sensitivity of these models with different transepithelial resistance to the studied absorption enhancers was investigated.

2. Materials and methods

2.1. Materials

Stable model hydrophilic drug ($c\log P = -1.87$; $pK_a = 2.8$ (acid)), was obtained from Lek (Ljubljana, Slovenia). Fluorescein sodium was obtained from Fluka (Deisenhofen, Germany). Laureth-6 (dodecyl hexaoxyethylene monoether) and sodium docusate (dioctylsulfosuccinate, sodium salt) were supplied by Sigma (Deisenhofen, Germany).

2.1.1. Cell culture chemicals

Dulbecco's modified eagles medium (DMEM) with supplements was used for the Caco-2 culture. DMEM, non-essential amino acids and gentamicine sulphate were obtained from Biochron KG (Berlin, Germany). Trypsin–EDTA solution was obtained from Sigma (Deisenhofen, Germany). Fetal calf serum was supplied by Greiner Labortechnik (Frickenhause, Germany). For the transport experiments Krebs–Ringer bicarbonate buffer with pH 7.4, containing 25 mM of D-glucose (KRB) was used.

2.2. Experiments with Caco-2 cells

Caco-2 cells were obtained from the German Cell Culture Collection DSMZ, DSMZ-no. ACC 169. The cells were cultured at 37 °C, 90% humidity and 10% CO_2. For the transport studies 10^4 cells/cm^2 were sown on clear polyester transwell filters (Costar, Germany) with an area of 1.13 cm^2 and 0.4 μm pore size.

Before the transport experiments, the cells were washed twice with KRB to remove the cell culture medium. Fresh pre-warmed KRB and the transport solution were added to the acceptor compartment (1.5 ml) and donor compartment (0.5 ml), respectively. The transport solution was prepared by dissolving the model drug (350 μM, final concentration) with and without surfactants in the DMSO (1% in the final volume) and diluted with fresh KRB. DMSO at such low concentrations is known to not affect the integrity of Caco-2 cells (Yamashita et al., 2000) or the integrity and viability of excised rat jejunal segments (Watanabe et al., 2000).

The monolayers were pre-incubated for 35 min to saturate the transport sites in the test system with the model drug. Afterwards, the KRB was removed completely from the acceptor compartment and replaced by fresh KRB (start of transport). Twenty-five millimolars D-glucose was present in the incubation medium at the apical and basolateral side throughout the experiments. During the transport study samples were taken from the acceptor compartments at defined time points (60, 120, 180 and 240 min). Between the

sampling points, the monolayers were incubated in a CO_2 incubator.

The trans-epithelial electrical resistance (TER) of Caco-2 cell monolayers were measured at the start and at the end of the experiments by EVOM® - measurement device.

2.3. Experiments with stripped porcine jejunum

The porcine jejunum was obtained from a local slaughterhouse. Immediately after slaughter of pigs (body weight: 80–100 kg), separate pieces were dissected from the jejunum, washed with a 0.9% NaCl and stored in ice cold KRB. The serosa and overlaying longitudinal and circular muscle layers were stripped off with blunt dissection. The stripped tissue was mounted between the two halves of six Ussing chambers (Scientific Instruments, Aachen, Germany) with an exposed area of 1 cm^2. Five milliliters of fresh warmed (37 °C) KRB was added to each chamber compartment. The solution in the chambers were oxygenated with carbogen gas (95% O_2/5% CO_2) and kept at 37 °C. A stabilization time of 10 min allowed the system to equilibrate. Afterwards, the superficial mucus was removed by incubating the apical side with 10 mM acetylcysteine for 10 min. Subsequently, the acetylcysteine solution was rinsed off twice by washing and total removing of the KRB. For the transport study the apical buffer was replaced by the transport solutions. The transport solution was prepared by dissolving the model drug (1.19 mM, final concentration) with (0.2 mg/ml) or without surfactants in the DMSO (1% in the final volume) and diluted with fresh KRB. Aliquots were sampled from the serosal (acceptor) side every 30 min during 120 min. The samples were replaced by an equal amount of fresh KRB. 25 mM D-glucose was present in the incubation medium at the mucosal and serosal side throughout the experiments.

For the determination of the tissue resistance a bipolar pulse (amplitude: 50 μA) was applied every 60 s for a time of 200 ms by computer controlled voltage–current clamp apparatus (Scientific Instruments, Aachen, Germany).

2.4. Experiments with rat ileo-jejunum

The experiments conform to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe No. 123, Strasbourg 1986).

The experiments with the rat ileo-jejunum were performed in a manner similar to that as described previously (Legen and Kristl, 2003). Ileo-jejunum (25 cm proximal to the ileocecal junction) was excised from male Wistar rats (250–20 g) which had free access to a standard laboratory chow and tap water until 18 h before the experiment. After decapitation, the small intestine was immediately excised and, prior to tissue preparation, placed into an ice-cold bubbled (carbogen, 95:5 O_2/CO_2) 10 mM solution of D-glucose in standard Ringer buffer pH 7.51. The tissue was rinsed with ice-cold standard ringer buffer to remove luminal contents and cut into 3 cm long segments, excluding visible Peyer's patches. The serosa and the overlaying longitudinal and circular muscle layers were not stripped off because these layers are too thin to be properly removed without damaging the mucosal layer. The intestinal segments were opened along the mesenteric border, mounted onto a special insert and placed between two EasyMount side-by-side diffusion chambers with an exposed tissue area of 1 cm^2 (Physiologic Instruments, San Diego, USA).

During the experiment the tissue was incubated on both sides with Ringer buffer containing 10 mM D-glucose at the serosal and 10 mM D-mannitol at the mucosal side and gassed with carbogen at 37 °C. After 25 min of equilibration the substance under investigation (2 mM) was added to the mucosal side with (0.2 mg/ml) or without the investigated surfactant. Samples of 250 μl were withdrawn from the acceptor compartment at 25 min intervals up to 175 min and replaced by fresh Ringer buffer containing 10 mM D-glucose.

The tissue viability and integrity were checked by monitoring potential difference (PD), short circuit current (I_{sc}) and trans-epithelial electrical resistance (TER) by a multichannel voltage–current clamp (model VCC MC6, Physiologic Instruments, USA) every 25 min and additionally by recording the increase of I_{sc} and PD after the addition of D-glucose (25 mM) to the mucosal compartment at the end of experiments. TER was determined according to Ohm's law.

The tissue integrity during transport studies was also validated by measuring the permeability of a hydrophilic transport marker, fluorescein sodium, with a donor concentration of 5 μM.

2.5. Analytical procedures

The concentrations of model drug in the samples from the transport experiments were analysed by validated HPLC methods with UV detection. In the case of Caco-2 cells and porcine jejunum Waters alliance 2790 with photodiode array detector was used. A column Symmetry C-18 (3.5 μm, 75 mm × 4.6 mm) (Waters, Eschborn, Germany) was used at 35 °C. The mobile phase consisted of NaH_2PO_4 (pH 3.5) and acetonitrile with a gradient delivery. In the case of rat ileo-jejunum Hewlett Packard Series 1100 was used. A Eurospher C-18 column (5 μm, 250 mm × 4 mm) (Bia Separations, Ljubljana, Slovenia) was used at 25 °C. The mobile phase consisted of 5% acetonitrile and 95% NaH_2PO_4 (pH 4.4) with an isocratic delivery. Concentrations of fluorescein were analyzed by fluorescence (λ_{EX} 485 nm, λ_{EM} 535 nm) using a microplate reader (Tecan, Salzburg, Austria).

2.6. Data analysis and statistics

Apparent permeability coefficients (P_{app}) were calculated according to the equation:

$$P_{app}\,(\mathrm{cm/s}) = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

where dQ/dt is the steady-state appearance rate on the acceptor side of the tissue, A is the exposed area of the tissue, and C_0 is the initial concentration of the drug in the donor compartment.

Results are expressed as mean ± S.E.M. Two-group comparisons were analysed by unpaired two-tailed *t*-test or paired Student's two-tailed *t*-test. In the case of unpaired *t*-test, F-test for variances was first applied. If the variances were equal, the standard Student's *t*-test was performed otherwise Behrens–Fisher test was used.

3. Results and discussion

Different surfactants are often included in the oral solid dosage forms in order to improve their properties, such as wetting and dissolution. It is often important to know whether these surfactants at concentrations that are achieved in the intestinal lumen enhance the permeability of the active ingredient. For the permeation enhancement studies, different models of the intestinal epithelium are being used such as Caco-2 cells and rat intestine (Sugiyama et al., 1997; Rege et al., 2002; Lo, 2003), but it seems that these models differ from each other regarding the sensitivity to the permeation enhancers. In the present study, we compared three different intestinal epithelia as models for the absorption enhancement studies.

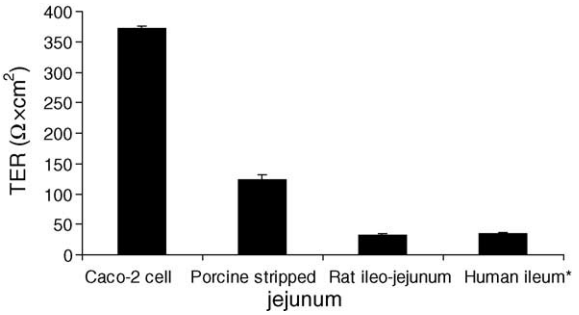

Fig. 1. Trans-epithelial electrical resistance (TER) of different intestinal epithelia. Results are expressed as the mean ± S.E.M. of five to twenty-one experiments. (*) Data are taken from Söderholm et al. (1998).

Among the studied intestinal epithelia the Caco-2 cell monolayers have the highest trans-epithelial electrical resistance (TER), followed by stripped porcine jejunum and rat ileo-jejunum (Fig. 1). The TER of the rat ileo-jejunum is similar to that of the human ileo-jejunum, indicating the similar tightness and/or number of the tight junctions. Since many absorption enhancers act by modulating tight junctions (Ward et al., 2000), the rat ileo-jejunum might, therefore be the most appropriate for the absorption enhancement studies among the studied intestinal epithelia.

It is very important that the permeation enhancement effect of surfactants is studied at the concentrations that are achieved by dissolving in gastrointestinal fluids after oral applications of solid dosage form. Usually, 250 ml is considered as an adequate volume of gastrointestinal fluids (Aungst, 2000). In the Caco-2 cell model laureth-6 and sodium docusate at concentrations 0.2 mg/ml (corresponding to 50 mg of the surfactant in the dosage form) markedly decreased TER to approximately 12% of the initial value (from 350 to 45 Ω cm^2 in 120 min), suggesting that the viability and consequently the integrity of Caco-2 cells were markedly affected. Therefore, the effects of these two surfactants on the permeability of the model drug were studied at

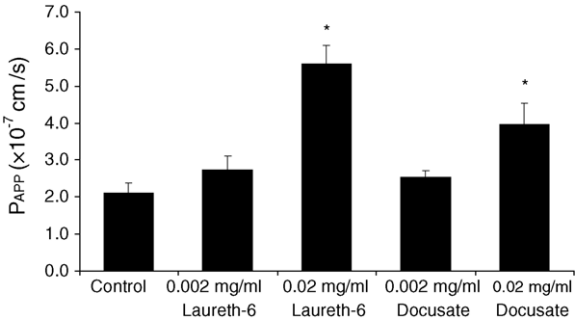

Fig. 2. Effects of different concentrations of laureth-6 and sodium docusate on the apparent permeability of a model drug across the Caco-2 cells. Results are expressed as the mean ± S.E.M. of three experiments. (*) Significantly different from the control experiment ($p < 0.05$).

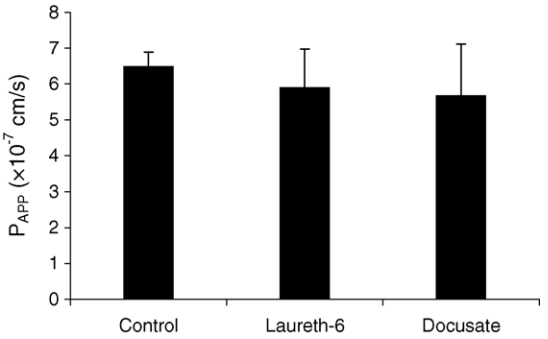

Fig. 4. Effects of laureth-6 (0.2 mg/ml) and sodium docusate (0.2 mg/ml) on the apparent permeability of a model drug across the stripped porcine jejunum. Results are expressed as the mean ± S.E.M. of three experiments.

much lower concentrations (Figs. 2 and 3). At concentrations 0.002 mg/ml both tested permeation enhancers, laureth-6 and docusate did not significantly increase the permeability of the model drug, while 10-times higher concentrations significantly increased the permeability compared to the control with the more pronounced effect observed for laureth-6 (Fig. 2). Correspondingly, lower concentrations of surfactants did not significantly affect TER compared to the control experiments, while higher concentrations significantly decreased TER (Fig. 3). Since the electrical conductivity measured across Caco-2 monolayers is almost entirely a result of paracellular ion flux (Brown et al., 2002), these results demonstrate that the investigated surfactants increased the transport of a model drug across

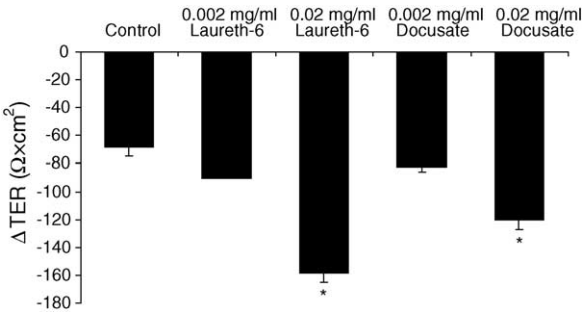

Fig. 3. Effects of different concentrations of laureth-6 and sodium docusate on the transepithelial electrical resistance of Caco-2 cells. Δ TER is the difference between the TER at the end (time 240 min) and at the start of the experiments (time 0 min). Results are expressed as the mean ± S.E.M. of three experiments. (*) Significantly different from the control experiment ($p < 0.05$).

Caco-2 monolayers by increasing the paracelullar permeability.

On the other hand, laureth-6 and docusate showed no significant effect on the permeability of a model drug across the stripped porcine jejunum although ten times higher concentrations (0.2 mg/ml) than in Caco-2 cells were used in these experiments (Fig. 4). This suggests that the native porcine jejunum is much more resistant to the action of surfactants than Caco-2 cells.

In accordance to the experiments with porcine jejunum, no significant effect of laureth-6 at high concentration (0.2 mg/ml) on the permeability of the model drug across the rat ileo-jejunum was observed (Fig. 5). Similarly, this surfactant did not affect the permeability of fluorescein (Fig. 5), which is a known marker for the paracellular permeability (Legen and Kristl, 2002). Ad-

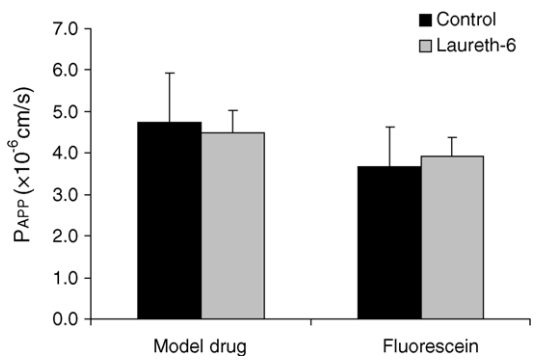

Fig. 5. Effects of laureth-6 (0.2 mg/ml) on the apparent permeability of a model drug and fluorescein across the rat ileo-jejunum. Results are expressed as the mean ± S.E.M. of three experiments.

ditionally, no influence on the TER of rat ileo-jejunum was observed due to the presence of laureth-6 at the mucosal solution ($31 \pm 6\,\Omega\,cm^2$, and $34 \pm 6\,\Omega\,cm^2$ for the control and laureth-6 treated tissue, respectively). Furthermore, this surfactant did not affect the tissue viability evaluated by measuring potential difference (-2.1 ± 0.8 mV and -1.8 ± 0.5 mV for the control and laureth-6 treated tissue, respectively) and short-circuit current ($63 \pm 15\,\mu A/cm^2$ and $54 \pm 9\,\mu A/cm^2$ for the control and laureth-6 treated tissue, respectively). These results clearly demonstrate that laureth-6 did not affect the paracellular permeability of the rat ileo-jejunum.

It must be pointed out that different buffer systems and different chambers were used in the experiments with Caco-2 cells, porcine jejunum and rat ileo-jejunum. The conditions for each experiment were such that enable the best performance of each intestinal epithelium in the light of its viability, based on the previous experiences with each system. It is not likely that different simple buffer systems or different chambers might affect the interaction of the investigated surfactants with the studied intestinal epithelia.

On the basis of the results from this study it can be concluded that Caco-2 cells are much more sensitive to the action of laureth-6 and docusate than the native intestinal segments. We showed that as low as 0.02 mg/ml of the investigated surfactants decreased TER of Caco-2 cells and increased the permeability of a model drug across these cells. On the other hand, ten times higher concentrations of surfactants have no significant effect on the permeability of a model drug across the native intestinal tissues. This is in accordance with the results of Tanaka et al. (1995) who showed that 10 mM EDTA, a calcium chelating agent, increased the permeability of FITC-dextran in Caco-2 cells much stronger than in the rat jejunum (35-times and 2.4-times in Caco-2 cells and rat jejunum, respectively). Therefore, the results obtained from the studies with Caco-2 cells might exaggerate the effects of the surfactants on the permeability compared to in vivo situation.

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