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Sucrose Esters Increase Drug Penetration, but Do Not Directly Inhibit Efflux Pumps in Caco-2 Intestinal Epithelial Cells

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ABSTRACT: Sugar fatty acid esters are increasingly used as excipients in pharmaceutical products, but few data are available on their toxicity profile, mode of action and efficacy on intestinal epithelial models. Three water soluble sucrose esters, palmitate (P-1695), myristate (M-1695), laurate (D-1216), and two reference absorption enhancers, Tween 80 and Cremophor RH40 were tested on Caco-2 cells. Caco-2 monolayers formed a good barrier as reflected by high transepithelial resistance and positive immunostaining for junctional proteins claudin-4, ZO-1 and β-catenin. Sugar esters in non-toxic concentrations significantly reduced resistance and impedance, and increased permeability for atenolol, fluorescein, vinblastine, caffeine, antipyrine and rhodamine 123 in Caco-2 monolayers. No junctional opening was induced by sucrose esters assessed by immunohistochemistry and electron microscopy, but some alterations were seen in the structure of F-actin microfilaments. It was demonstrated for the first time that sucrose esters fluidize the plasma membrane of living epithelial cells in low concentrations. They enhanced the accumulation of efflux transporter ligands rhodamine 123 and calcein AM in cells when co-administered, but not in pretreatment. These data indicate that in addition to their dissolution increasing properties sugar esters can enhance drug permeability through both the transcellular and paracellular routes without direct effect on efflux pumps.

Keywords: absorption enhancer, Caco-2 cells, drug permeability, efflux pumps, epithelial, excipients, membrane fluidity, sucrose ester, tight junction, toxicity

INTRODUCTION

Drug delivery across biological barriers, like intestine or blood-brain barrier remains a great challenge in pharmaceutical research. One of the options to improve permeability of low penetrating active agents is the use of absorption enhancers.[1] Among the non-ionic surface active agents used for this purpose ethoxylated sorbitans (e.g. Tween 80) and castor oil esters (e.g. Cremophor RH40) have been widely investigated and were demonstrated to enhance the solubility or absorption of drugs.[2,3] Clinically employed surfactants applied in pharmaceutical industry as absorption enhancers have several advantageous properties[4] but can also cause side-effects *in vivo*.[4-6] There is a need for new, innovative absorption enhancers with more favourable profile and fever drawbacks.

Sucrose fatty acid esters are non-ionic surfactants which are present naturally in plants and microorganisms.[7] For industrial purposes they are synthesized by esterification of fatty acids with sucrose.[8] Food industry applies them as food emulsifiers and food additives for breads, cakes, pastas, noodles, baby's foods, ice creams in the last 40 years.[9,10] Cosmetic industry also employs them because of low skin irritation, excellent emulsification and solubilizing properties.[11]

Sugar esters are promising candidates for improving the solubility and permeability of active agents in pharmaceutical technology as recently reviewed by Szűcs and Szabó-Révész.[12] In recent decades sucrose esters due to their excellent emulsifier and surfactant properties were tested for transdermal drug delivery using various formulations including microemulsions and reversed vesicles.[13-17] These surfactants form micelles[18] and have also been used to prepare nanovehicles, like nanodispersions, nanosuspensions and niosomes.[19-22] The use of sucrose esters for oral application was less studied. Controlled release from matrix tablets containing sucrose esters was demonstrated.[23] Daunomycin accumulation was effectively enhanced by sugar esters in Caco-2 intestinal cells.[24] In animal studies sucrose esters improved the intestinal permeability of different types of active agents, like the polypeptide hormone calcitonin,[25] lidocaine hydrochloride[26] and cyclosporine A.[27] The intestinal absorption of water soluble paracellular marker dextran was also enhanced by L-1695 laurate ester treatment in rats.[28] Although the paracellular barrier in intestinal cells is regulated by intercellular junctions[4] changes in tight or adherens junctions following sucrose fatty acid ester treatments have not been investigated yet, and the affected transport pathways were not elucidated. Sugar esters were also described to inhibit efflux pump proteins, mainly P-glycoprotein (P-gp, ABCB1) in epithelial cells and in animal experiments.[29,30] While the

effect of sucrose esters on cell membranes was hypothesized[24] it was not investigated in living cells yet.

The human Caco-2 cell line is a widely used model of the epithelium of small intestine. Cells are polarised, grow in monolayer, possess microvilli, make tight connections with adjacent cells by their tight and adherens junction structures and express many nutrient and efflux transporters such as P-gp.[31-33] Caco-2 cells are widely used to measure drug permeability and data correlate well with *in vivo* drug absorption, mostly in case of passive transcellular pathway.[31,34,35]

Our aim was to test the effects of three different, palmitate (P-1695), myristate (M-1695) and laurate (D-1216) sucrose esters on the viability, passive permeability, junctional and cytoskeletal morphology, membrane fluidity and efflux pump activity of Caco-2 intestinal epithelial cells and compare with reference absorption enhancers Tween 80 and Cremophor RH40. The toxicity of surfactants was determined by standard colorimetric end-point assays. The paracellular barrier properties were tested by resistance and real-time impedance measurements. To assess passive as well as efflux transport across epithelial monolayers the following markers and drugs were examined in the presence or absence of surfactants: fluorescein, atenolol, caffeine, antipyrine, vinblastine and rhodamine 123. Cellular and junctional morphology in sugar ester-treated Caco-2 cells was monitored by immunostaining for junctional proteins claudin-1, zonula occludens protein-1 (ZO-1), β-catenin and transmission electron microscopy for the first time to our best knowledge. Arrangement of filamentous actin (F-actin) a main cytoskeletal protein contributing to normal organization of tight and adherens junctions was also investigated after treatments with absorption enhancers. Plasma membrane fluidity in living Caco-2 cells in the presence of surfactants was also checked for the first time. Rhodamine 123 and calcein AM fluorescent markers were used for efflux pump experiments.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich Ltd., Hungary, unless otherwise indicated. Laurate sucrose ester (D-1216) was of pharmaceutical grade, palmitate (P-1695) and myristate (M-1695) sucrose esters were of analytical grade (Mitsubishi Kagaku Foods Co., Tokyo Japan). Tween 80 and Cremophor RH40 (BASF, Ludwigshafen am Rhein, Germany)

were of pharmaceutical grade. Table S1 summarizes some of properties of the surfactants employed in the study.

Cell culture

Human intestinal epithelial Caco-2 cell line (ATCC cat.no. HTB-37, USA) was used in the experiments. To obtain a more uniform morphology and higher efflux pump expression cells were treated with vinblastine (10 nM) for at least 6 passages as described by Hellinger et al. (2010).[34] Cells were grown in Eagle's minimal essential medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Lonza, Basel, Switzerland), sodium-pyruvate (Gibco, Life Technologies, Carlsbad, CA, USA) and 50 µg/ml gentamicin in a humidified 37°C incubator with 5 % CO_2. Cells were seeded in culture dishes at a density of 5×10^4 cells/cm^2 and the medium was changed every 2 days. When cells reached approximately 80 - 90 % confluence in the dish they were subcultured with 0.05 % trypsin-EDTA solution. For the cytotoxicity assays cells were cultured in 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and for real-time cell electronic sensing (RT-CES) 96-well plates with gold electrodes (E-plate 96, ACEA Biosciences, San Diego, USA) were used. For electric resistance measurements and permeability studies Caco-2 cells were cultured on Transwell inserts (polycarbonate membrane, 0.4 µm pore size, 1.12 cm^2 surface area, Corning Life Sciences, Tewksbury, MA, USA). For staining of nuclei, junctions and F-actin cells were grown on glass coverslips (Menzel-Gläser, Braunschweig, Germany). All surfaces were coated with 0.05 % rat tail collagen before cell seeding, unless otherwise indicated.

Cell viability measurements

Treatment doses of sucrose esters P-1695, M-1695 and D-1216 varied between 3-3,000 µg/ml. The reference absorption enhancers were used in 1-100,000 µg/ml concentrations. Treatment solutions were prepared in Dulbecco's Modified Eagle Medium without phenol red (DMEM; Life Technologies, Carlsbad, CA, USA). Triton X-100 in 10 mg/ml dose was used as a toxicity control.

MTT dye (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide) conversion was used to measure cell metabolic activity and viability. Caco-2 epithelial cells were cultured in 96-well plates for 3 days. Confluent cultures were treated for 1 or 24-hours, washed with phosphate buffered saline (PBS; pH: 7.4) and incubated with 0.5 mg/ml MTT solution for 3 hours in a CO_2 incubator. The amount of blue formazan crystals converted by

cells was dissolved in dimethyl-sulfoxide and determined by measuring absorbance at 595 nm wavelength with a microplate reader (Fluostar Optima, BMG Labtechnologies, Ortenberg, Germany). Cell viability was calculated as percentage of dye conversion by non-treated cells.

Lactate dehydrogenase (LDH) release, the indicator of cell membrane damage, was determined from culture supernatants by "Cytotoxicity detection kit (LDH)" (Roche, Basel, Switzerland). Caco-2 cells cultured in 96-well plates for 3 days were treated for 1 or 24-hours with absorption enhancers, then 50 µl samples from culture supernatants were incubated with equal amounts of reaction mixture for 15 minutes. The enzyme reaction was stopped by 0.1 M HCl. Absorbance was measured at a wavelength of 492 nm with a microplate reader (Fluostar Optima, BMG Labtechnologies, Ortenberg, Germany). Cell death was calculated as percentage of the total LDH release from cells treated by 10 mg/ml Triton X-100. The non toxic concentrations (TC0), 50 % toxic concentrations (TC50) and concentrations causing death in all cell (TC100) were calculated from fitted curves (GraphPad Prism 5.0, GraphPad Software Inc., San Diego, CA, USA).

Measurement of electrical resistance and impedance of cell layers

Transepithelial electrical resistance (TEER), representing the permeability of tight junctions for ions, mostly sodium in culture conditions, was measured by an EVOM resistance meter using STX-2 electrodes (World Precision Instruments Inc., Sarasota, FL, USA), and it was expressed relative to the surface area ($\Omega \times cm^2$). The TEER of intestinal epithelial cell monolayers was 800±138 $\Omega \times cm^2$ (mean±SD; n=158) after 3 weeks of culture. Resistance measurements were carried out before and after permeability studies to check the barrier integrity.

RTCA SP (ACEA Biosciences, San Diego, USA) instrument was used for the measurement of impedance at 10 kHz. The interaction between cells and gold electrodes of E-plates generates impedance response which correlates with ion flux.[36] Impedance non-invasively quantifies proliferation, viability or permeability of adherent cells in real-time.[37,38,6] E-plates were coated with 0.2 % gelatine-PBS solution for 20 minutes at 37°C. Culture medium (80 µl) was added to each well for background readings then 80 µl cell suspension was dispensed at the density of 6×10^3 cells/well. The cells were kept in incubator at 37°C for 48 hours and monitored every 5 minutes. The impedance at each time point was defined as $R_n - R_b$, where R_n is the cell-electrode impedance of the well when it

contains cells and R_b is the background impedance of the well with the medium alone. During treatments cells impedance was measured every 2 minutes.

Permeability study

The flux of the drugs caffeine, antipyrine, atenolol, vinblastine and fluorescent dyes fluorescein and rhodamine 123 across epithelial cell layers was determined in permeability studies. Caco-2 cells were seeded onto Transwell filter inserts and the resistance of the cell layers was checked twice a week. After 21 days when the resistance was high and stable, the inserts were transferred to 12-well plates containing 1.5 ml Ringer–Hepes solution (150 mM NaCl, 6 mM $NaHCO_3$, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 2.8 mM D-glucose, 5 mM Hepes, pH 7.4) in the basolateral compartments. In apical chambers culture medium was replaced by 500 µl Ringer–Hepes containing drugs or marker molecules with or without absorption enhancers. The drugs and rhodamine 123 were used at the dose of 10 µM, fluorescein was administered at 10 µg/ml (30 µM) concentration. The concentrations of absorption enhancers chosen for permeability assays were in the non-toxic range: Tween 80 and Cremophor RH40 were used at 30, 60, 100, 1000 µg/ml, P-1695 and M-1695 at 30, 60 µg/ml, and D-1216 at 30, 60, 100 µg/ml. The plates were kept in a CO_2 incubator for 1 hour on a rocking platform (100 rpm). For rhodamine 123 bidirectional transport was determined. After the incubation samples from the upper and lower compartments were collected and the concentrations of fluorescein and rhodamine 123 were determined by a fluorescent microplate reader (Fluostar Optima) while drug concentrations were measured by HPLC, as detailed below. The apparent permeability coefficient (P_{app}) in apical to basal (AB) direction was calculated by this equation:

$$P_{app}\ (\text{cm/s}) = \frac{[C]_B \times V_B}{A \times [C]_A \times t}$$

where $[C]_B$ is the concentration of the tracer in the basal compartment after 1 hour; $[C]_A$ is the concentration in the apical compartment at 0 hour; V_B is the volume of the basal compartment (1.5 ml); A is the surface area available for transport (1.12 cm^2); *t* is the length of the time of the transport assay (1 hour). To calculate the apparent permeability of the opposite transport direction (BA) the following equation was used:

$$P_{app}\ (\text{cm/s}) = \frac{[C]_A \times V_A}{A \times [C]_B \times t}$$

where $[C]_A$ was the concentration of the tracer in apical compartment after 1 hour; $[C]_B$ is the concentration in basal compartment at 0 hour, and V_A is the volume of the apical compartment (0.5 ml).

HPLC analytical procedures

Analytical measurements were performed on a Merck-Hitachi LaChrom HPLC system equipped with UV and fluorescence detector (Merck, Darmstadt, Germany). Antipyrine and vinblastine were determined by using Purospher C18e 125 mm×3 mm (5 µm) column (Merck, Darmstadt, Germany) operated at 0.5 ml/min flow rate, maintained at 40 ºC. Mobile phase for antipyrine consisted of 40 % methanol in 0.1 M ammonium acetate with isocratic elution. UV detection was at 250 nm wavelength. For vinblastine, elution was applied to a mixture of 250 ml methanol, 200 ml 25 nM ammonium-acetate and 3 ml 10 % trifluoroacetic acid. Vinblastine was quantified at 275/360 nm excitation/emission wavelengths. HPLC measurement of caffeine was performed using Purospher C18e 125 mm×4 mm (5 µm) column (Merck, Darmstadt, Germany) operated at 0.8 ml/min flow rate, maintained at 35 ºC. Mobile phase was 15 % methanol in 0.1 M ammonium acetate. UV detection was at 275 nm. Samples with atenolol were injected onto a Gemini C18e 150 mm×3 mm (5 µm) column (Phenomenex Inc., Aschaffenburg, Germany) operated at 35°C and at an eluent flow rate of 0.5 ml/min. Elution was applied to a mixture of 300 ml methanol, 400 ml 0.1 M ammonium-acetate, 20 ml 10 % ammonium hydroxide and 2 ml 0.1 M Na_2EDTA. Atenolol was quantified at 230/300 nm excitation/emission wavelengths.

Fluorescent and immunostainings

Morphology and cell-cell connections of Caco-2 cells were confirmed by immunostaining for tight junction protein claudin-1, cytoplasmic linker protein zonula occludens 1 (ZO-1) and adherens junction protein β-catenin. Filamentous actin was stained by fluorescently labelled phalloidin, and cell nuclei by bis-benzimide dye (Hoechst dye 33342). Cell layers were grown on glass coverslips and treated with absorption enhancers (P-1695 30 µg/ml, M-1695 60 µg/ml, D-1216 100 µg/ml, Tween 80 and Cremophor RH40 1000 µg/ml) for 1 hour. Cytochalasin D, an inhibitor of actin polymerization was applied at 2 µg/ml concentration. After treatments the cultures were washed twice in PBS and fixed with 4 % paraformaldehyde-PBS for 30 minutes. Cells were blocked with 3 % bovine serum albumin in PBS and incubated with primary antibodies anti-claudin-1, anti-ZO-1 and anti-β-catenin (Life Technologies, Carlsbad, CA, USA) overnight. Incubation with secondary antibody Cy3-

labeled anti-rabbit IgG, Alexa Fluor 488 Phalloidin (Life Technologies, Carlsbad, CA, USA) and bis-benzimide lasted for 1 hour. Between and after incubations cells were washed three times with PBS. Coverslips were mounted by Fluoromount and staining was examined by a Leica SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Electron microscopy

Cells grown on culture inserts were treated with absorption enhancers (P-1695 30 µg/ml, M-1695 60 µg/ml, D-1216 100 µg/ml, Tween 80 and Cremophor RH40 1000 µg/ml) for 1 hour. After treatments cells were washed by PBS and fixed with 3% paraformaldehyde containing 0.5% glutaraldehyde in cacodylate buffer (pH 7.4) for 30 min at 4°C. After washing with the buffer several times, cells were postfixed in 1% OsO_4 for 30 min. Following a rinse with distilled water, the cells were dehydrated in graded ethanol, block-stained with 1% uranyl acetate in 50% ethanol for 1 h. After the last step of dehydration, inserts were placed in the 1:1 mixture of ethanol and Taab 812 (Taab; Aldermaston, Berks, UK) for 30 min at 30°C. Finally, the membranes of the culture inserts with the cells were removed from their support and embedded in Taab 812. Polymerization was performed overnight at 60°C. Ultrathin sections were cut perpendicularly for the membrane using a Leica UCT ultramicrotome (Leica Microsystems, Milton Keynes, UK) and examined using a Hitachi 7100 transmission electron microscope (Hitachi Ltd., Tokyo, Japan)

Measurement of plasma membrane fluidity in Caco-2 cells

Caco-2 cells were collected by trypsinization, washed twice and resuspended in PBS. The density of cells was set by absorbance measurement at 360 nm to OD_{360}=0.1 (Hewlett Packard 8452A Diode Array Spectrophotometer). Cells were labeled with 0.2 µM TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; Molecular Probes, Life Technologies) for 5 minutes. Fluorescence anisotropy was measured on a T-format fluorescence spectrometer (Quanta Master QM-1, Photon Technology International, Princeton, NJ, USA). Excitation and emission wavelengths were 360 and 430 nm, respectively (5-nm slits). Cells were kept at 37°C under stirring conditions.[39,40] Anisotropy data were acquired in every second. After 5 minutes the lowest concentrations of treatment solutions were added rapidly to the cell suspension and every 3 minutes the treatment concentration was increased during anisotropy measurements. Control cells received only vehicle. A strong membrane fluidizer, benzyl alcohol (30 mM) (Merck, Darmstadt, Germany) was used as a positive control in the experiments. The following treatment concentrations

were applied: 1, 10, 30, 60, 100 μg/ml for sucrose ester, and 1, 10, 100, 1000 μg/ml for Tween 80 and Cremophor RH40. The average of 50 anisotropy measurements in the last 1 minute of each treatment concentration was calculated and compared to the anisotropy of the vehicle-treated cells at the same time point.

Measurement of efflux pump activity

The activity of efflux pumps was determined by cellular accumulation of rhodamine 123 and calcein AM (Life Technologies, Carlsbad, CA, USA) in Caco-2 epithelial cells grown in 24-well plates for 2 days (seeding density: 10^5 cells/well). In the pre-treatment experiments cells were incubated with surfactants P-1695 (30 μg/ml), M-1695 (60 μg/ml), D-1216 (100 μg/ml), Tween 80 (1000 μg/ml), Cremophor RH40 (1000 μg/ml) or efflux pump inhibitors verapamil (100 μM) and cyclosporin A (10 μM) for 1 hour, then the cell layers were washed and incubated with 10 μM rhodamine 123 in Ringer-Hepes solution for another 1 hour. In the co-treatment experiments, cells were pre-incubated with Ringer-Hepes buffer for 20 minutes. Treatments with surfactants or efflux pump inhibitors were done in the presence of rhodamine 123 for 1 hour. Following incubations cells were washed three times with ice cold PBS and lysed with 0.1 M NaOH in both types of experiments. Rhodamine 123 concentration was determined by a fluorescent microplate reader (excitation wavelength: 485 nm, emission wavelength: 520 nm; Fluostar Optima).

The calcein AM assay can be used for the measurement of the activity of efflux transporters P-gp and multidrug resistance protein-1 or -2 (MRP-1, ABCC1).[41,30] The cellular transport of the non-fluorescent calcein AM is inhibited by efflux transporters, therefore only limited amount gets into the cells where intracellular esterases convert the dye to a fluorescent metabolite. For calcein AM assay Caco-2 cells were seeded on 96-well plate (cell density: 7×10^4 cells/well) and grew for 24 hours. In the pre-treatment experiments cells were incubated for 1 hour with surfactants or efflux pump inhibitors. After the treatments cells were washed and the buffer solution was replaced with 1.25 μM calcein AM in Ringer-Hepes, and the plate was immediately placed in a microplate reader (Fluostar Optima). The fluorescence was measured at 5 minutes interval for 1 hour (excitation wavelength: 485 nm; emission wavelength: 520 nm). In the co-treatment experiments cells were pre-incubated with Ringer-Hepes for 20 minutes. Treatments with surfactants or efflux pump inhibitors were done in the presence of 1.25 μM calcein AM. The fluorescence was immediately measured by a microplate reader at 5 minutes interval for 1 hour. The fluorescence intensity was calculated as percentage of the control group.

Statistical analysis

All data presented are means ± SD. Values were compared using analysis of variance followed by Dunnett's test (GraphPad Prism 5.0, GraphPad Software Inc., San Diego, CA, USA). In case of anisotropy measurements two-way analysis of variance followed by Bonferroni posttest was applied. Changes were considered statistically significant at $P < 0.05$. All experiments were repeated at least two times, the number of parallel samples varied between 4 and 12.

RESULTS

Effect of sucrose esters on the viability of cultured epithelial cells

Treatment of Caco-2 cells with sucrose esters dose- and time-dependently decreased MTT dye conversion indicating reduced cell viability (Fig. 1, Table 1). The doses above 30 µg/ml of palmitate or 60 µg/ml of myristate esters significantly reduced metabolic activity of Caco-2 cells, while concentrations above 200 µg/ml resulted in cell death (Fig. 1a). D-1216 laurate ester was the least toxic among the investigated sucrose esters, the 100 µg/ml dose did not decrease epithelial cell viability, while 200 µg/ml to 600 µg/ml concentrations reduced MTT conversion in epithelial cells after 1 hour. The reference surfactants showed lower toxicity on epithelial cells. Tween 80 or Cremophor RH40 at 3,000 µg/ml concentration did not reduced cell viability of epithelial cells for 1 hour (Fig. 1a). Similar results were obtained by measurements of LDH enzyme release from treated cells indicating plasma membrane damage. The non-toxic concentrations for all sucrose esters were the same with both cell viability methods. Cell death was observed after 1-hour treatment above 300 µg/ml sucrose esters, but Tween 80 or Cremophor RH40 did not increase the enzyme release at any tested concentrations (Fig. 1b). Based on these results the following concentrations were considered as safe for 1 hour-treatments and applied in further experiments on Caco-2 epithelial cells: P-1695 30 µg/ml, M-1695 60 µg/ml, D-1216 100 µg/ml, Tween 80 and Cremophor RH40 1000 µg/ml.

The concentrations of surfactants causing 50 % or complete cell death (TC 50 and TC 100) at 24 hours were lower than for the 1-hour treatments (Table 1). The non-toxic doses of P-1695, M-1695 and D-1216 were about 20, 20 and 100 µg/ml, respectively, by both assays. The toxic concentrations of reference absorption enhancers were more than one order of magnitude higher than that of sucrose esters. The TC 0 values for Tween 80 were calculated as 473 µg/ml (MTT test) and 1068 µg/ml (LDH assay). The non-toxic concentrations of

Cremophor RH40 on Caco-2 cells for 24 h were 30,000 µg/ml and 2067 µg/ml determined by by MTT test and LDH assay, respectively (Table 1).

Effect of sucrose esters on the electrical resistance and impedance of epithelial cell layers

Non-toxic doses of sucrose esters but not of reference surfactants increased the ionic permeability across Caco-2 monolayers monitored by impedance and TEER measurements. Sugar esters reduced the impedance of epithelial cell layers measured by RTCA SP (Fig. 2a). The impedance decreased rapidly from the baseline of 125 Ω to 90 Ω within 30 minutes by P-1695 (30 µg/ml) and D-1216 (100 µg/ml) treatments. M-1695 (60 µg/ml) also significantly reduced the impedance of Caco-2 layers. No significant change was caused by Tween 80 or Cremophor RH40. In accordance with the impedance measurements P-1695, M-1695 and D-1216 reduced the resistance of the cell layers to 25 %, 55 % and 62 % as compared to control at 1-hour treatment (Fig. 2b).

Effect of sucrose esters on Caco-2 permeability for drugs and fluorescein

The permeability of hydrophilic atenolol (0.17×10^{-6} cm/s) and efflux pump ligand lipophilic vinblastine (0.62×10^{-6} cm/s) was low across Caco-2 cells (Table 2). Sucrose esters and reference surfactants significantly enhanced the penetration of atenolol and vinblastine across intestinal epithelial cell layers. The P_{app} of passive lipophilic drugs antipyrine (80.67×10^{-6} cm/s) and caffeine (87.59×10^{-6} cm/s) was high and the absorption enhancers did not increase it further (Table 2).

The average apparent permeability coefficient of fluorescein was also low (0.78×10^{-6} cm/s) in the control group. Using previously selected non-toxic concentrations for each surfactants, all investigated compounds caused significant increase in fluorescein flux across Caco-2 layers after 1-hour treatment (Fig. 2c). Myristate (60 µg/ml) and laurate sugar ester (100 µg/ml) doubled the flux of the marker molecule at 1-hour application. The increase in paracellular transport caused by palmitate sucrose ester (30 µg/ml) was smaller. Tween 80 (1000 µg/ml) tripled the flux of fluorescein. Cremophor RH40 at similar dose enhanced the dye permeability to 164 %. Sucrose esters and reference absorption enhancers were also tested at 30, 60, and 100 µg/ml doses for 1 hour (Fig. S1a-c). The effect of sugar esters on the marker permeability was dose dependent (Fig. S1a-c). At 30 µg/ml concentration P-1695 and

M-1695 sucrose esters, but not the other surfactants (Fig. S1a), at 60 µg/ml concentration both myristate and laurate sugar esters increased the permeability of the marker dye (Fig. S1b). D-1216 at 100 µg/ml raised the fluorescein flux two times higher compared to control (Fig. S1c). In contrast to sucrose esters reference absorption enhancers in the concentration range of 30-100 µg/ml did not increase the permeability of fluorescein.

Effects of sucrose esters on the epithelial intercellular junctions

Claudin-1 transmembrane tight junction protein, ZO-1 cytoplasmic junctional linker protein, and β -catenin adherens junction protein all appeared at the cell-cell borders in a continuous, belt-like manner. Treatments with sucrose esters or reference surfactants for 1 hour did not affect the gross morphology of cells or intercellular junctions assessed by these immunostainings (Fig. 3a). No rupture of the interjunctions or fragmentation of the pericellular immunostaining was seen.

F-actin structure was investigated in different regions of epithelial cells. At the level of tight junctions the cortical actin ring was sharply delineated in the control cells mimicking tight junction protein immunostainings (Fig. 3a). Following treatments with sucrose esters and reference absorption enhancers this F-actin staining near the tight junction region became wider and more blurred compared to control images. At the apical part of the cells the structure of microvilli was well shown by F-actin staining, which was preserved in all treatment groups (Fig. S2) At the basal part of cells staining of F-actin revealed long filaments organized in bunches (Fig. S2). This filamentous organization was less observed in the treatment groups, except for P-1695. Cytochalasin D, which inhibits actin polymerization changed the F-actin staining: dot-like aggregations appeared in the actin ring at the level of tight junctions and in the basal area (Fig. S2). Triton X-100 surfactant (1 %) disrupted the plasma membrane and the structure of microvilli at apical surface (Fig. S2). The junctional actin ring was mostly preserved, but aggregations were observed in the junctional and basal areas.

The ultrastructure of tight intercellular junctions between Caco-2 cells was preserved in all treatment groups (Fig. 3b). No morphological change was seen by electron microscopy in the structure of apical microvilli, tight junctions, desmosomes, or interdigitations of adjacent epithelial cells. No open tight junctions were observed in the control or treatment groups by checking 140 electron micrographs (19-28 images/treatment group).

Effects of sucrose esters on plasma membrane fluidity in Caco-2 cells

The membrane fluidity of living epithelial cells was determined by the measurement of fluorescence anisotropy of the cationic membrane probe TMA-DPH (Fig. 4). The anisotropy slightly decreased from 0.293±0.002 to 0.286±0.001 in vehicle-treated Caco-2 cells after 20 minutes. The membrane fluidizer benzyl alcohol quickly and greatly reduced the anisotropy: TMA-DPH fluorescence anisotropy fell to 90.2% compared to control after 3 minutes. Sucrose esters fluidized the plasma membrane of Caco-2 cells at all investigated concentrations (Fig. 4a). A U-shaped dose-response curve was observed for all sucrose esters. The biggest change in anisotropy compared to the control group was measured at 10 μg/ml of P-1695 (92.0%), 30 μg/ml of M-1695 (91.9%) and 60 μg/ml of D-1216 (96.1%). Concentrations above these most effective doses resulted in higher anisotropy indicating reduced membrane fluidity.

The reference absorption enhancers at 1 μg/ml concentration did not alter significantly membrane fluidity in contrast to sucrose esters (Fig. 4b). Tween 80 at 10 μg/ml was also ineffective, but at 100 μg/ml concentration and above statistically significantly fluidized the plasma membrane of epithelial cells with lowest anisotropy at 1000 μg/ml (94.2%). Cremophor RH40 was the most effective at 10 μg/ml dose (94.0%). Higher concentrations of the reference surfactants were also less effective to reduce membrane anisotropy.

Effect of sucrose esters on efflux pump activity in Caco-2 cells

Efflux transporter activity in Caco-2 cells (Fig. 5 and Fig. S3) was measured by the cellular uptake of calcein AM and the cellular uptake and bidirectional transport of rhodamine 123, a ligand of P-gp and BRCP.[42] Co-administration of surfactants and rhodamine 123 resulted in statistically significant uptake of rhodamine (Fig. 5a). M-1695 and Tween 80 caused the highest, six-fold accumulation of the marker molecule compared to control. Simultaneous administration of sucrose esters and calcein AM significantly elevated the uptake of the ligand (200 – 245 %) (Fig. 5b). Co-treatment with Tween 80 (1000 μg/ml) also significantly increased the cellular accumulation of calcein AM. In contrast to rhodamine 123 uptake, Cremophor RH40 had no effect on calcein AM uptake (Fig. 5b). Verapamil (100 μM) and cyclosporin A (10 μM) significantly raised the level of both ligands in Caco-2 cells (Fig. 5a-b).

Pre-treatment with sucrose esters did not change the accumulation of rhodamine 123 in Caco-2 cells, but Tween 80 and Cremophor RH40 raised slightly the dye uptake to 133 %

and 117 %, respectively (Fig. S3a). Pre-treatment with surfactants did not increase significantly the uptake of calcein AM except for Tween 80 (113 %). Verapamil and cyclosporin A pre-treatment increased both rhodamine 123 and calcein AM accumulation in Caco-2 cells (Fig. S3).

Rhodamine 123 permeability in apical-to-basal (AB) direction was significantly enhanced by both absorption enhancers and efflux pump inhibitors as compared to control group ($0.82 \pm 0.18 \times 10^{-6}$ cm/s) (Fig. 5c-e). Treatments with sugar esters resulted in several fold elevated AB flux of the marker molecule as compared to reference surfactants or efflux pump inhibitors. The transport of rhodamine 123 in basal-to-apical (BA) direction was unchanged by P-1695 and D-1216, but increased by M-1695. In contrast, reference absorption enhancers and efflux pump inhibitors significantly decreased rhodamine 123 permeability (Fig. 5d). Efflux ratios were calculated from the apparent permeability coefficients measured in BA and AB directions (BA/AB) (Fig. 5e). The ratio in the control group was 20.9 and all treatments decreased it significantly.

DISCUSSION

The potential use of sucrose esters as pharmaceutical excipients to improve drug solubilization and absorption has been recently reviewed.[12] Several studies examined their application for transdermal drug delivery but there are only few papers on oral formulations using *in vivo*[25-30] or *in vitro*[24,29] models. The present study described the effect of three sugar esters with same hydrophilic-lipophilic balance (HLB) value of 16 but different fatty acid chain length (C12-C16) (Table S1) on cellular toxicity, drug permeability, intercellular junction morphology, efflux pump activity and plasma membrane fluidity, giving a more complex and detailed view on their action on cultured intestinal epithelial cells than previous works.

Sucrose esters and viability of cultured epithelial cells

Among the applied sucrose esters D-1216 laurate ester (C12) was the least toxic to human Caco-2 intestinal cells while myristate (C14) and palmitate (C16) ester had higher toxicity at both 1- or 24-hour treatments. The same results were obtained by these sugar esters in our previous works on Caco-2 human intestinal[43] and on RPMI 2650 human nasal epithelial cell lines.[38] This observation is also in agreement with a previous work in which an unspecified sucrose monoester at 50 µg/ml concentration was non-toxic to Caco-2 cells.[44] The toxicity of sucrose laurate, Tween 80 and Cremophor RH40 was also studied on MDCK dog kidney

cells and two MDCK derived cell lines with high P-gp or MRP2 expression: laurate sucrose ester above 100 µg/ml caused cell damage, while Tween 80 and Cremophor RH40 were non-toxic below 5000 µg/ml for 1.5 hour.[30] In our previous study on Cremophor RH40 and EL similar results were obtained, treatment with Cremophor RH40 above 5000 µg/ml induced LDH release in intestinal epithelial and brain endothelial cells after 24 h.[6] The toxic concentration of the reference surfactants was at least one order of magnitude higher in both our studies and in the literature indicating that Tween 80 and Cremophor RH40 have a safer toxicity profile on cultured cells than sucrose esters.

In contrast to cell culture works *ex vivo* investigation of food grade sugar esters found no damages on palatal and buccal pork tissues.[26] Animal studies indicate that sucrose fatty acid esters are hydrolysed by intestinal enzymes into sucrose and fatty acids in physiological conditions prior absorption and that their metabolites are not toxic.[45-47] In addition sugar esters are chemically hydrolyzed under acidic or basic conditions[48,49] and can be disintegrated by bacterial lipases.[50] Tween 80 and Cremophor RH40 are also degraded in the intestine or in the blood,[51,52] but the effects of the metabolites are unknown. A recent review drew the attention to the dangers of using surfactants as food additives: the permeability enhancing and efflux pump inhibitor properties of these emulsifiers may cause intestinal barrier dysfunction and increase the incidence of allergic and autoimmune disease,[53] but conclusive animal studies and human data are missing. Despite the results of in vitro cell culture tests and the hypothesized dangers of surfactants ingested as additives in large quantities from foodstuff, the animal studies suggest that the toxicity of sucrose esters might be lower, if any, when given orally in small quantities as excipients. However, further chronic toxicity experiments are needed to prove the safety of sucrose esters as potential oral excipients.

Sucrose esters and the permeability of intestinal epithelial cells

The recommended HLB values for absorption enhancers in pharmaceutical applications is high.[12,26] The tested surfactants possess high HLB (15-16), indicating a good potential penetration increasing effect. To determine the effect of sucrose esters on the para- and transcellular permeability and reveal the extent of the permeability increase different methods were used.

The flux of ions, one of the most sensitive markers of paracellular permeability, can be measured by electrical resistance or impedance across epithelial cell layers. Both the resistance and impedance of epithelial cell layers were decreased by all three sucrose esters in non-toxic doses. Data from other studies also indicate that resistance of RPMI 2650 or Caco-

2 cell layers dropped after treatment with sugar esters.[38,44] We observed no significant changes in TEER or impedance by the reference surfactants in Caco-2 monolayers. Furthermore, two independent studies confirmed that lower and higher concentrations than in the present study did not decrease the resistance of Caco-2 cell layers.[54,55] These data are in agreement with our previous works on RPMI 2650 [38] and Caco-2 cells[6]. Based on the results of electrical resistance and impedance measurements sugar esters enhance the ion flux through cultured epithelial cell layers.

Sucrose esters and reference absorption enhancers significantly increased the penetration of paracellular marker molecule fluorescein in a dose dependent manner. We described for the first time that sugar esters elevated the flux of the hydrophilic drug atenolol and the lipophilic efflux pump ligand vinblastine through Caco-2 cell layers, indicating the absorption enhancer properties of these surfactants. The penetration of the passive lipophilic drugs caffeine and antipyrine across Caco-2 layers was not changed by any of the tested surfactants. These data are in accordance with the study of Takaishi et al., in which the efflux pump ligand daunomycin permeability was also enhanced by sucrose esters in Caco-2 cells.[24] The absorption enhancing effects of sugar esters, especially laurate was demonstrated in several *in vivo* investigations.[25-28] Moreover, many studies propose the use of sugar esters to enhance the penetration of molecules by the transdermal, nasal and ocular routes as reviewed by Szűts and Szabó-Révész.[12]

Tween 80 and Cremophor RH40 did not influence fluorescein permeability in same concentrations as sugar esters, only in higher doses, indicating that sugar esters are more effective in small concentrations. Both reference surfactants enhanced the permeability of atenolol and vinblastine across epithelial cell layers. Other studies also showed that the application of Tween 80 increased the penetration of efflux pumps ligands,[3] peptide molecules,[56] dexamethasone and lucifer yellow[55] through Caco-2 cell monolayers. Tween 80 improves the absorption of drugs and is clinically applied in medicines as excipient (Fluxarix, Boostrix, Tubersol, and Tripedia). Our group demonstrated that Cremophor RH40 increases dextran permeability in nasal epithelial cell layers *in vitro*[38] and *in vivo*[57]. Cremophor RH40 is also used clinically in Neoral as an inactive ingredient beside cyclosporine A.

In this study immunostaining of junctional proteins, fluorescent labeling of F-actin and transmission electron microscopy were used to visualize intercellular connections and demonstrate the integrity of the paracellular barrier. Cytoskeletal F-actin changes caused by an undefined sucrose ester were observed in Caco-2 cells,[44] but junctional proteins or ultrastructure were not investigated. To our best knowledge it was observed for the first time

that sucrose esters, Tween 80 and Cremophor RH40 did not cause any major change in the distribution of immunostaining for junctional proteins claudin-1, ZO-1 and β-catenin on cultured Caco-2 cells at the applied concentrations. Our previous work showed a similar effect for Cremophor RH40 on Caco-2 cells.[6] Surfactants slightly changed the organization of F-actin at the junctional (cortical actin ring) and basal part of the cells, but did not cause visible opening of the junctions. This F-actin redistribution can be linked to changes in the permeability of the junctions.[4] The ultrastructure of Caco-2 cells was also investigated by electron microscopy, and the tested surfactants did not change the morphology of tight junctions. No disruption of junctions or cell layers were observed by these morphological examinations confirming the safety of the selected surfactant concentrations and that sugar esters do not cause visible damage of paracellular junctions.

Surfactants modify the fluidity of cellular plasmamembranes, and this can contribute to enhanced membrane permeability and changes in the activity of membrane transporters and efflux pumps.[58,59] Sucrose monoester of palmitic and stearic acid mixture decreased the membrane fluidity in vesicles of intestinal brush border membranes,[60] but no data were available on cultured cells. We tested and compared the effects of sugar esters and reference surfactants on membrane fluidity in living Caco-2 cells. Sucrose esters fluidized the plasma membrane of cells at lower concentrations than reference absorption enhancers, and caused stronger TMA-DPH fluorescence anisotropy reduction than Tween 80 or Cremophor RH40. Sugar esters containing longer fatty acid chain, like palmitate and myristate, increased better the membrane fluidity at lower concentrations than laurate, indicating a correlation between the length of fatty acid chain of sugar esters and their effects on membrane fluidity.

Considering together the results of resistance, impedance, permeability, morphology and membrane fluidity measurements we propose that several mechanisms contribute to the absorption enhancer effect of sugar esters (Fig. 6). All excipients increased the permeability of the tested passive permeability markers and drugs in cultured epithelial cells. Since plasma membrane fluidity was elevated by the surfactants, we hypothesize that this effect increase the transcellular passage of molecules. Tween 80 and Cremophor RH40 did not change the morphology of intercellular junctions visualized by immunostainings and did not modify epithelial resistance and impedance measurements suggesting that an increase in the paracellular transport of molecules can be excluded as a major mechanism of action. Sugar esters decreased both resistance and impedance indicating an effect on the function of intercellular junctions and the paracellular pathway. Thus it is proposed, that sugar esters enhance drug permeability through both the trans- and paracellular routes (Fig. 6).

Effects of sucrose esters on efflux pumps

Efflux transporters hinder drug delivery across biological barriers and blocking these pumps is considered as a way to increase drug penetration.[61] Surfactants were described to inhibit efflux pumps.[62,63] The effect of sucrose esters on efflux pumps is contradictory with studies describing inhibitory[29,30] or no effect[24]. In the present experiments sugar esters, unlike inhibitors and reference surfactants, increased the permeability of rhodamine 123 in apical to basal, but not in the other direction, suggesting no direct effect on efflux pumps. Sucrose esters elevated rhodamine 123 and calcein AM accumulation in co-treatment, but this effect alone do not indicate a direct inhibition of efflux transporters. We suggest that this increase in accumulation may be due to permeabilization of the plasma membrane or indirect inhibition of efflux pumps by changing the fluidity of cell membranes. Indeed, membrane cholesterol modulation by excipients is able to inhibit P-glycoprotein.[64] Takaishi et al. supposed that elevated daunomycine flux in Caco-2 cells caused by sugar esters is due to permeabilization of the cellular membrane,[24] but the present study is the first to experimentally prove it.

Reference surfactants significantly increased the penetration of rhodamine 123 from apical to basal compartment but reduced the permeability in the other direction, similarly to inhibitors verapamil and cyclosporine A. Tween 80 and Cremophor RH40 also significantly increased the accumulation of rhodamine 123 in both pre- and co-treatment conditions. The uptake of calcein AM was increased by Tween 80 and the efflux pump inhibitors, but not by Cremophor RH40. The difference in the rhodamine and calcein AM uptake assays may be explained by the different specificity of the dyes: rhodamine 123 is a ligand of P-gp and BRCP while calcein AM is a ligand of P-gp and MRP-1 and -2.[30,42,65] Our data indicate a direct inhibitory effect of these surfactants on efflux pump activity (Fig. 6) in agreement with other studies on Tween 80 [3,29,30,62] and Cremophor RH40[30,66].

Conclusion

Due to their favourable properties sugar esters are increasingly used as inactive ingredients in pharmaceutical products. Our study on cultured epithelial cells confirmed, that sucrose esters are effective absorption enhancers for both hydrophilic drugs and efflux pump ligands. Sugar esters enhance drug penetration by the trans- and paracellular routes, but do not directly inhibit efflux pumps. It was demonstrated for the first time that sucrose esters fluidize the plasma membrane of epithelial cells in low concentrations. Reference surfactants Tween 80 and Cremophor RH 40 increase drug penetration by the transcellular pathway via membrane

fluidization and direct inhibition of efflux pump activity and not by acting on intercellular junctions. These data indicate that sucrose esters as oral excipients may act differently than the reference absorption enhancers, therefore further studies are needed to optimize oral dosage forms with these surfactants.

References

1. Strickley RG. 2004. Solubilizing excipients in oral and injectable formulations. Pharm Res 21:201-230.

2. Zhao X, Liu JP, Zhang X, Li Y. 2006. Enhancement of transdermal delivery of theophylline using microemulsion vehicle. Int J Pharm 327:58-64.

3. Rege BD, Yu LX, Hussain AS, Polli JE. (2001. Effect of common excipients on Caco-2 transport of low-permeability drugs. J Pharm Sci 90:1776–1786.

4. Deli MA. 2009. Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery. Biochim Biophys Acta 1788:892-910.

5. Gelderblom H, Verweij J, Nooter K, Sparreboom A. 2001. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur J Cancer 37:1590–1598.

6. Kiss L, Walter FR, Bocsik A, Veszelka S, Ózsvári B, Puskás LG, Szabó-Révész P, Deli MA. 2013. Kinetic analysis of the toxicity of pharmaceutical excipients Cremophor EL and RH40 on endothelial and epithelial cells. J Pharm Sci 102: 1173-1181.

7. Daudé D, Remaud-Siméon M, André I. 2012. Sucrose analogs: an attractive (bio)source for glycodiversification. Nat Prod Rep 29:945-960.

8. Polat T, Linhardt R. 2001. Syntheses and applications of sucrose-based esters. J Surfact Deterg 4:415–421.

9. Chung O, Pomeranz Y, Goforth DR, Shogren MD, Finney KF. 1976. Improved sucrose esters in breadmaking. Cereal Chem 53:615-625.

10. Garti N, Aserin A, Fanun M. 2000. Non-ionic sucrose esters microemulsions for food applications. Part 1. Water solubilization. Colloids Surf A Physicochem Eng Asp 164:27–38.

11. Muratsubaki Y. 2006. Cosmetics and materials. Application expansion in bidirection with other fields. Applied technology from cosmetics: Applied technology of sucrose fatty acid ester to cosmetics. Engineering Materials 54:48–51.

12. Szűts A, Szabó-Révész P. 2012. Sucrose esters as natural surfactants in drug delivery systems - A mini-review. Int J Pharm 433:1–9.

13. Yamato K, Takahashi Y, Akiyama H, Tsuji K, Onishi H, Machida Y. 2009. Effect of penetration enhancers on transdermal delivery of propofol. Biol Pharm Bull 32:677–683.

14. Thevenin M, Grossiord JL, Poelman M. 1996. Sucrose esters/cosurfactant microemulsion systems for transdermal delivery: assessment of bicontinuous structures. Int J Pharm 137:177–186.

15. Lehmann L, Keipert S, Gloor M. 2001. Effects of microemulsions on the stratum corneum and hydrocortisone penetration. Eur J Pharm Biopharm 52:129–136.

16. Garti N, Clement V, Leser M, Aserin A, Fanun M. 1999. Sucrose ester microemulsions. J Mol Liq 80:253–296.

17. Mollee HM, Steenvoorden DP, De Vringer T, Crommelin DJ. 2001. The influence of the incorporation of cholesterol and water on the particle size, bilayer thickness, melting behavior, and relative sucrose ester composition of reversed vesicles. J Pharm Sci 90:588–598.

18. Sanchez SA, Gratton E, Zanocco AL, Lemp E, Gunther G. 2011. Sucrose monoester micelles size determined by Fluorescence Correlation Spectroscopy (FCS). PLoS One 6:e29278.

19. Ullrich S, Metz H, Mäder K. 2008. Sucrose ester nanodispersions: microviscosity and viscoelastic properties. Eur J Pharm Biopharm 70:550–555.

20. Li W, Das S, Ng K, Heng PWS. 2011. Formulation, biological and pharmacokinetic studies of sucrose ester-stabilized nanosuspensions of oleanolic acid. Pharm Res 28:2020–2033.

21. Muzzalupo R, Tavano L, Cassano R, Trombino S, Ferrarelli T, Picci N. 2011. A new approach for the evaluation of niosomes as effective transdermal drug delivery systems. Eur J Pharm Biopharm 79:28–35.

22. Rentel C-O, Bouwstra JA, Naisbett B, Junginger HE. 1999. Niosomes as a novel peroral vaccine delivery system. Int J Pharm 186:161–167.

23. Chansanroj K, Betz G. 2010. Sucrose esters with various hydrophilic-lipophilic properties: novel controlled release agents for oral drug delivery matrix tablets prepared by direct compaction. Acta Biomat 6:3101–3109.

24. Takaishi N, Satsu H, Shimizu M. 2006. Enhanced daunomycin accumulation in human intestinal Caco-2 cells from non-ionic food emulsifiers unrelated to the P-glycoprotein inhibitory mechanism. Biosci Biotechnol Biochem 70:2703–2711.

25. Nakada Y, Awata N, Nakamichi C, Sugimoto I. 1988. The effect of additives on the oral mucosal absorption of human calcitonin in rats. J Pharmacobiodyn 11:395–401.

26. Ganem-Quintanar A, Quintanar-Guerrero D, Falson-Rieg F, Buri P. 1998. Ex vivo oral mucosal permeation of lidocaine hydrochloride with sucrose fatty acid esters as absorption enhancers. Int J Pharm 173:203–210.

27. Lerk P, Sucker H. 1993. Application of sucrose laurate, a new pharmaceutical excipient, in peroral formulations of cyclosporin A. Int J Pharm 92:197–202.

28. Onishi H, Imura Y, Uchida M, Machida Y. 2012. Enhancement potential of sucrose laurate (L-1695) on intestinal absorption of water-soluble high molecular weight compounds. Curr Drug Deliv 9:487–494.

29. Cornaire G, Woodley J, Hermann P, Cloarec A, Arellano C, Houin G. 2004. Impact of excipients on the absorption of P-glycoprotein substrates in vitro and in vivo. Int J Pharm 278:119–131.

30. Hanke U, May K, Rozehnal V, Nagel S, Siegmund W, Weitschies W. 2010. Commonly used nonionic surfactants interact differently with the human efflux transporters ABCB1 (p-glycoprotein) and ABCC2 (MRP2). Eur J Pharm Biopharm 76:260–268.

31. Artursson P, Palm K, Luthman K. 2001. Caco-2 monolayers in experimental and theoretical predictions of drug transport. Adv Drug Deliv Rev 46:27–43.

32. Englund G, Rorsman F, Rönnblom A, Karlbom U, Lazorova L, Gråsjö J, Kindmark A, Artursson P. 2006. Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. Eur J Pharm Sci 29:269–277.

33. Hayeshi R, Hilgendorf C, Artursson P, Augustijns P, Brodin B, Dehertogh P, Fisher K, Fossati L, Hovenkamp E, Korjamo T, Masungi C, Maubon N, Mols R, Müllertz A, Mönkkönen J, O'Driscoll C, Oppers-Tiemissen HM, Ragnarsson EG, Rooseboom M, Ungell AL. 2008. Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. Eur J Pharm Sci 35:383-396.

34. Hellinger E, Bakk ML, Pócza P, Tihanyi K, Vastag M. 2010. Drug penetration model of vinblastine-treated Caco-2 cultures. Eur J Pharm Sci 41:96-106.

35. Hellinger E, Veszelka S, Tóth AE, Walter F, Kittel Á, Bakk ML, Tihanyi K, Háda V, Nakagawa S, Duy TDH, Niwa M, Deli MA, Vastag M. 2012. Comparison of brain capillary endothelial cell-based and epithelial (MDCK-MDR1, Caco-2, and VB-Caco-2) cell-based surrogate blood-brain barrier penetration models. Eur J Pharm Biopharm 82:340-351.

36. Atienza JM, Yu N, Kirstein SL, Xi B, Wang X, Xu X, Abassi YA. 2006. Dynamic and label-free cell-based assays using the real-time cell electronic sensing system. Assay Drug Dev Technol 4:597–607.

37. Ózsvári B, Puskás LG, Nagy LI, Kanizsai I, Gyuris M, Madácsi R, Fehér LZ, Gerő D, Szabó C. 2010. A cell-microelectronic sensing technique for the screening of cytoprotective compounds. Int J Mol Med 25:525–530.

38. Kürti L, Veszelka S, Bocsik A, Dung NTK, Ózsvári B, Puskás LG, Kittel Á, Szabó-Révész P, Deli MA. 2012. The effect of sucrose esters on a culture model of the nasal barrier. Toxicol In Vitro 26:445–454.

39. Török Z, Horváth I, Goloubinoff P, Kovács E, Glatz A, Balogh G, Vígh L. 1997. Evidence for a lipochaperonin: association of active protein-folding GroESL oligomers with lipids can stabilize membranes under heat shock conditions. Proc Natl Acad Sci USA 94:2192-2197.

40. Balogh G, Maulucci G, Gombos I, Horváth I, Török Z, Péter M, Fodor E, Páli T, Benkő S, Parasassi T, De Spirito M, Harwood JL, Vígh L. 2011. Heat stress causes spatially-distinct membrane re-modelling in K562 leukemia cells. PLoS One 6:e21182.

41. Juvale K, Pape VF, Wiese M. 2012. Investigation of chalcones and benzochalcones as inhibitors of breast cancer resistance protein. Bioorg Med Chem 20:346-355.

42. Schinkel AH, Jonker JW. 2003. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev 55:3-29.

43. Szűts A, Láng P, Ambrus R, Kiss L, Deli MA, Szabó-Révész P. 2011. Applicability of sucrose laurate as surfactant in solid dispersions prepared by melt technology. Int J Pharm 410:107-110.

44. Mine Y, Zhang JW. 2003. Surfactants enhance the tight-junction permeability of food allergens in human intestinal epithelial Caco-2 cells. Int Arch Allergy Immunol 130:135–142.

45. Berry J, Turner D. 1960. The enzymatic hydrolysis and tissue oxidation of fatty acid esters of sucrose. J Am Oil Chem Soc 37:302-305.

46. Shigeoka T, Izawa O, Kitazawa K, Yamauchi F, Murata T. 1984. Studies on the metabolic fate of sucrose esters in rats. Food Chem Toxicol 22:409–414.

47. Noker PE, Lin TH, Hill DL, Shigeoka T. 1997. Metabolism of 14C-labelled sucrose esters of stearic acid in rats. Food Chem Toxicol 35:589–595.

48. Christiansen A, Backensfeld T, Kühn S, Weitschies W. 2011. Investigating the stability of the nonionic surfactants tocopheryl polyethylene glycol succinate and sucrose laurate by HPLC-MS, DAD, and CAD. J Pharm Sci 100:1773–1782.

49. Okumura H, Kitazawa N, Wada S, Hotta H. 2011. Stability of sucrose fatty acid esters under acidic and basic conditions. J Oleo Sci 60:313–320.

50. Marciello M, Mateo C, Guisan JM. 2011. Full enzymatic hydrolysis of commercial sucrose laurate by immobilized-stabilized derivatives of lipase from Thermomyces lanuginosa. Colloids Surf B Biointerfaces 84:556–560.

51. Christiansen A, Backensfeld T, Weitschies W. 2010. Effects of non-ionic surfactants on in vitro triglyceride digestion and their susceptibility to digestion by pancreatic enzymes. Eur J Pharm Sci 41:376–382.

52. van Tellingen O, Beijnen JH, Verweij J, Scherrenburg EJ, Nooijen WJ, Sparreboom A. 1999. Rapid esterase-sensitive breakdown of polysorbate 80 and its impact on the plasma pharmacokinetics of docetaxel and metabolites in mice. Clin Cancer Res 5:2918–2924.

53. Csáki KF. 2011. Synthetic surfactant food additives can cause intestinal barrier dysfunction. Med Hypotheses 76:676–681.

54. Yu H, Hu YQ, Ip FCF, Zuo Z, Han YF, Ip NY. 2011. Intestinal transport of bis(12)-hupyridone in Caco-2 cells and its improved permeability by the surfactant Brij-35. Biopharm Drug Dispos 32:140–150.

55. Takahashi Y, Kondo H, Yasuda T, Watanabe T, Kobayashi S, Yokohama S. 2002. Common solubilizers to estimate the Caco-2 transport of poorly water-soluble drugs. Int J Pharm 246:85–94.

56. Nerurkar MM, Ho NF, Burton PS, Vidmar TJ, Borchardt RT. 1997. Mechanistic roles of neutral surfactants on concurrent polarized and passive membrane transport of a model peptide in Caco-2 cells. J Pharm Sci 86:813–821.

57. Horvát S, Fehér A, Wolburg H, Sipos P, Veszelka S, Tóth A, Kiss L, Kurunczi A, Balogh G, Kürti L, Erős I, Szabó-Révész P, Deli MA. 2009. Sodium hyaluronate as a mucoadhesive component in nasal formulation enhances delivery of molecules to brain tissue. Eur J Pharm Biopharm 72:252–259.

58. Aungst BJ. 2000. Intestinal permeation enhancers. J Pharm Sci 89:429-442.

59. Rege BD, Kao JPY, Polli JE. 2002. Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers. Eur J Pharm Sci 16:237–246.

60. Koga K, Murakami M, Kawashima S. 1998. Effects of fatty acid sucrose esters on ceftibuten transport by rat intestinal brush-border membrane vesicles. Biol Pharm Bull 21:747-751.

61. Darby RA, Callaghan R, McMahon RM. 2011. P-glycoprotein inhibition: the past, the present and the future. Curr Drug Metab 12:722-731.

62. Hugger ED, Novak BL, Burton PS, Audus KL, Borchardt RT. 2002. A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity in vitro. J Pharm Sci 91:1991–2002.

63. Bogman K, Erne-Brand F, Alsenz J, Drewe J. 2003. The role of surfactants in the reversal of active transport mediated by multidrug resistance proteins. J Pharm Sci 92:1250-1261.

64. Fenyvesi F, Fenyvesi É, Szente L, Goda K, Bacsó Z, Bácskay I, Váradi J, Kiss T, Molnár E, Janáky T, Szabó GJr, Vecsernyés M. 2008. P-glycoprotein inhibition by membrane cholesterol modulation. Eur J Pharm Sci 34:236–242.

65. Molinas A, Sicard G, Jakob I. 2012. Functional evidence of multidrug resistance transporters (MDR) in rodent olfactory epithelium. PLoS One 7:e36167.

66. Yamagata T, Kusuhara H, Morishita M, Takayama K, Benameur H, Sugiyama Y. 2007. Effect of excipients on breast cancer resistance protein substrate uptake activity. J Control Release 124:1–5.

67. Plou FJ, Cruces MA, Ferrer M, Fuentes G, Pastor E, Bernabé M, Christensen M, Cornelles F, Parra JL, Ballesteris A. 2002. Enzymatic acylation of di- and trisaccharides

with fatty acids: choosing the appropriate enzyme, support and solvent. J Biotechnol 96:55-66.

68. Ayala-Bravo HA, Quintanar-Guerrero D, Naik A, Kalia YN, Cornejo-Bravo JM, Ganem-Quintanar A. 2003. Effects of sucrose oleate and sucrose laureate on in vivo human stratum corneum permeability. Pharm Res 20:1267–1273.

Table 1. Cellular toxicity of sugar esters and reference molecules

Toxicity of P-1695, M-1695, D-1216 sucrose fatty acid esters and Tween 80, Cremophor RH40 reference absorption enhancers on human Caco-2 intestinal epithelial cells was measured by MTT dye conversion and LDH release methods after 24 hours of incubations. The MTT values were compared to non-treated group (100% viability). For LDH measurement groups were compared to the Triton-X 100 treated group (100% toxicity). TC 0, non toxic concentration; TC 50, caused 50 % toxicity, TC 100, 100% cell death; n.a., not applicable.

Table 2. Effect of surfactants on drug permeability

Fold changes in the apparent permeability coefficients of atenolol, vinblastine, caffeine and antipyrine measured on confluent human Caco-2 intestinal epithelial cell layers after 1-hour treatment with P-1695, M-1695, D-1216 sucrose fatty acid esters and Tween 80, Cremophor RH40 reference absorption enhancers. Data are presented as mean ± S.D., n = 3; statistical analysis: ANOVA followed by Dunnett test; $*P < 0.05$, $***P < 0.001$, all doses were compared to control.

Table S1. Properties of surfactants

R- carbon chain length, carbon chain length of fatty acid residues; CMC: critical micelle concentration; HLB: hydrophilic-lipophilic balance; US DMF, US drug master file.; Ph.Eur., European Pharmacopoea.

Legend to figures

Figure 1. Effect of sucrose esters and reference surfactants on cellular viability

Toxicity of M-1695, P-1695, D-1216 sucrose fatty acid esters (10 - 3000 µg/ml) and Tween 80, Cremophor RH40 reference absorption enhancers (10 – 3000 µg/ml) on human Caco-2 intestinal epithelial cells measured by (a) MTT dye conversion and (b) LDH release methods. The MTT values were compared to non-treated group (100% viability). For LDH measurement values were compared to the Triton-X 100 treated group (100% toxicity). Data are presented as mean ± S.D., n = 6; statistical analysis: ANOVA followed by Dunnett test; statistically significant differences ($P < 0.05$) were detected in the control group compared to the values measured in a: P-1695 group; b: M-1695 group; c: D-1216 group; e: Cremophor RH40 group.

Figure 2. The effect of sucrose esters on impedance, resistance and fluorescein permeability of cell layers

Effects of P-1695 (30 µg/ml), M-1695 (60 µg/ml), D-1216 (100 µg/ml) sucrose fatty acid esters and Tween 80 (1000 µg/ml), Cremophor RH40 (1000 µg/ml) reference absorption enhancers on (a) the impedance, (b) transepithelial electrical resistance and (c) fluorescein permeability on Caco-2 epithelial cells layer. Data are presented as mean ± S.D., n = 4; statistical analysis: ANOVA followed by Dunnett test. In case of (a) impedance measurement statistically significant differences were detected compared to the values measured in control group after treatment with P-1695 (# $P < 0.05$, ### $P < 0.001$); M-1695 (*$P < 0.05$, **$P < 0.01$); D-1216 (+ $P < 0.05$, +++ $P < 0.001$). Statistical analysis was also done at (b) TEER and (c) fluorescein permeability measurements by ANOVA followed by Dunnett test, ***$P < 0.001$, all groups were compared to control. Abbreviations: C, control; P, P-1695; M, M-1695; D, D-1216; Tw, Tween 80; CR, Cremophor RH40.

Figure 3. Effect of surfactants on cellular and junctional morphology, fluorescent immunostainings and electron microscopy

(a) Immunostaining for tight and adherens junction membrane proteins claudin-1, zonula occludens-1 and β-cathenin and fluorescent staining for F-actin microfilaments in human Caco-2 intestinal epithelial cells after 1 hour treatment with sucrose esters and reference absorption enhancers. Bar for claudin-1, ZO-1 and β-cathenin is 20 µm and for F-actin is 15

µm. (b) Transmission electron microscopy at cell-cell connections after 1 hour treatment with sucrose esters and reference absorption enhancers; bar = 400 nm. Applied concentrations: M-1695 at 60 µg/ml; P-1695 at 30 µg/ml; D-1216 at 100 µg/ml; Tween 80 at 1000 µg/ml; Cremophor RH40 at 1000 µg/ml. Abbreviations: C, control; M, M-1695; P, P-1695; D, D-1216; Tw, Tween 80; CR, Cremophor RH40; ZO-1, zonula occludens protein-1; arrow: tight junction; AJ: adherence junction; DE: desmosome; ID: interdigitation.

Figure 4. Effect of sucrose esters and reference surfactants on plasma membrane fluidity

The effect on plasma membrane fluidity of (a) sucrose esters, (b) reference absorption enhancers and benzyl alcohol measured by TMA-DPH fluorescence anisotropy on living Caco-2 cell suspensions. The anisotropy values in control cells were between 0.293±0.002 and 0.286±0.002 during the observation, and control value was considered as 100%. Data are presented as mean ± S.D., n = 6; statistical analysis: ANOVA followed by Dunnett test; ***$P < 0.001$, all groups were compared to control. Applied concentrations of sugar esters: 1, 10, 30, 60, 100 µg/ml; treatment doses of reference surfactants: 1, 10, 100, 1000 µg/ml. Benzyl alcohol was used at 30 mM concentration. Abbreviations: P, P-1695; M, M-1695;D, D-1216; Tw, Tween 80; CR, Cremophor RH40; BA, benzyl alcohol.

Figure 5. Effect of surfactants on efflux pump activity

The effects of absorption enhancers on efflux pump activity measured by accumulation and permeability studies. The accumulation of efflux pump ligands (a) rhodamine 123, and (b) calcein AM was determined after the treatment with sucrose esters, reference absorption enhancers, verapamil and cyclosporin A on confluent Caco-2 cells. Treatments were carried out simultaneously. The effects of sucrose esters and reference absorption enhancers on the apparent permeability coefficients of rhodamine 123 in (c) apical-to-basal, (d) basal-to-apical directions and on the (e) efflux ratio of rhodamine 123 were measured on confluent Caco-2 cell layers. Data are presented as mean ± S.D., n = 3-6; statistical analysis: ANOVA followed by Dunnett test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, all groups were compared to control. Applied concentrations: M-1695 at 60 µg/ml; P-1695 at 30 µg/ml; D-1216 at 100 µg/ml; Tween 80 at 1000 µg/ml; Cremophor RH40 at 1000 µg/ml; Verapamil at 100 µM; CycA, cyclosporine A at 10 µM. Abbreviations: P_{app}, apparent permeability coefficient; C, control; M, M-1695; P, P-1695; D, D-1216; Tw, Tween 80; CR, Cremophor RH40; Ver, verapamil; Cyc A, cyclosporine A.

Figure 6. How sucrose esters enhance permeability in epithelial cells?

Sucrose esters and reference absorption enhancers may increase the penetration of molecules in several ways. (1) Surfactants enhance the dissolution of molecules and change plasma membrane fluidity which contribute to enhanced delivery of agents. (2) Reference surfactants, but not sugar esters, directly inhibit efflux transporters. (3) Sucrose esters may alter the function of cellular junctions, but reference molecules do not. Abbreviations: ABC tp, ABC efflux transporters; CR, Cremophor RH40; SE, sucrose esters; Tw, Tween 80; efflux pumps.

Figure S1. Effect of sucrose esters and reference surfactants on fluorescein permeability

The effect of sucrose esters and reference absorption enhancers on the apparent permeability coefficients of fluorescein on confluent Caco-2 cell layers applied in the same doses of (a) 30 µg/ml, (b) 60 µg/ml, (c) 100 µg/ml. Data are presented as mean ± S.D., n = 3; statistical analysis: ANOVA followed by Dunnett test; ***$P < 0.001$, all groups were compared to control. Abbreviations: P_{app}, apparent permeability coefficient; C, control; P, P-1695; M, M-1695; D, D-1216; Tw, Tween 80; CR, Cremophor RH40.

Figure S2. Effects of surfactants, cytochalasin D and Triton X-100 on actin cytoskeleton

Fluorescent staining for F-actin cytoskeletal protein with phalloidin labelled by Alexa Fluor 488 in human Caco-2 intestinal epithelial cells after 1 hour treatment with sucrose esters, reference absorption enhancers, cytochalasin D or Triton X-100. Images were captured from two regions of cells after treatment with absorption enhancers: apical and basal part; and three different region of cells with treatment by cytochalasin D and Triton X-100: apical, junctional and basal part. Abbreviations: C, control; M, M-1695; P, P-1695; D, D-1216; Tw, Tween 80; CR, Cremophor RH40; CCD, cytochalasin D;TX, Triton X-100. Bar = 15 µm.

Figure S3. Effect of surfactants on efflux pump activity at pre-treatment

The pre-treatment effect of sucrose esters, reference absorption enhancers, verapamil and cyclosporin A on the accumulation of efflux pump ligand (a) rhodamine 123 and (b) calcein AM in confluent Caco-2 cell layers. Data are presented as mean ± S.D., n = 6; statistical analysis: ANOVA followed by Dunnett test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, all groups were compared to control. Applied concentrations: M-1695 at 60 µg/ml; P-1695 at 30 µg/ml;

D-1216 at 100 µg/ml; Tween 80 at 1000 µg/ml; Cremophor RH40 at 1000 µg/ml; Verapamil at 100 µM; CycA, cyclosporine A at 10 µM. Abbreviations: C, control; M, M-1695; P, P-1695; D, D-1216; Tw, Tween 80; CR, Cremophor RH40; Ver, verapamil; Cyc A, cyclosporine A.

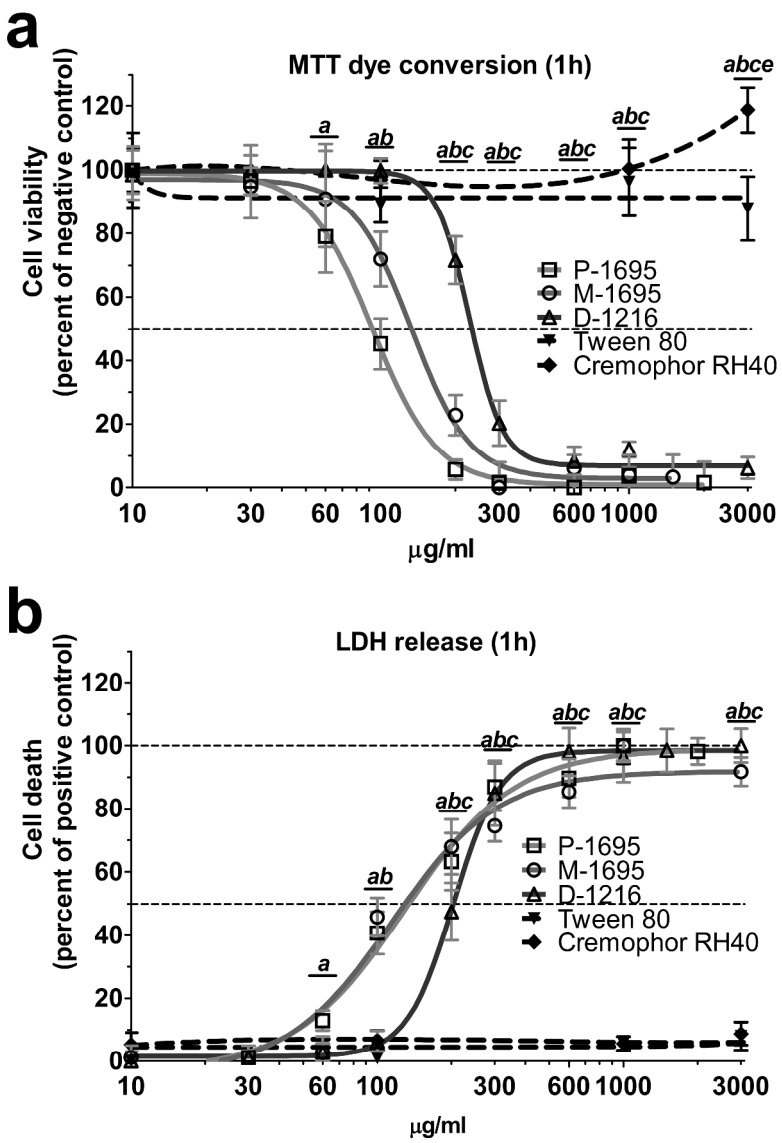
Table 1. Cellular toxicity of sugar esters and reference molecules

	MTT dye conversion				LDH release		
Absorption enhancers	**TC 0 (µg/ml)**	**TC 50 (µg/ml)**	**TC 100 (µg/ml)**		**TC 0 (µg/ml)**	**TC 50 (µg/ml)**	**TC 100 (µg/ml)**
P-1695	19	49	128		20	72	260
M-1695	18	62	211		20	61	189
D-1216	99	189	360		92	162	283
Tween 80	473	1073	2432		1068	1229	1621
Cremophor RH40	30900	33884	n.a.		2067	11810	44668

n.a.: not applicable

Table 2. Effect of surfactants on drug permeability

	Passive hydrophilic	Efflux substrate	Passive lipophilic	
	Atenolol	Vinblastine	Caffeine	Antipyrine
P-1695 30μg/ml	1.46±0.02 ***	4.84±0.79 ***	1.01±0.01	0.95±0.06
M-1695 60μg/ml	2.37±0.63 ***	25.92±2.74 ***	0.99±0.01	1.00±0.02
D-1216 100μg/ml	1.58±0.08 ***	9.74±0.31 ***	0.98±0.03	0.99±0.03
Tween 80 1000μg/ml	2.11±0.02 ***	10.74±0.67 ***	1.06±0.02	1.00±0.02
Cremophor RH40 1000μg/ml	1.20±0.15 ***	3.96±0.59 *	1.00±0.03	0.98±0.01



Effect of sucrose esters and reference surfactants on cellular viability
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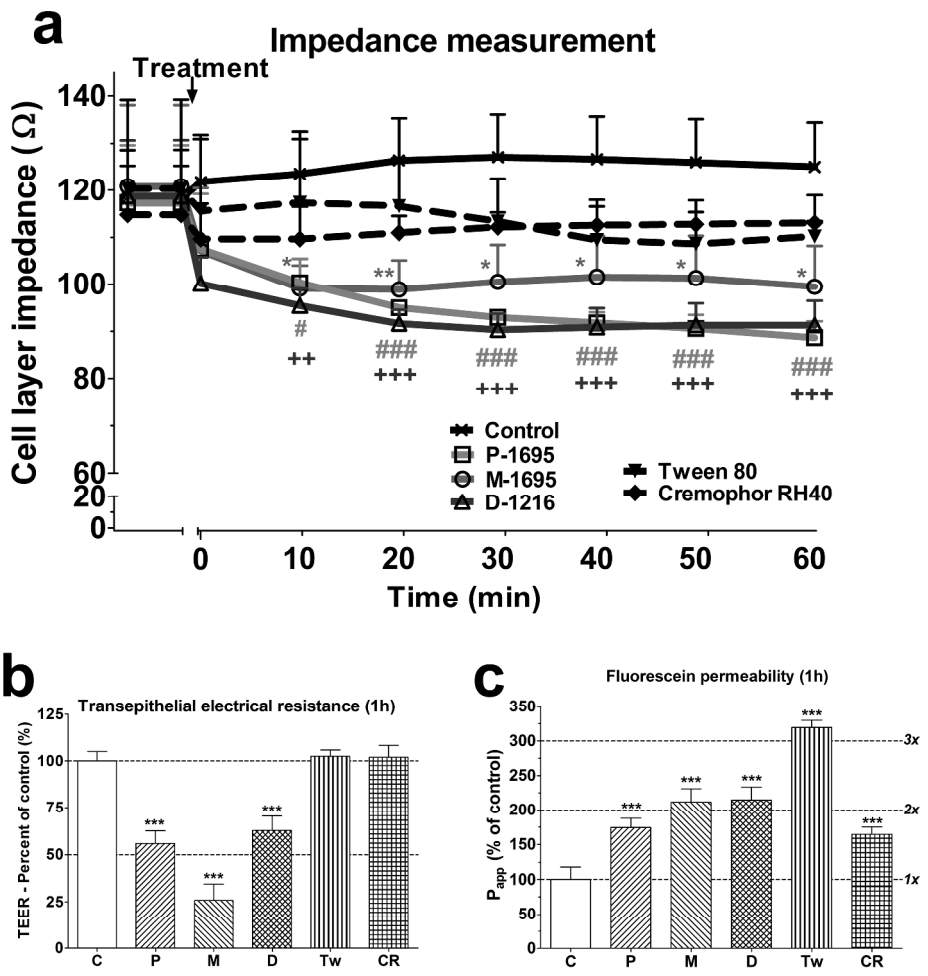

The effect of sucrose esters on impedance, resistance and fluorescein permeability of cell layers
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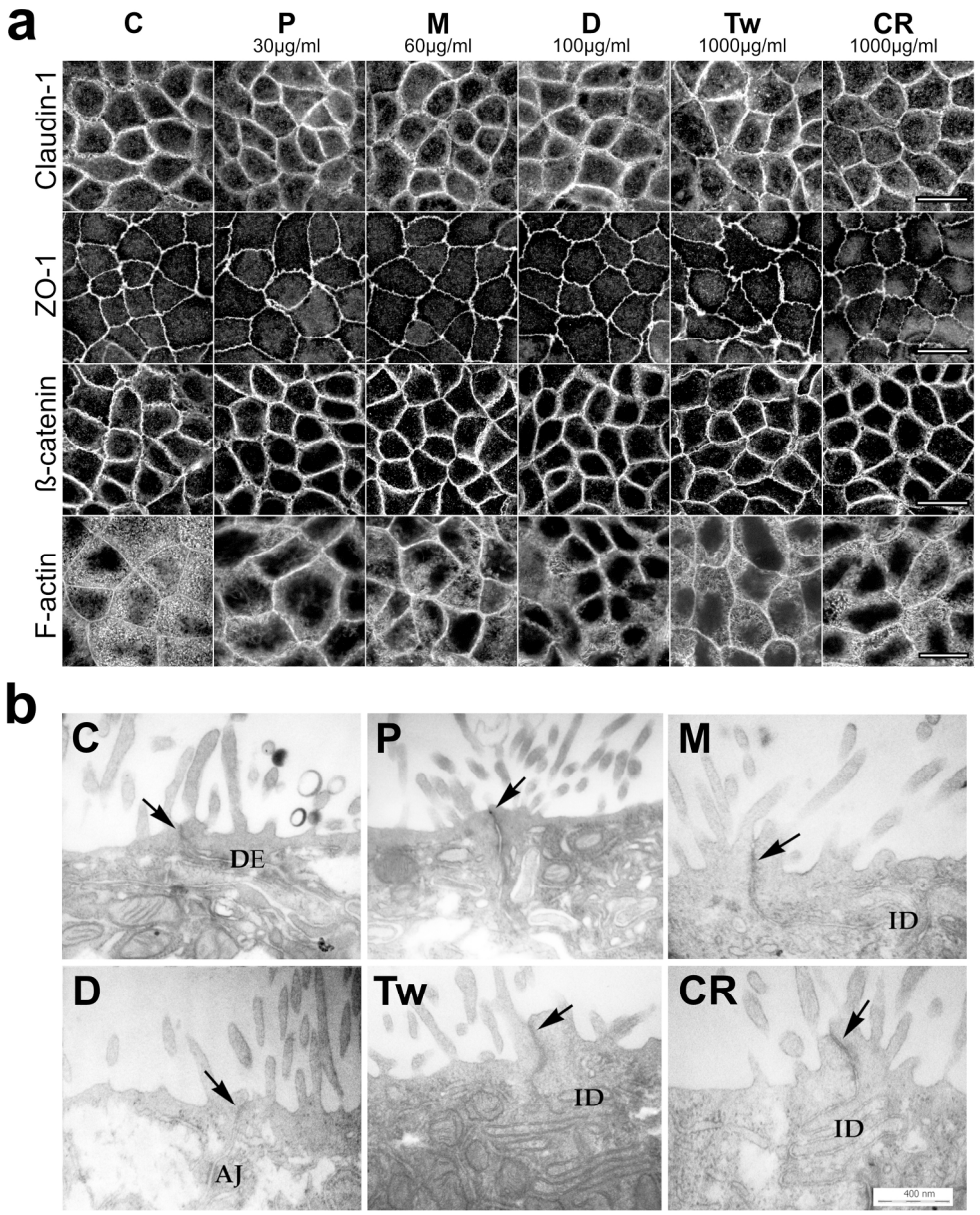

Effect of surfactants on cellular and junctional morphology, fluorescent immunostainings and electron microscopy
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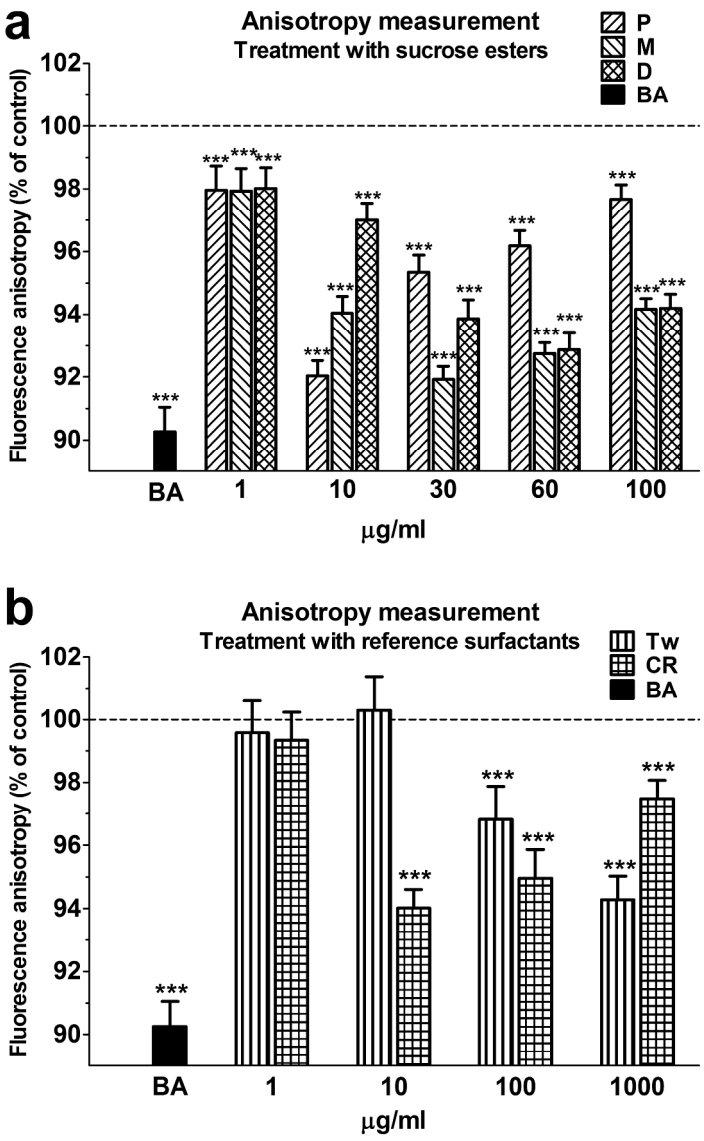

Effect of sucrose esters and reference surfactants on plasma membrane fluidity
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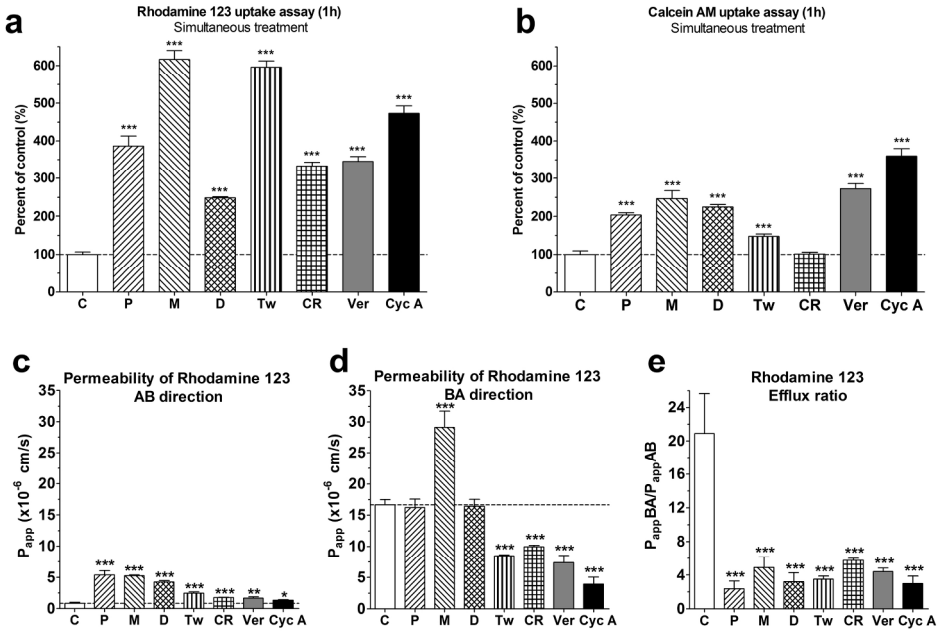

Effect of surfactants on efflux pump activity
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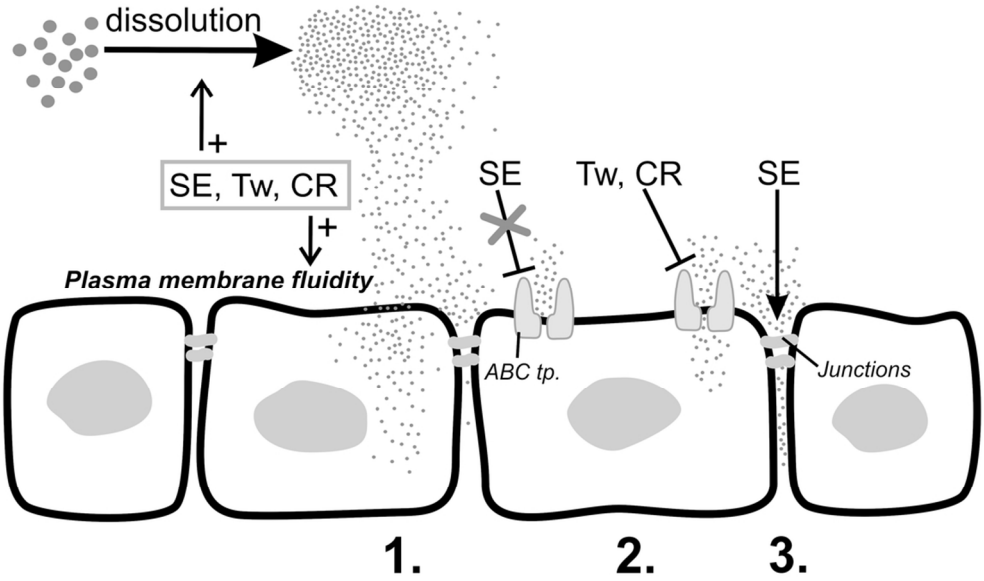

How sucrose esters enhance permeability in epithelial cells?
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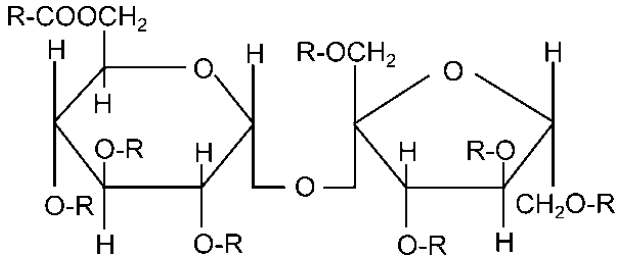
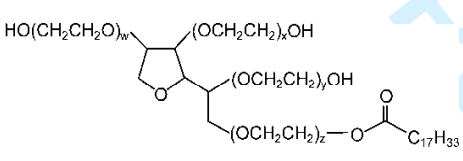
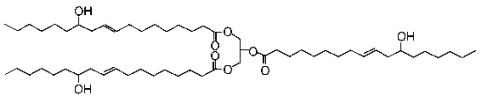
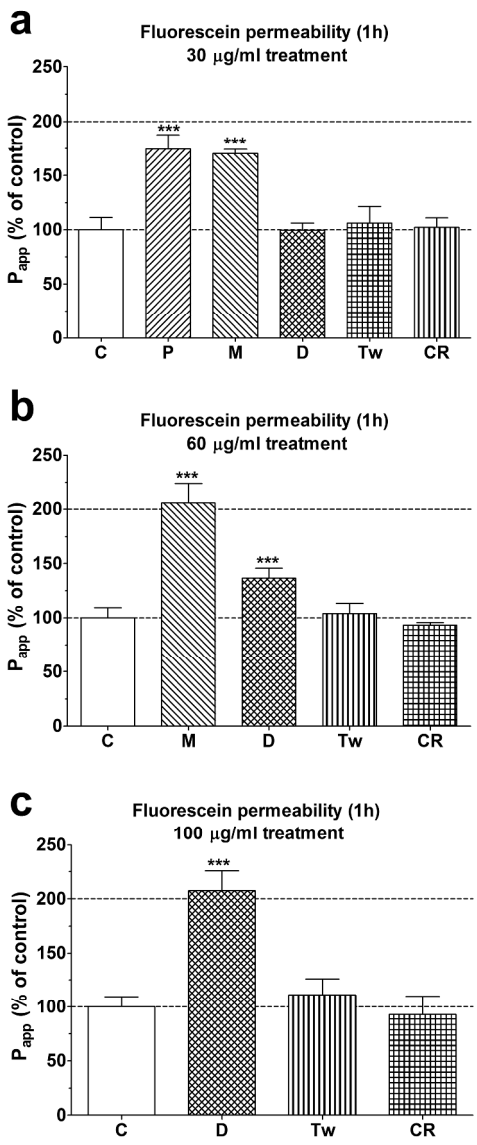
Table S1. Properties of surfactants

	P-1695	M-1695	D-1216
Chemical structure			
R- carbon chain lenght	16	14	12
HLB	16	16	16
CMC	28-250 µM [67]	28-250 µM [67]	500 µg/ml [68]
Trade name	Ryoto sugar ester P-1695	Ryoto sugar ester M-1695	Surfhope SE Pharma D-1216 (US DMF)
Chemical name	palmitate sucrose ester	myristate sucrose ester	laurate sucrose ester
Solubility	water soluble	water soluble	water soluble

	Polysorbate 80 (Ph.Eur.)	Macrogol-glycerolhydroxystearate 40 (Ph.Eur.)
Chemical structure		
R- carbon chain lenght	18	18
HLB	15	15
CMC	50-63µg/ml [51,56]	390 µg/ml [48]
Trade name	Tween 80	Cremophor RH40
Chemical name	polyethoxylatedsorbitan and oleic acid	polyethoxylated 40 hydrogenated castor oil
Solubility	water soluble	water soluble



Effect of sucrose esters and reference surfactants on fluorescein permeability
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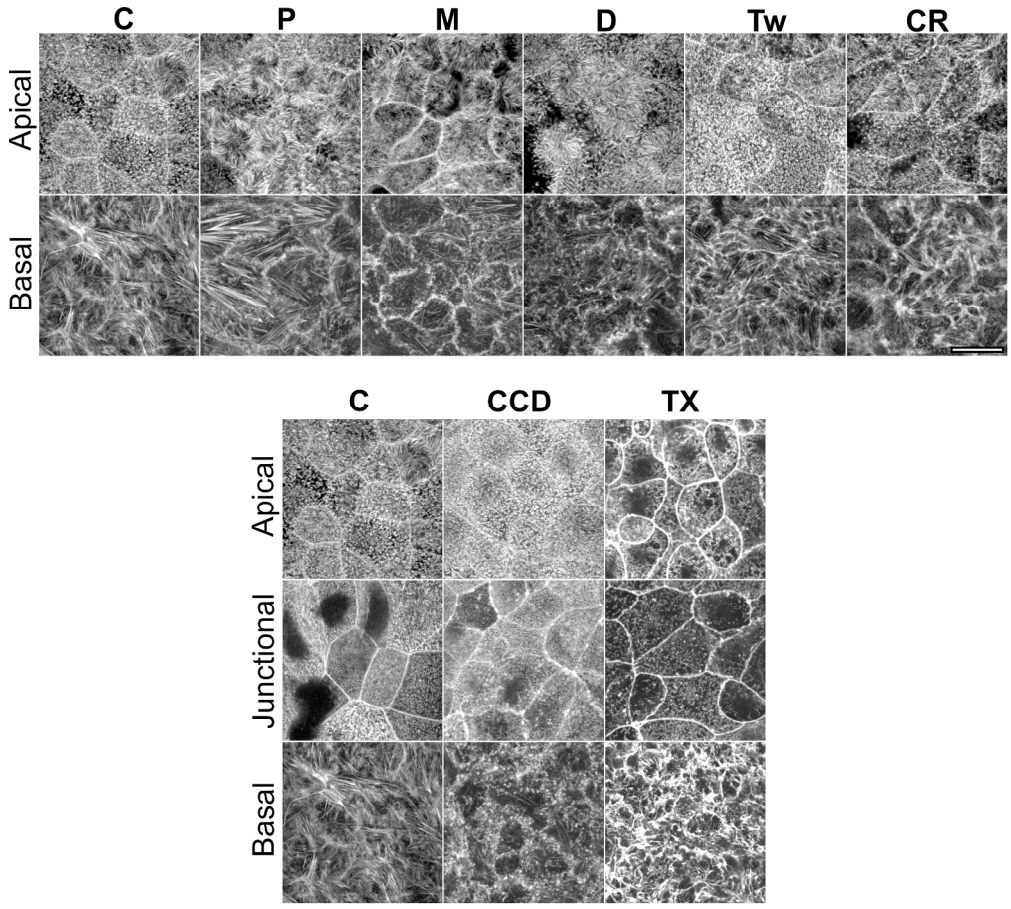

Effects of surfactants, cytochalasin D and Triton X-100 on actin cytoskeleton
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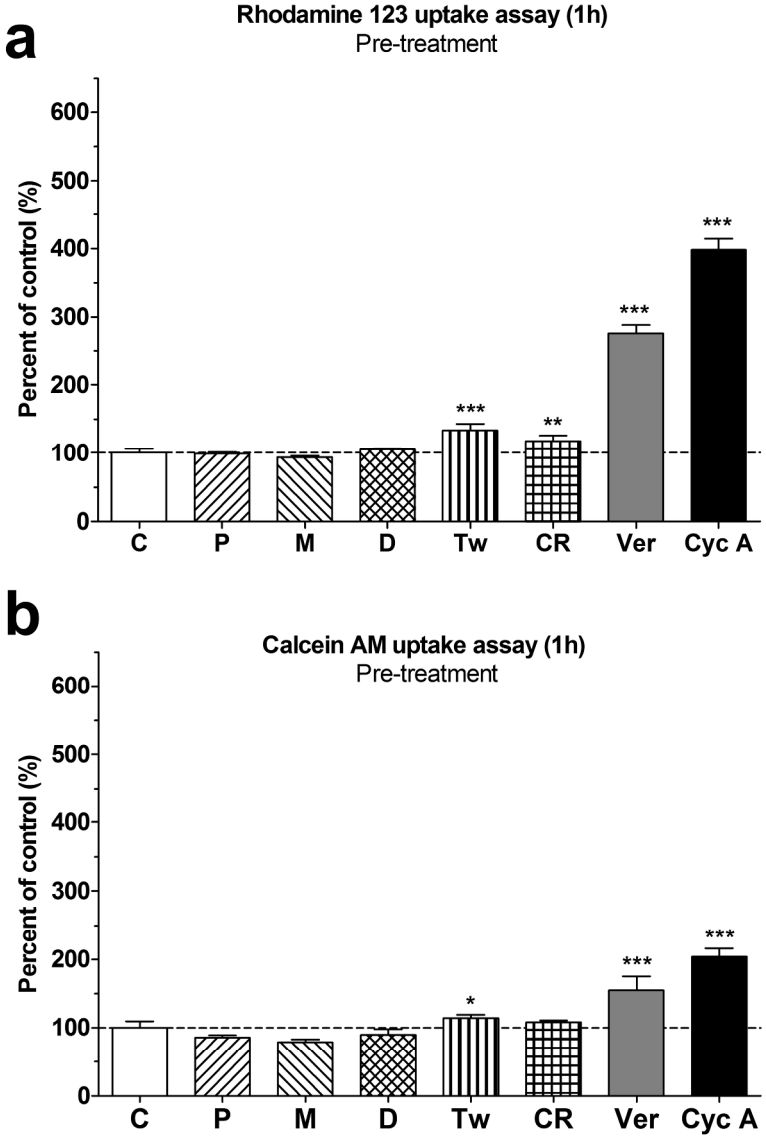

Effect of surfactants on efflux pump activity at pre-treatment
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