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Antibiotic-free nanotherapeutics: Ultra-small, mucus-penetrating solid lipid nanoparticles enhance the pulmonary delivery and anti-virulence efficacy of novel quorum sensing inhibitors

Noha Nafee [a,c,d,e,*], Ayman Husari [a,c,d], Christine K. Maurer [b], Cenbin Lu [b], Chiara de Rossi [c], Anke Steinbach [b], Rolf W. Hartmann [b,d], Claus-Michael Lehr [c,d], Marc Schneider [a,*]

[a] Pharmaceutics and Biopharmacy, Philipps University Marburg, Marburg, Germany
[b] Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Department of Drug Development and Optimization (DDOP), Saarland University, Saarbrücken, Germany
[c] Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS), Department of Drug Delivery (DDEL), Saarland University, Saarbrücken, Germany
[d] Department of Pharmacy, Saarland University, Saarbrücken, Germany
[e] Department of Pharmaceutics, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt

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ABSTRACT

Cystic fibrosis (CF) is a genetic disease mainly manifested in the respiratory tract. *Pseudomonas aeruginosa* (*P. aeruginosa*) is the most common pathogen identified in cultures of the CF airways, however, its eradication with antibiotics remains challenging as it grows in biofilms that counterwork human immune response and dramatically decrease susceptibility to antibiotics. *P. aeruginosa* regulates pathogenicity via a cell-to-cell communication system known as quorum sensing (QS) involving the virulence factor (pyocyanin), thus representing an attractive target for coping with bacterial pathogenicity. The first *in vivo* potent QS inhibitor (QSI) was recently developed. Nevertheless, its lipophilic nature might hamper its penetration of non-cellular barriers such as mucus and bacterial biofilms, which limits its biomedical application.

Successful anti-infective inhalation therapy necessitates proper design of a biodegradable nanocarrier allowing: 1) high loading and prolonged release, 2) mucus penetration, 3) effective pulmonary delivery, and 4) maintenance of the anti-virulence activity of the QSI.

In this context, various pharmaceutical lipids were used to prepare ultra-small solid lipid nanoparticles (us-SLNs) by hot melt homogenization. Plain and QSI-loaded SLNs were characterized in terms of colloidal properties, drug loading, *in vitro* release and acute toxicity on Calu-3 cells. Mucus penetration was studied using a newly-developed confocal microscopy technique based on 3D-time-lapse imaging. For pulmonary application, nebulization efficiency of SLNs and lung deposition using next generation impactor (NGI) were performed. The anti-virulence efficacy was investigated by pyocyanin formation in *P. aeruginosa* cultures.

Ultra-small SLNs (<100 nm diameter) provided high encapsulation efficiency (68–95%) according to SLN composition, high burst in phosphate buffer saline compared to prolonged release of the payload over >8 h in simulated lung fluid with minor burst. All types and concentrations of plain and QSI-loaded SLNs maintained the viability of Calu-3 cells. 3D time-lapse confocal imaging proved the ability of SLNs to penetrate into artificial sputum model. SLNs were efficiently nebulized; NGI experiments revealed their deposition in the bronchial region. Overall, nanoencapsulated QSI showed up to sevenfold superior anti-virulence activity to the free compound. Most interestingly, the plain SLNs exhibited anti-virulence properties themselves, which was shown to be related to anti-virulence effects of the emulsifiers used. These startling findings represent a new perspective of ultimate significance in the area of nano-based delivery of novel anti-infectives.

1. Introduction

Cystic fibrosis (CF) is one of the life-threatening genetic disorders attacking the respiratory tract as a result of mutations in the CF Transmembrane Conductance Regulator (CFTR) gene, which encodes a membrane-bound adenosine 3′,5′-cyclic monophosphate (cAMP)-regulated chloride channel [1]. In consequence, a decrease in epithelial chloride secretion and an increase in sodium absorption across the cell

* Corresponding authors at: Pharmaceutics and Biopharmacy, Philipps University Marburg, Ketzerbach 63, D-35037 Marburg, Germany. Tel.: +49 6421 282 5885; fax: +49 6421 282 7016.
E-mail addresses: noha.nafee@mx.uni-saarland.de, n.nafe3@gmail.com (N. Nafee), Marc.Schneider@pharmazie.uni-marburg.de (M. Schneider).

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membrane take place. In addition, water is not distributed leading to airway surface liquid depletion and failure of normal mucociliary clearance. This in turn causes thickened and viscous mucus that adheres to the airway surface causing increased sputum production, shortness of breath, chest pain and lung deterioration [2]. Patients with CF are susceptible to opportunistic bacteria, most notably *P. aeruginosa* that protect themselves from attacks of the immune system by forming slimy colonies in 3D-polymeric networks known as biofilms [3]. These are thought to play a key role in the ability of this species to tolerate antibiotics, to protect against host immune defenses and to survive in the lungs of CF-patients representing an important cause of mortality [4].

P. aeruginosa uses a cell density-dependent cell-to-cell communication system that is referred to as "quorum sensing" (QS) to coordinate group behavior such as the production of virulence factors. Within the *Pseudomonas quinolone signal* (*pqs*) QS system, PqsR is a key DNA-binding receptor that is specific to *P. aeruginosa* and a critical regulator that fine-tunes a set of genes encoding for virulence factors such as pyocyanin, elastase B and hydrogen cyanide [5,6]. PQS and 2-heptyl-4-hydroxyquinoline (HHQ) are the natural ligands and agonists of this receptor and function as signal molecules of *pqs* QS [7,8]. The virulence regulator PqsR is, thus, considered an attractive target for attenuating bacterial pathogenicity without eliciting resistance.

Treatment of CF-related infections remains yet very challenging; while long term treatment with antibiotics and antibiotic combinations proved to be insufficient for bacterial eradication, repairing of the defected genes was also found to be of limited *in vivo* performance [9]. Thus, the interest in the development of novel antibiotic-free therapeutics rather than new antibiotic entities is growing. An upcoming treatment strategy focuses on developing anti-infectives with novel modes of action with special highlight on QS inhibitors (QSIs) as potential powerful agents for anti-virulence therapy. First attempts resulted in *pqs* QSI with low efficiency in an animal model [10]. A ligand-based drug design approach led to the discovery of the first antagonist of PqsR [11]. Further ligand- and fragment-based strategies resulted in PqsR antagonists with moderate cellular activity [12–14]. Recently, a highly affine PqsR antagonist (2-heptyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxamide), Fig. S1 (supp. materials), was identified that strongly inhibited the virulence of *P. aeruginosa in cellulo* (IC_{50} of 2 μM towards pyocyanin) and *in vivo* in two animal infection models [15,16]. However, the lipophilicity of this promising QSI limits its biomedical application. First attempts to improve the physicochemical properties of this compound class using medicinal chemistry strategies resulted in compounds with enhanced water solubility that, however, turned out to be less potent PqsR antagonists [16]. The effectiveness of the top compound in CF-patients will thus depend on a suitable delivery strategy, a delivery system likely to improve the solubility of QSI, control its release rate and target the infected mucus in the bronchial area without negatively affecting its anti-virulence potency is of ultimate demand.

The use of biocompatible, biodegradable nanoparticles for controlled drug/gene delivery at mucosal sites proved to be an effective therapeutic strategy [17]. Improving the delivery of anti-infectives is still a rather new paradigm in drug delivery and in nanomedicine in particular. Over the last 20 years, several nano-sized delivery systems like fusogenic liposomes, PLGA nanoparticles and lipid-polymer hybrid nanoformulations have shown to be promising carriers for targeting drugs to the site of infection [18]. Several nanomedicines for the treatment of infectious diseases have already reached market authorization such as liposomes (e.g. AmBisome®) and protein–polymer conjugates (e.g. Intron® A). In addition, numerous preclinical nano-delivery systems, e.g. polymeric nanoparticles, drug–polymer conjugates and complexes, dendrimers, niosomes and lipid nanoparticles are in clinical or preclinical investigation for delivery of anti-infectives [19]. Solid lipid nanoparticles (SLNs) composed of physiological lipid, dispersed in aqueous surfactant solution represent the most interesting class of nanocarriers [20] as they offer the advantages of ability of readily incorporating lipophilic candidates, improved drug stability, possibility of controlled release, and a higher safety threshold due to avoidance of organic solvents [21,22]. However, some limitations are always associated with SLNs including low drug loading, risk of gelation and drug leakage during storage owing to lipid polymorphism [23].

The application of SLNs gave promises in various routes of administration, especially the pulmonary route [22,24]. Notably, SLNs typically possess a particle size >150 nm; the lipidic nature makes the particles prone to immediate recrystallization into larger size SLNs. Therefore, the production of ultra-small SLNs (<100 nm) remains challenging and was rarely reported [25]. Us-SLNs are expected to improve drug loading, mucus penetration as well as internalization by bacterial targets [26].

Back to the fact that mucus, especially when pathologically changed under the condition of the disease, probably represents a major barrier against efficient CF therapy [27], engineering mucus-penetrating nanoparticles (MPP) able to cross the thick mucus layer, protect the payload and improve its intracellular uptake by the bacterial cells would offer the prospect of novel opportunities for CF therapy [28]. Efforts to develop MPP – avoiding adhesion to mucin fibers and being small enough to avoid significant steric inhibition by the dense fiber mesh – are ongoing [28]. Coating polymeric particles with hydrophilic polymers (e.g., polyethylene glycol (PEG), Pluronic) was found to improve mucus penetration [29,30]. Similar investigations related to SLNs coated with various hydrophilic stabilizers are still lacking.

On this basis, the objective of our study implies for the first time – to the best of our knowledge – the preparation of us-SLNs to improve the pulmonary delivery of novel QSI. Multiple challenges are to be overcome so far. The delivery system is to be optimized in terms of size (<100 nm), surface hydrophilicity, highest loading, prolonged QSI release and efficient nebulization. In addition, several biological aspects are to be fulfilled including safety of epithelial bronchial tissue, efficient mucus penetration as well as maintained anti-virulence activity represented by inhibition of pyocyanin formation.

2. Materials and methods

2.1. Materials

Glyceryl palmitostearate (Precirol ATO 5, Pre), glyceryl behenate (Compritol 888 ATO, GB) were kindly donated by Gattefossé, Saint-Priest, France. Tristearin (Dynasan 118, Tri) was a gift from Cremer Oleo GmbH & Co. KG, Hamburg, Germany. Nile Red (NR), mucin from porcine stomach-Type II, DNA (low molecular weight from salmon sperm, Fluka), Poloxamer 407 (Pluronic® F-127, P) and Polysorbate 80 (Tween 80, Tw) were purchased from Sigma-Aldrich, Steinheim, Germany. Polyvinyl alcohol (Mowiol 4-88, PVA) was obtained from Kuraray Europe GmbH, Hattersheim am Main, Germany. AlexaFluor-labeled wheat germ agglutinin was received from Invitrogen, Oregon, USA. *P. aeruginosa* strain PA14 was obtained from Susanne Häussler, Twincore, Hannover and stored in glycerol stocks at −80 °C. Other reagents are described in the supp. materials.

2.2. Methods

2.2.1. Preparation of SLNs (plain, QSI-loaded & labeled SLNs)

SLNs were prepared by hot melt homogenization in which the lipid (GB, Pre or Tri) was first melted at 10 °C above its melting point, then emulsified with the surfactant aqueous phase (PVA, Poloxamer or Tween 80) [31]. Probe sonication and high shear homogenization were used for droplet size reduction. SLNs were allowed to solidify by cooling under gentle stirring. Production of us-SLNs was optimized by varying the lipid/emulsifier type and concentration, temperature, sonication/homogenization time and speed. Optimized formulations, Table 1, were selected for the preparation of QSI-loaded and NR-labeled SLNs. In this case, either QSI (100 μM) or NR (5 μg/mL) in DMSO was added to the molten lipid and proceeded as above.