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Research paper

Rhamnolipids as epithelial permeability enhancers for macromolecular therapeutics

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ABSTRACT

The use of surfactants as drug permeability enhancers across epithelial barriers remains a challenge. Although many studies have been performed in this field using synthetic surfactants, the possibility of employing surfactants produced by bacteria (the so called biosurfactants") has not been completely explored. Among them, one of the most well characterized class of biosurfactants are rhamnolipids. The aim of the study was to investigate the effect of rhamnolipids on the epithelial permeability of fluorescein isothiocyanate-labelled dextrans 4 kDa and 10 kDa (named FD4 and FD10, respectively) as model for macromolecular drugs, across Caco-2 and Calu-3 monolayers. These cell lines were selected as an *in vitro* model for the oral and respiratory administration of drugs. Before performing permeability studies, rhamnolipids mixture was analysed in terms of chemical composition and quantification through mass analysis and HPLC. Cytotoxicity and transepithelial electrical resistance (TEER) studies were also conducted using Caco-2 and Calu-3 cell lines. A dose-dependent effect of rhamnolipids on TEER and FD4 or FD10 permeability across both cell lines was observed at relatively safe concentrations. Overall, results suggest the possibility of using rhamnolipids as absorption enhancers for macromolecular drugs through a reversible tight junction opening (paracellular route), despite more investigations are required to confirm their mechanism of action in term of permeability.

1. Introduction

The use of biosurfactants, as well as surfactants from renewable sources, for drug delivery applications has become increasingly appealing in recent years [1,2]. One such class of surfactants is that of rhamnolipids. These have attracted significant attention as promising surface-active excipients for cosmetic and pharmaceutical applications, owing to their good emulsification, foaming and wetting properties, as well as their excellent surface activity [3]. Rhamnolipids are biosurfactants belonging to the class of glycolipids, made up of one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnose moieties, linked to a large variety of 3-(hydroxyalkanoyloxy)alkanoic acids (generally from C8 to C16 carbon chain). They are predominantly produced from different strains of *Pseudomonas aeruginosa* by a fermentation process, which gives rise, after purification, to a mixture of mono-and di-rhamnolipids with different lengths of the hydrophobic tails. As with other biosurfactants, rhamnolipids offer advantages over known synthetic surfactants, including a potentially low toxicity and high level of environmental biodegradability, in addition to favourable intrinsic biological properties such as antimicrobial activity against several Gram positive and Gram negative bacteria, and fungi [4–6]. Rhamnolipids have already been investigated as an alternative to synthetic surfactants in several pharmaceutical formulations, including microemulsions [7], nanoemulsions [8] or topical formulations administered to the skin [9].

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A key application of surfactants in the pharmaceutical field is their ability to act as drug absorption enhancers. Therapeutically acceptable absorption of therapeutic biomacromolecules such as proteins and peptides across the mucosal surfaces of the intestinal and the respiratory system remains a challenge and injection-mediated administration remains the default option for these therapeutics. Several classes of synthetic surfactants have been shown to increase mucosal drug permeability *in vitro* and *in vivo*, including polysorbates [10,11], sodium dodecyl sulphate [12] sodium caprate [13,14], through different selective or non-specific mechanisms [15]. The interaction of surfactants with the epithelial tissue is thought to modulate the barrier property, e.g. through a tight junction opening mechanism. However, key to the safe and effective use of these materials is an acceptable toxicity profile, which has often been raised as an issue with the use of some surfactants.

Research to date demonstrates that rhamnolipids possess a favourable toxicological profile [16,17] and, being secreted from *Pseudomonas aeruginosa* as virulence factors to promote the infiltration across epithelia [18], could display an improved performance (at optimal doses) as permeability enhancers in comparison to synthetic surfactants. Rhamnolipids have previously been evaluated as permeability enhancers [19,20]. Specifically, the effect of different concentrations of rhamnolipid mixtures on the permeability of small molecules such as phenol red, propranolol [19] and [^{14}C] mannitol across Caco-2 (intestinal epithelial) monolayers has been investigated [20]. However, it is not known (not reported in the literature) whether these materials can enhance the epithelial permeability of macromolecules. The aim of this study is to investigate the effect of a rhamnolipid mixture on the epithelial permeability of macromolecules. Fluorescently-labelled dextrans of two different molecular weights, namely 4 kDa and 10 kDa (FD4 and FD10), were used as model macromolecular drugs. The commercial mixture of rhamnolipids was initially analysed to identify, through mass analysis and high-performance liquid chromatography (HPLC), the main components. This was followed by assessment of rhamnolipid effects on cytotoxicity, transepithelial electrical resistance (TEER) and permeability in Caco-2 and Calu-3 polarised monolayers as models for the intestinal and airway epithelia, respectively.

2. Materials

Rhamnolipids from *Pseudomonas aeruginosa* (90% purity) were purchased from Sigma-Aldrich (Poole, UK). Caco-2 and Calu-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and used at passages 32–36 for Calu-3 and 62–68 for Caco-2. Hank's Balanced Salt Solution (HBSS) with sodium bicarbonate and without phenol red, trypsin, antibiotic/antimycotic solution and Foetal Bovine Serum (FBS) were all obtained from Sigma-Aldrich (Poole, UK). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Thermo Fisher Scientific (Waltham, USA) and supplemented with penicillin, streptomycin and amphotericin at final concentrations of 100 units/mL, 100 μg/mL and 0.25 μg/mL, respectively, and FBS at 10% (v/v). Transwell® permeable supports of 12 mm diameter, 0.4 μm pore size, were obtained from Corning Life Sciences (Tewksbury, MA, USA). MTS reagent (commercially known as "CellTiter 96 AQueous One Solution Cell Proliferation Assay") was purchased from Promega (Madison, Wisconsin). LDH assay kit (Pierce LDH Cytotoxicity Assay Kit) was purchased from Thermo Scientific (Waltham, MA, USA). Fluorescein isothiocyanate (FITC)-labelled dextran of approximate average MW of 4 kDa (FD4) and 10 kDa (FD10) were purchased from Sigma-Aldrich (Poole, UK).

3. Methods

3.1. Mass analysis

For mass analysis, approximately 2.5 mg of the rhamnolipid mixture was dissolved in 1 mL of water and analysed by direct injection in an electrospray ionisation (ESI) mass apparatus (HP 1100 LC/MSD, Agilent), equipped with a single quadrupole detector. The sample (1 μL) was analysed in negative mode at different fragmentor voltages (0, 30, 60, 90 V).

3.2. HPLC analysis

HPLC runs were performed using a HPLC system (HP1100, Agilent Technologies) equipped with a photodiode array detector (DAD). Rhamnolipids were analysed after derivatization using 2-bromoacetophenone [21]. The separation of rhamnolipids was achieved using a reverse phase liquid chromatography with a C18 Discovery column (5 μm, 15 cm × 4.6 mm) (Supelco, USA). The elution was made using water/acetonitrile mixture at 70:30 ratio. Flow rate was 0.8 ml/min. Estimation of the relative amount (as a percentage) of the mono-and di-rhamnolipids in the mixture was performed by the ratio of the integrated area over time relative to the chromatographic peaks measured at 248 nm (ChemStation software, Agilent Technologies).

3.3. Surface tension measurements

Surface tension measurements were performed at 37 °C by tensiometry, using the Du-Noüy ring method (DCA-100 contact angle tensiometer; First Ten Angstrom, USA). A stock solution of rhamnolipids was prepared in HBSS and then diluted to obtain different concentrations. Surface tension of each solution was measured, controlling the temperature of the vessel (Lauda E300 circulating thermostat). The critical micelle concentration (CMC) and the surface tension at CMC (γCMC) were determined from the breakpoint of the surface tension versus log surfactant concentration (mM). Measurements were performed in triplicates.

3.4. Dynamic light scattering

Dynamic light scattering measurements were performed using a Malvern Zetasizer NanoS instrument (Malvern, Worchestershire, UK). Counts (Kcps) of different concentration of rhamnolipids solutions were recorded as previously reported [22]. CMC was determined by the straight-line interception method. Measurements were performed in triplicates at 37 °C.

3.5. Cytotoxicity assay

Caco-2 and Calu-3 cells were seeded on 96-well plates at 10,000 cells per well and cultured in DMEM for 48 h. Prior to the assay, culture medium was removed and replaced with different concentrations of rhamnolipids dissolved in HBSS. Triton X-100 (0.1% v/v in HBSS) and HBSS were used as the positive and negative control, respectively. Cells were incubated (at 37 °C, 5% CO_2) with samples and controls for 3 h. Samples and controls were then removed and the tests were subsequently conducted according to the manufacturers' instructions for both MTS and LDH assay, with at least four repeats for each sample. The absorbance of the formazan product in both cytotoxicity assays was measured at 490 nm using a plate reader (Tecan M200 Pro). The EC_{50} values (concentration of surfactants inducing 50% cell death from MTS and concentration of surfactants inducing 50% of LDH release from LDH assays) were