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A permeation enhancer for increasing transport of therapeutic macromolecules across the intestine

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ABSTRACT

Delivery of therapeutic macromolecules is limited by the physiological limitations of the gastrointestinal tract including poor intestinal permeability, low pH and enzymatic activity. Several permeation enhancers have been proposed to enhance intestinal permeability of macromolecules; however their utility is often hindered by toxicity and limited potency. Here, we report on a novel permeation enhancer, Dimethyl palmitoyl ammonio propanesulfonate (PPS), with excellent enhancement potential and minimal toxicity. PPS was tested for dose- and time-dependent cytotoxicity, delivery of two model fluorescent molecules, sulforhodamine-B and FITC-insulin *in vitro*, and absorption enhancement of salmon calcitonin (sCT) *in vivo*. Caco-2 studies revealed that PPS is an effective enhancer of macromolecular transport while being minimally toxic. TEER measurements in Caco-2 monolayers confirmed the reversibility of the effect of PPS. Confocal microscopy studies revealed that molecules permeate *via* both paracellular and transcellular pathways in the presence of PPS. *In vivo* studies in rats showed that PPS enhanced relative bioavailability of sCT by 45-fold after intestinal administration. Histological studies showed that PPS does not induce damage to the intestine. PPS is an excellent permeation enhancer which provides new opportunities for developing efficacious oral/intestinal delivery systems for therapeutic macromolecules.

1. Introduction

Despite its convenience and compliance, oral delivery of macromolecules has remained a distant clinical reality due to enzymatic degradation of macromolecules in the gastrointestinal tract and limited permeability across intestinal epithelium [1,2]. These limitations result in poor drug absorption into systemic circulation and insignificant bioavailability of macromolecules *via* the oral route. A variety of innovative approaches have been developed to tackle these challenges. These include: (i) strategies to avoid gastrointestinal degradation such as the use of enzyme inhibitors [3,4], encapsulation into microspheres [5,6], nanoparticles [7,8], and liposomes [9,10]; and (ii) strategies to enhance intestinal permeability by chemical modification of the macromolecules [11,12], and/or the use of chemical permeation enhancers (CPEs) to increase the uptake of therapeutic macromolecules across intestinal epithelium [13,14].

Chemical permeation enhancers (CPEs) are chemical agents that interact with the cellular structure of the epithelium and modify it so as to increase its permeability. CPEs have been thoroughly investigated for their efficacy in enhancing drug delivery across the epithelial membranes of oral, buccal, nasal, pulmonary, and vaginal tissues [15–18]. CPEs are capable of increasing oral bioavailability of poorly permeated therapeutic molecules by the transcellular route, *i.e.*, by enhancing the transport across the epithelial membrane or by the paracellular route, *i.e.*, by modifying the epithelial intercellular tight junctions [13,19].

A variety of CPEs have been tested for their efficacy in enhancing macromolecular permeability across the intestinal epithelium. These include surfactants, bile salts and fatty acids, which operate through several mechanisms including partial solubilization of cell membrane and increase in local calcium concentration [20–22]. In spite of their promise, one of the main concerns associated with CPEs is an arguably direct correlation between potency and toxicity. Based on a study of a large number of CPEs, Whitehead et al. previously reported that certain CPEs deviate from the correlation between potency and toxicity [23–25]. A leading example of such CPEs was a zwitterionic surfactant, Dimethyl palmitoyl ammonio propanesulfonate (PPS) [23]. PPS was shown to be non-toxic to Caco-2 monolayers *in vitro* while enhancing transport of small molecules such as mannitol *in vitro* [23]. Here, we report based on *in vitro* and *in vivo* studies that PPS is capable of safely enhancing intestinal absorption of therapeutic macromolecules including insulin and salmon calcitonin (sCT).

2. Materials and methods

2.1. Materials

Palmitoyl dimethyl ammonio propane sulfonate (PPS), FITC labeled bovine insulin, and sulforhodamine B were obtained from Sigma Aldrich (St. Louis, MO, USA). Salmon calcitonin was obtained

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from Anaspec, Inc. (Fremont, CA, USA). The transwell Caco-2 system was set up using 24 well BD-Biocoat™ HTS Caco-2 assay system (fibrillar collagen coated, 1 μm pore size) obtained from BD Biosciences (Bedford, MA, USA). Extraction-free ELISA kit for analysis of salmon calcitonin was obtained from Bachem Americas, Inc. (Torrance, CA, USA). Colorimetric assay kit for calcium measurements was obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). Supplies for Caco-2 culture, cell viability studies and for confocal imaging were obtained from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals used were of analytical grade and were obtained from various vendors.

2.2. Cell culture and transwell assay system set-up

Human colorectal adenocarcinoma Caco-2 cell line (HTB-37) obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) was used for all cell culture experiments. Cell lines were maintained as per the provider's protocol with slight modifications. Cell culture was performed in Hyclone® DMEM high glucose media supplemented with 50 IU/ml of penicillin, 50 mg/l of streptomycin, and 100 ml/l of fetal bovine serum at 37 °C in a humidity-controlled 5% CO_2 cell culture incubator. Cells were split at a ratio of 1:3 after reaching 90% confluence.

2.3. Cell viability studies with Methyl Thiazole Tetrazolium (MTT) assay

Toxicity of PPS was tested on Caco-2 cell line (HTB-37) using an MTT assay. Briefly, Caco-2 cells were plated in a 96 well plate at a density of 50,000 cells per well in flat-bottom 96-well microtiter tissue culture plates and allowed to grow overnight in DMEM medium as described earlier. Immediately prior to the start of the experiment, the medium was removed from the wells, the cells were washed twice with sterile saline, 100 μL of PPS test solutions (concentrations ranging between 0.0005% and 0.03% w/v) were added, and the plates were incubated at 37 °C for predetermined time points (10 min, 1 h, and 5 h). Following the incubation, the test samples were removed from the wells, the bottom of each well was washed with saline, 10 μL of MTT solution (5 mg/ml) was added to each well, and the cells were again incubated at 37 °C for 4 h. After 4 h, the MTT solution was removed carefully, 100 μL of 100% DMSO was added, and the plates were incubated for 1 h with moderate shaking. Absorbance was measured at 570 nm using a Tecan Saffire™ fluorescent microplate reader (Tecan Group Ltd, Mannedorf, Switzerland). Each assay was performed on 8 samples ($n = 8$), and cell viability was expressed as the % of cells without treatment (negative controls).

2.4. Transwell assay set-up

For the development of the transwell assay system for permeability experiments, Caco-2 cells were seeded onto fibrillar collagen coated polyethylene terephthalate (PET) filter supports (1 μm pore size) of BD Biocoat™ HTS Caco-2 assay system (BD Biosciences, Bedford, MA, USA) according to the manufacturer's protocol with slight modifications. All the transwell experiments were performed with cells between 5th and 14th passages due to possible phenotypic differences between cells from high and low passage intervals. Cells were cultivated for at least 2 passages before seeding onto transwell filter supports to stabilize the cell phenotype [26]. Briefly, Caco-2 cells were seeded onto the filter supports at a concentration of 200,000 cells/insert (6.6×10^5 cells/cm^2) using basal seeding medium (with MITO + serum extender) provided in the HTS system, and were incubated in cell culture incubator (37 °C, 5% CO_2). Cells were fed from both sides using the Multiwell feeder tray. After 24 h, the feeding media was replaced with MITO + serum extender supplemented Entero-STIM media, and growing monolayers were incubated as described earlier. Upon incubation for 48–72 h, Caco-2 cells developed a tight junction monolayer, integrity of which was determined by TEER measurements.

2.5. Transepithelial electrical resistance (TEER) measurements

The integrity of Caco-2 monolayer was determined by measuring the transepithelial electrical resistance (TEER) of the cell monolayer grown on filter supports using Millicell-ERS electrical resistance measuring system (Millipore, Bedford, MA) using chopstick electrodes. Briefly, the Caco-2 inserts were transferred to a 24-well culture plate with 1400 μl media in the feeding well, and 500 μl in culture inserts. The electrodes were immersed in a way that shorter electrode was in the insert and longer electrode in the outer well. Care was taken that the electrode did not touch the monolayer. A resistance reading of 150–200 Ω cm^2 was considered as indicative of a confluent Caco-2 monolayer with tight junctions.

2.6. Efficacy of PPS in enhancing transport of sulforhodamine B (SRB) and FITC-insulin (FITC-I)

Efficacy of PPS in enhancing transport of both small and large molecular weight pharmaceutical moieties, transport of 2 fluorescent compounds, Sulforhodamine B (MW 558 Da) and FITC-insulin (Bovine insulin labeled with fluorescein isothiocynate, MW ~6000 Da) was tested across Caco-2 monolayers. Briefly, cells were pre-conditioned with basal seeding medium for 30 min before starting the experiment at 37 °C. Both FITC-insulin and sulforhodamine-B were loaded onto the individual Caco-2 monolayer filter supports at a concentration of 0.15 mg/well. PPS was added to the apical chamber loading solutions at concentrations of 0.01% and 0.03% w/v, dissolved in 500 μl of basal seeding medium. The basolateral chamber consisted 1400 μl of the same growth media as per manufacturer's protocol. The plates were incubated for 5 h with gentle shaking. TEER measurements were performed at predetermined time intervals (0, 0.25, 0.5, 1, 2, 3, and 5 h). At the same time-points, 100 μl sample was withdrawn from the basolateral chamber to quantify the total amount of FITC-insulin/sulforhodamine-B transported across the monolayer. The withdrawn sample was immediately replaced with an equivalent amount of the experimental media. Withdrawn samples were analyzed using a Tecan Safire™ fluorescent microplate reader (Tecan Group Ltd, Mannedorf, Switzerland) at respective wavelengths for FITC-insulin (Ex 488 nm; Em 525 nm) and sulforhodamine-B (Ex 560 nm and Em 590 nm).

2.7. Determination of apparent permeability (P_{app}) and enhancement ratio (ER)

The apparent permeability coefficients (P_{app}) of all the pharmaceutical molecules were calculated using the following equation [27]:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A.C_o} \quad (1)$$

where dQ is the amount of solutes transported across the Caco-2 barrier in time dt, C_o is the solute concentration in apical compartment at time zero, and A is the cross-sectional area of the epithelium in contact with apical solution. Total percent drug transport was calculated by dividing the cumulative amount of molecules transported with the original loading concentrations.

Transport enhancement ratios were calculated according to the following equation [28]:

$$ER = \frac{P_{app-Enhancer}}{P_{app-Control}}. \quad (2)$$

All P_{app} and % transport data were calculated using cumulative amount of FITC-I/SRB transported across the monolayers in 5 h.