Sucrose Esters Increase Drug Penetration, But Do Not Inhibit P-Glycoprotein in Caco-2 Intestinal Epithelial Cells

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ABSTRACT: Sucrose 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-1, ZO-1, and β-catenin. Sucrose esters in nontoxic concentrations significantly reduced resistance and impedance, and increased permeability for atenolol, fluorescein, vinblastine, and rhodamine 123 in Caco-2 monolayers. No visible opening of the tight junctions was induced by sucrose esters assessed by immunohistochemistry and electron microscopy, but some alterations were seen in the structure of filamentous actin microfilaments. Sucrose esters fluidized the plasma membrane and enhanced the accumulation of efflux transporter ligands rhodamine 123 and calcein AM in epithelial cells, but did not inhibit the P-glycoprotein (P-gp)-mediated calcein AM accumulation in MES-SA/Dx5 cell line. These data indicate that in addition to their dissolution-increasing properties sucrose esters can enhance drug permeability through both the transcellular and paracellular routes without inhibiting P-gp. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3107–3119, 2014
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INTRODUCTION

Drug delivery across biological barriers 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] Nonionic surface-active agents, Tween 80, an ethoxylated sorbitan and Cremophor RH40, a castor oil ester, have been widely investigated and were demonstrated to enhance the solubility or absorption of drugs.[2,3] Clinically employed surfactants have advantageous properties as absorption enhancers,[4] but can also cause side effects.[4–6] There is a need for new, innovative absorption enhancers with more favorable profile and fever drawbacks.

Sucrose fatty acid esters are nonionic surfactants that are present naturally in plants and microorganisms.[7] Food industry applies them as food emulsifiers and food additives.[8,9] Because of low-skin irritation, excellent emulsification, and solubilizing properties, they are also used in cosmetics.[10] Sucrose esters are promising candidates for improving the solubility and permeability of drugs as recently reviewed by Szűcs and Szabó-Révész.[11] Because of their excellent emulsifier and surfactant properties, sucrose esters were tested for transdermal drug delivery in microemulsions and reversed vesicles.[12–16] The use of sucrose esters for oral application was less studied. Controlled release from matrix tablets containing sucrose esters was demonstrated.[17] Daunomycin accumulation was effectively enhanced by sucrose esters in Caco-2 intestinal cells.[18] In animal studies, sucrose esters improved the intestinal permeability of the polypeptide hormone calcitonin,[19] lidocaine hydrochloride,[20] and cyclosporine A.[21] The intestinal absorption of paracellular marker dextran was also enhanced by L-1695 laurate ester treatment in rats.[22] Although the paracellular barrier in intestinal cells is regulated by intercellular junctions,[4] changes in tight or adherens junctions and transport pathways following sucrose fatty acid ester treatments have not been investigated yet. Sucrose esters were also described to inhibit efflux pumps, mainly P-glycoprotein (P-gp, ABCB1) in epithelial cells and in animal experiments.[23,24] Although the effect of sucrose esters on cell membranes was suggested,[24] it was not investigated in living cells.

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The human Caco-2 cell line is a widely used culture model for intestinal drug absorption showing good correlation with *in vivo* data, which structurally and functionally resembles small intestinal epithelium.[25–27] Cells are polarized, grow in monolayer, possess microvilli, form tight intercellular connections by apical junctional complexes, and express solute carriers, and efflux transporters such as P-gp, BCRP, or MRP-2.[25,28,29]

Our aim was to test the effects of three different sucrose esters, namely, palmitate (P-1695), myristate (M-1695), and laurate (D-1216) esters, on the viability, passive permeability, junctional and cytoskeletal morphology, membrane fluidity, and efflux pump activity of Caco-2 intestinal epithelial cells in relation to reference absorption enhancers Tween 80 and Cremophor RH40. The toxicity of surfactants was determined by standard colorimetric end-point assays. The barrier properties were tested by resistance and impedance measurements. To assess passive and efflux transport across epithelial monolayers atenolol, caffeine, antipyrine, vinblastine, fluorescein, and rhodamine 123 were examined in the presence or absence of surfactants. Junctional morphology monitored by immunostaining for claudin-1, zonula occludens protein-1 (ZO-1), β-catenin, and transmission electron microscopy, and plasma membrane fluidity were investigated for the first time in sucrose ester-treated Caco-2 cells. Filamentous actin (F-actin), a main cytoskeletal protein contributing to normal organization of junctions was also studied. The effect of the sucrose esters on efflux pump activity was determined in Caco-2 cells; P-gp functionality was measured on MES-SA and MES-SA/Dx5 cell lines.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma–Aldrich Ltd. (Budapest, 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 Company, Tokyo, Japan). Tween 80 and Cremophor RH40 (BASF, Ludwigshafen am Rhein, Germany) were of pharmaceutical grade. Supplementary Table S1 summarizes some of the properties of the surfactants employed in the study.

Cell Culture

Human intestinal epithelial Caco-2 (ATCC catalog no. HTB-37, USA) cell line 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 six passages as described by Hellinger et al.[26] Cells were grown in Eagle's Minimal Essential Medium (Gibco, Life Technologies, Carlsbad, California) supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), sodium-pyruvate (Gibco, Life Technologies), 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 – Ethylenediaminetetraacetic acid 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, California) 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, Massachusetts). 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.

Human uterine sarcoma lines MES-SA, and its doxorubicin-selected derivative expressing high levels of P-gp (MES-SA/Dx5) were used for testing P-gp functionality.[30] Cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, Life Technologies) supplemented with 10% fetal bovine serum, 5 mM L-glutamine, and 50 unit/mL penicillin/streptomycin. To maintain homogenous P-gp expression, MES-SA/Dx5 cells were treated with 500 nM doxorubicin for at least two passages before the experiments.

Cell Viability Measurements

Treatment concentrations of sucrose esters P-1695, M-1695, and D-1216 varied between 3 and 3000 μg/mL. The reference absorption enhancers were used in 1–100,000 μg/mL concentrations. Treatment solutions were prepared in DMEM without phenol red. Triton X-100 (10 mg/mL) was used as a toxicity control.

(3-(4,5-Dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) dye 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 h, washed with phosphate-buffered saline (PBS; pH 7.4) and incubated with 0.5 mg/mL MTT solution for 3 h 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 nontreated 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 h with absorption enhancers, and then 50 μL samples from culture supernatants were incubated with equal amounts of reaction mixture for 15 min. 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). Cell death was calculated as percentage of the total LDH release from cells treated by 10 mg/mL Triton X-100. The nontoxic 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, California); equations are shown in Supporting Information 1.

Measurement of Electrical Resistance and Impedance of Cell Layers

Transepithelial electrical resistance (TEER) was measured by an EVOM resistance meter using STX-2 electrodes (World Precision Instruments Inc., Sarasota, Florida) and expressed relative to the surface area ($\Omega \times$ cm^2). TEER represents the paracellular permeability of cell layers for ions. The TEER of Caco-2

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