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Research paper

Calcifediol-loaded liposomes for local treatment of pulmonary bacterial infections

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ABSTRACT

The influence of vitamin D3 and its metabolites calcifediol (25(OH)D) and calcitriol on immune regulation and inflammation is well described, and raises the question of potential benefit against bacterial infections. In the current study, 25(OH)D was encapsulated in liposomes to enable aerosolisation, and tested for the ability to prevent pulmonary infection by *Pseudomonas aeruginosa*. Prepared 25(OH)D-loaded liposomes were nanosized and monodisperse, with a negative surface charge and a 25(OH)D entrapment efficiency of approximately 23%. Jet nebulisation of liposomes was seen to yield an aerosol suitable for tracheo-bronchial deposition. Interestingly, 25(OH)D in either liposomes or ethanolic solution had no effect on the release of the proinflammatory cytokine KC from *Pseudomonas*-infected murine epithelial cells (LA-4); treatment of infected, human bronchial 16-HBE cells with 25(OH)D liposomes however resulted in a significant reduction in bacterial survival. Together with the importance of selecting an application-appropriate *in vitro* model, the current study illustrates the feasibility and practicality of employing liposomes as a means to achieve 25(OH)D lung deposition. 25(OH)D-loaded liposomes further demonstrated promising effects regarding prevention of *Pseudomonas* infection in human bronchial epithelial cells.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease, characterised by persistent and recurring infection of the lungs [1,2]. A chronic inflammation in response to the presence of pathogens also develops in CF patients, which is mainly characterised by the accumulation of neutrophils [3–6]. Continuing infection and inflammation lead to a progressive destruction of the lung tissue, with subsequent respiratory failure being the ultimate outcome [7]. The causative agents of infection in CF are limited to a relatively narrow spectrum of pathogens, with *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* being the most prevalent organisms [1,6,8]. Despite progress in the development of antibiotic therapy, pulmonary infections still dictate the fate of most CF patients. Effective antimicrobial treatment of CF-associated infection is presently limited by several factors, including development of bacterial resistance against the antibiotics commonly in use (exacerbated by the common need for regular or prophylactic antibiotic therapy), as well as a lack of novel anti-infectives currently in the pharmaceutical pipeline [9–11].

The role of vitamin D3 in the regulation of immune and host defence reactions is well described, as is its influence on the release of inflammatory mediators from neutrophils and macrophages [12–15]. In recent years a connection between vitamin D3 and pulmonary diseases such as asthma and chronic obstructive lung disease has been suggested, and a clear link between vitamin D3 deficiency and respiratory tract infections in patients has been postulated [16,17]. Interestingly, low serum levels of vitamin D3 have been found specifically in CF patients, probably as a result of malabsorption [18]. Therefore, it may be hypothesised that the administration of vitamin D3 or its metabolites directly to the lung of CF patients could lead to an improved clinical outcome. Unfortunately

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however, the poor water solubility of these compounds necessitates dissolution in organic solvents such as ethanol, which limits administration *in vivo*. Therefore, to enable pulmonary delivery of vitamin D3 and to study its potential effects on CF-relevant infections, aerosolisable liposomes of the vitamin D3 metabolite calcifediol (25(OH)D) were developed and characterised in the current work. The potential of 25(OH)D liposomes to act as a local delivery system to prevent *P. aeruginosa* infection was then tested *in vitro* in two different cell models.

2. Materials and methods

2.1. Material

Dipalmitoylphosphatidylcholine (DPPC) was obtained as a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Calcifediol (25(OH)D, Ph.Eur/USP) was provided by Dishman Netherlands (Veenendaal, The Netherlands). 1,2 dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (Rh-DPPE) was purchased from Avanti Polar Lipids (Alabaster AL, USA). Distilled de-ionised water having a conductivity of less than 18.2 MΩ/cm at 25 °C was used throughout the study. All the other solvents and chemicals used were of at least analytical grade. For cell cultivation, Ham's F12 medium containing 15% or DMEM-HamF12 (1:1) with foetal bovine serum (FBS) (all from Life Technologies, Darmstadt, Germany), penicillin and streptomycin (both Life Technologies, Darmstadt, Germany) and Ultroser-G (Pall, Fribourg, Switzerland) were used.

2.2. Liposome preparation and characterisation

Liposome formulations were prepared based on a modified version of the lipid film hydration method [19]. Briefly, DPPC and 25(OH)D or DPPC alone (total weight 75 mg) were dissolved in 5 ml of ethyl acetate/methanol (4:1 v/v) in a round-bottomed flask. Following dissolution, 0.1 ml of a 0.5 mg/ml solution of Rh-DPPE was added and mixed. The organic solvent mixture was then evaporated under reduced pressure and with a rotation speed of 145 rpm at 70 °C using a rotavapor (Büchi, Essen, Germany). The resulting homogenous thin lipid film was then re-hydrated by the addition of 5 ml of deionised water, followed by further rotation at 60 °C for 1 h. The formed liposomal dispersion was sonicated in a sonication bath (Bandelin Sonorex, Berlin, Germany) for 10 min and then extruded (LiposoFast extruder, Avestin, Mannheim, Germany) repeatedly through 200 nm pore size membranes (AMD Manufacturing Inc., Ontario, Canada) to achieve size reduction and uniformity. Liposomes were then diluted 1:10 with deionised water and stored at 4 °C under nitrogen until further use. Physical characterisation of diluted liposomal formulations was performed by dynamic light scattering (size and size distribution) and electrophoretic mobility (zeta potential) at 25 °C using a Zetasizer Nano (Malvern Instruments Ltd, Worcestershire, United Kingdom).

2.3. Determination of liposomal DPPC and 25(OH)D content

The amount of 25(OH)D incorporated within liposomes was determined via HPLC, performed on a Dionex HPLC system (Thermo Scientific, Bremen, Germany) composed of a P680 pump, an Elite degassing System, an Asta-medica AG 80 column oven and a UV detector. A LiChrospher® RP-18 (5 µm, 125 × 4 mm) column (Merck KGaA, Darmstadt, Germany) was employed. A mobile phase of methanol/acetonitrile (30:70 v/v) was used, with an injection volume of 100 µl, a flow rate of 2 ml/min and a temperature of 30 °C. For sample analysis, liposomes were first dissolved in a mixture of 50% ethyl acetate/methanol (4:1) and 50% acetonitrile. The 25(OH)D content of dissolved liposome samples was determined using UV detection at a wavelength of 265 nm, and calculated in reference to standard solutions of 25(OH)D. The determined amount of 25(OH)D was then used to calculate the encapsulation efficiency (EE) of liposomes, defined as the measured amount of 25(OH)D as percentage of the initially added amount [20].

The amount of DPPC present in liposome formulations was assessed according to the Bartlett assay [21]. Briefly, a calibration curve was constructed from a stock solution of 0.05 mg/ml potassium phosphate (Sigma-Aldrich, St. Louis, Missouri, USA) diluted as required with deionised water to produce standards of known concentration. Both liposome samples and standards were dried completely in a sand bath at 180 °C prior to any analysis. A 450 µl volume of 70% perchloric acid (AppliChem, Darmstadt, Germany) was then added to both samples and standards, followed by incubation at 250–260 °C for 30 min. After cooling, 3.5 ml of deionised water, 500 µl of 2.5% w/v ammonium molybdate solution and 500 µl of 10% w/v ascorbic acid solution (both from VWR BDH Prolabo, Darmstadt, Germany) were added to vials of sample and standards, to initiate the colorimetric reaction. The final mixtures were vortexed and incubated in a water bath at 100 °C for 7 min. The reaction was then stopped by placing the vials in an ice bath. Subsequently, the UV absorbance of standard solutions and samples was measured at 820 nm (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer, Waltham, USA).

With the determined amounts of DPPC and 25(OH)D a loading efficiency (LE) was calculated, expressed as the quantified drug/lipid molar concentration ratio as percentage of the initial drug/lipid molar concentration ratio [22].

2.4. Aerosolisation of liposomes

Prior to deposition studies, the effect of the nebulisation process on the colloidal stability of liposomes was assessed. Liposomes were dispersed in water and nebulised using an electronic vibrating membrane inhaler (eFlow, PARI Medical Holding GmbH Starnberg, Germany). For stability, nebulised liposome samples were collected and the diluted liposomal aerosol was measured for size and zeta potential, as mentioned in the previous section.

For investigating the aerodynamic properties of nebulised liposomes, a next-generation impactor (NGI, Copley Scientific, Nottingham, UK) was used. Deposition experiments were conducted according to the procedure specified in the European Pharmacopoeia [23] and as detailed further in the supplementary material. The amount of deposited liposomes in each NGI stage was determined by measuring the fluorescence of Rh-DPPE using a plate reader (Genios Pro Tecan, Männedorf, Switzerland, excitation wavelength = 560 nm, emission wavelength = 662 nm). To predict pulmonary deposition *in vivo*, parameters of Mass Median Aerodynamic Diameter (MMAD), Geometric Standard Deviation (GSD) and Fine Particle Fraction (FPF) were calculated. For determination of the MMAD and GSD, probit analysis [24] was employed. FPF was defined as the mass of aerosolised material with an aerodynamic diameter of less than 5 µm.

2.5. Bacteria cultivation

To determine the influence of 25(OH)D on the immune response to infection, heat inactivated or viable *P. aeruginosa* PAO1 cultured as described previously [25] were used. The viable bacterial suspension was diluted 1:10 in phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}, pH 7.4, Life Technologies, Darmstadt, Germany) prior to application. For heat inactivation the undiluted bacterial suspension was incubated for 10 min at 95 °C, and subsequently stored in aliquots corresponding to 3×10^7 colony-