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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

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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 the 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 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 newly-developed confocal microscopy technique based on 3D-time laps 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 SLNs 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 laps 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 the nano-based delivery of novel anti-infectives.

keywords: Solid lipid nanoparticles; cystic fibrosis; *Pseudomonas aeruginosa*; quorum sensing inhibitors; anti-infectives; anti-virulence agents.

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 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 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 as 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 for 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* (IC₅₀ 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 authorisation 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 fibres and being small enough to avoid significant steric inhibition by the dense fibre 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 on epithelial bronchial tissue, efficient mucus penetration as well as maintained anti-virulence activity represented by inhibition of pyocyanin formation.

Materials and methods:

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.

Methods:

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 μ M) or NR (5 μ g/ml) in DMSO was added to the molten lipid and proceeded as above.

Table 1: Composition of SLNs:

	SLNs composition		
	Lipid	Emulsifier	Lipid:Emulsifier ratio
GB-Tw SLNs	Glyceryl Behenate	Tween	4:5
GB-PVA SLNs	Glyceryl Behenate	PVA	1:5
Tri-Tw SLNs	Tristerain	Tween	4:5
Tri-P SLNs	Tristerain	Poloxamer	4:5
Pre-Tw SLNs	Precirol	Tween	4:5

Pre-PVA SLNs	Precirol	PVA	1:5
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2. Characterization of SLNs:

The colloidal properties for plain, QSI-loaded and NR-labeled SLNs (diluted 1:10 in deionized water) were determined using the Malvern Zetasizer Nano, Malvern Co, UK. The colloidal stability of SLNs in release and culture media was verified. Particle morphology was examined by SEM and TEM. The thermal properties of pure lipids, QSI, plain and loaded SLNs were determined by differential scanning calorimetry (DSC). For this, 3–5 mg powder samples were sealed in aluminium pans and heated from 10 to 260 °C at a heating rate of 5 °C/min followed by a cooling cycle from 260 to 10 °C at the same rate in presence of an empty aluminium pan as reference.

3. Drug loading and Encapsulation Efficiency (EE):

QSI-loaded SLNs were separated from free QSI by centrifugal ultrafiltration using Centriscart-I[®], MWCO 10 kDa, Sartorius AG, Goettingen, Germany. Encapsulated QSI was extracted from purified loaded SLNs using methanol/dichloromethane solvent mixture and quantified by liquid chromatography coupled with mass spectroscopy (LC-MS). For detailed procedure of the LC-MS technique and chromatogram of QSI, refer to supp. materials (Fig. S1).

4. *In vitro* release & release kinetics:

Release of QSI from SLNs was tested in phosphate buffer saline (PBS) and simulated lung fluid (SLF) (refer to composition in supp. materials) [32], pH 7.4, 50 ml, at 37 °C over 48 h. Samples from the release medium were withdrawn and filtered using Millex[®]-LG syringe filter units containing low protein binding hydrophilic LCR (PTFE) membrane (0.2 µm), EMD-Millipore Co., Tokyo, Japan. QSI released at predetermined intervals was analyzed by LC-MS. The release profiles were fitted to different kinetic models.

5. Stability on storage:

Plain and loaded SLNs were stored at 4 °C up to 2 months. The colloidal properties as well as drug content were monitored to check for possible signs of agglomeration and/or drug leakage.

6. SLNs-mucus interaction:

Measurement of colloidal properties and absorbance:

GB-based SLNs were incubated with mucin solution (0.8 % w/v) up to 4 h at 37 °C. At predetermined time intervals, the size, PDI and zeta potential were monitored. In addition, the absorbance was measured at 650 nm to record any signs of turbidity/agglomeration [33].

Confocal laser scanning microscopy (CLSM):

A model system was set up in house to study and visualize particle diffusion in mucus. Artificial sputum medium (ASM) stained with AlexaFluor-wheat germ agglutinin was prepared [34]. The time-dependent diffusion of NR-labeled SLNs in ASM was verified by 3D time laps imaging.

7. Cytotoxicity assay:

Different types of plain and QSI-SLNs in the concentration range (0.1 - 5 mg/ml) were incubated with Calu-3 cells (2×10^5 cell/ml) for 4 h, washed then replaced with fresh culture medium. Cell viability was determined by MTT assay (supp. materials).

8. Nebulization and lung deposition experiments:

NR-labeled SLNs were nebulized using an ultrasonic nebulizer (eFlow, PARI Pharma GmbH, Starnberg, Germany). Nebulization efficiency (output rate and residual volume) as well as particle stability were verified. The deposition pattern as well as the aerodynamic properties of the nebulized particles were determined by the next generation impactor (NGI, Copley Scientific, Shoreview, USA) at a flow rate of 15 L/min.

9. Pyocyanin assay:

Pyocyanin produced by *P. aeruginosa* PA14 was determined as previously described [12] according to the method of Essar et al. [35]. In short, cultures inoculated with a starting OD₆₀₀ of 0.02 were grown in the presence of free QSI, plain SLNs, QSI-loaded SLNs, or a mixture of free QSI and plain SLNs in PP GAS medium (refer to composition in supp. materials) at 37 °C, 200 rpm, and a humidity of 75% for 16 h. For pyocyanin determination, cultures were extracted with chloroform and re-extracted with 0.2 M HCl. The OD₅₂₀ was determined using FLUOstar Omega (BMG Labtech) and normalized to cell growth measured as OD₆₀₀. For each sample, cultivation and extraction were performed at least in triplicates.

10. Statistical analysis:

All measurements were performed in replicates ($n = 3 - 5$); results were represented as mean $\pm$ standard deviation. Statistical analysis was done using Two-way ANOVA, Holm-Sidak method for comparison, with $p = 0.001 - 0.05$ as level of significance.

Results & discussion:

Inhaled nanocarrier-mediated anti-infective therapy is an area of great activity in many disciplines especially CF clinical research [36, 37]. Apart from the metallic nanoparticles exerting their own antimicrobial action, special attention to lipid and polymeric nanocarriers is currently growing due to their attractive properties like biocompatibility, versatility of materials and surface modifications, possibility for targeting and triggered release, ability to incorporate lipophilic as well as hydrophilic drugs and a reduction of unwanted side effects of the drug [18]. A liposomal nanoparticle formulation of amikacin (300 nm in diameter) was developed for once-daily delivery using the PARI eFlow® nebulizer system [38]. The neutral liposomal shell was suggested to allow the drug to penetrate into bacterial biofilms and to be released slowly over time. Phase 2 studies and preliminary results indicate that this formulation results in significant improvement in lung function and reductions of sputum *P. aeruginosa* density [39]. Similar liposomal formulation have been developed for ciprofloxacin, gentamicin and tobramycin [18, 40]. Nanocarriers can overcome existing drug resistance mechanisms, including decreased uptake and increased efflux of drug from the microbial cell, biofilm formation, and intracellular bacteria [41].

Even though the therapeutic efficacy of the addressed QSI has been well established, inefficient delivery could result in inadequate therapeutic index and side effects.

1. Physicochemical characterization of SLNs:

Optimized us-SLNs were all < 100 nm in diameter with PDI < 0.2 , except for Pre-PVA SLNs (~ 200 nm), Fig. 1A. Preliminary trials reflected the significant role of lipid/emulsifier type and concentration as well as the process parameters on the colloidal properties of the resulting SLNs. The high temperature applied during preparation resulted in lower particle size due to reduction in viscosity of the inner phase [42]. PVA was used in a higher concentration compared to tween and poloxamer; PVA is a viscosity imparting stabilizer which negatively affects the size reduction process [22]. Previous reports suggested high heterogeneity of SLNs prepared from lipids with a high viscosity and/or high content of monoglycerides [43]. Nevertheless, emulsification of extremely lipophilic lipids is more challenging. Precirol is a glyceryl distearate containing a C18 fatty acid, while GB composed of behenate (C22) is more lipophilic. In comparison, tristearin is a model of pure triglyceride that is expected to be the most lipophilic candidate. The solubility parameter is a measure of cohesive energy and could be closely linked to polarity. Jensen et al. determined the solubility parameter for precirol, compritol and tristearin to be 9.5, 9.3 and 8.9, respectively [43]. Accordingly, more homogenous SLNs would be obtained using precirol $>$ compritol $>$ tristearin.

The hot homogenization technique was reported to be suitable for many drugs especially lipophilic and insoluble drugs such as the QSI used in this study [21, 24]. Loading with QSI did not significantly change the particle size but rather increased the polydispersity of some samples, Fig. 1A (Mann-Whitney Rank Sum Test, $p = 0.818$).

Both plain and loaded SLNs were negatively charged (zeta potential between -15 and -35 mV). The negative charge together with the steric stabilization provided by the emulsifiers resulted in stable colloidal dispersion of SLNs. An overall yield of SLNs between 73.4 - 99.4 % was determined gravimetrically.

From the DSC thermograms, Fig. 1 B and Fig. S2 (Supp. materials), it can be deduced that the lipids undergo some polymorphic changes to less ordered structures during particle preparation allowing better incorporation of the drug in the lipid matrix. In most cases, the melting peak corresponding to the QSI disappeared together with the appearance of small exothermic peak in the heating curve (Fig S2, Supp. materials); this might be attributed to either loss of crystalline structure, complete dissolution of the drug in the lipid matrix or undetectable peaks due to very low concentration of the loaded drug. The thermal properties including melting peak (T_m), recrystallization temperature (T_c) and corresponding enthalpies are represented in Table S2 A and B (Supp. materials). The reduction in T_m of the lipid after particle preparation could be related to the colloidal nature of the sample, the small particle size, the high specific surface area, and the presence of emulsifier [44]. Following the Kelvin effect and the Thomson equation, small particles would melt at temperature lower than the melting temperature of bulk materials [45, 46]. The lower melting enthalpy values recorded for plain and QSI-SLNs also suggest a less ordered lattice arrangement of the lipid within the SLNs compared to the bulk materials [31]. Similar to the melting process, broader recrystallization peaks (sometimes with shoulders)

could be observed. The crystallization enthalpy is generally lower than the melting enthalpy, suggesting that crystallization and melting may occur in different polymorphs [44].

SEM and TEM micrographs, Fig. 1 C and D illustrate the ultra-small size of SLNs that is in accordance with the size measurements, with spherical shape, smooth surface and homogeneous distribution.

2. Encapsulation efficiency and loading:

The lipophilic nature of QSI facilitated its encapsulation in lipidic particles showing an EE of 68 - 95 % (Fig. 2A) and a loading range between 0.4 - 1.54 mg/g SLNs depending on the lipid-emulsifier combination. In fact, EE can be influenced by several factors including the chemical nature of the lipid, its chain length and crystalline structure, the HLB value of lipid and emulsifier, the type and concentration of the emulsifier in addition to the preparation technique that allows minimum leakage of the drug during size reduction processes [42]. Hot melt homogenization technique is not suited for incorporation of hydrophilic drugs into SLNs because of the higher partition of drug into water during homogenization resulting in low entrapment efficiency [24].

Figure 1

In our case, highest EE were recorded for precirol-based SLNs; during particle preparation precirol exists in a less ordered arrangement, forming crystals with many imperfections, thus, offering spaces to accommodate more drug [31]. In contrast, DSC thermograms showed the very stable crystalline structure of tristearin that would relatively lead to drug expulsion. However, when poloxamer was used as surfactant, QSI-loaded Tri-SLNs had high EE, which underlines the complexity of the system. During the preparation process, the lipid is in the liquid phase, allowing high mobility of the drug, that may partition between the liquid lipid and the aqueous surfactant solution according to drug lipophilicity. The incorporation efficiency is therefore likely to strongly depend on the degree of lipophilicity and drug solubility in the two phases, keeping in mind that, the solubility of the drug in the liquid lipid during processing may be higher than in the solid lipid [47].

3. *In vitro* release of QSI:

The release of QSI from different SLNs was studied in PBS and in SLF, of which the latter is considered to be the more relevant medium for the *in vivo* conditions. A controlled release of QSI in PBS could be observed; 60 - 95 % released over 8 h with an initial high burst of 60 % within the first 2 h, except for GB-Tw SLNs (< 20 % burst), Fig. 2B. The high burst might be related to the ultra-small particle size, very large surface area per se and the short diffusion distance for the drug together with the high temperature during production, high diffusion coefficient due to small molecular size, low viscosity in the matrix [48]. The biphasic release profiles described by high burst followed by a very slow release phase like a plateau can be attributed to an enrichment of drug in the outer region of the SLN. During the cooling step, the lipid might start crystallizing first, forming an inner core of pure lipid, that would force part of the drug to be entrapped in the periphery of the particles or adsorbed to the surface [47]. This amount would be thus prematurely liberated in the release medium in the initial stage. In contrast, GB-Tw and Pre-PVA SLNs exhibited more

uniform QSI release over time. Lipids with lower melting points are expected to ensure higher release rate of the drug, owing to relatively higher mobility at the temperature used in the release experiment [43], which was not clear in the current study. It is well known and also shown by DSC measurements that the melting point of colloidal structures is lower than that of the bulk due to the influence of surface energy [44]. A difference in release profiles caused by a difference in lipid melting points was also suggested by Paolicelli et al. [49].

The release of QSI was more prolonged in SLF showing < 20 % burst in all SLNs, Fig. 2C. In this case, no biphasic profiles could be noted; between 10 - 70 % QSI was uniformly released over 8 h. GB-based SLNs ensured QSI release of 60 - 65 % over 8 h regardless of the emulsifier. More prolonged release (~ 30 %) was shown in case of precirol-based SLNs, whereas only 10 % QSI was released from tristearin-based SLNs. Interestingly, almost all of the incorporated QSI was released from all formulations within 48 h.

A correlation between the size of SLNs and the amount of QSI released could not be deduced. The release profiles cannot simply be explained by a single factor or correlated to only lipid or emulsifier type. Indeed, the physicochemical characteristics of QSI, particle size, lipid/surfactants used, method of preparation, production parameters as well as the release medium are all contributing to such release behavior [42].

Comparing Fig. 2 B and C reveals the crucial role of the release medium on the amount of QSI released from the same particles. SLF contains a very high amount and variety of salts. Nevertheless, the colloidal stability of SLNs in SLF was maintained over the study period with no signs of agglomeration or aggregation (Fig. S3, Supp. materials). This means that the variation in release was not due to change in particle size or total surface area of particles exposed to the release medium. Notably, the zeta potential was dramatically reduced due to the extensive amounts of surrounding electrolytes. These excessive amounts of salts in the release medium might obviously affect the swelling behavior of the surfactant layer and likely compete with the lipophilic drug, thus, retarding drug diffusion from the particles and limiting its incorporation in water.

The release profiles were fitted to different kinetic models to identify possible release mechanisms from the SLNs, Table S2 (supp. materials). According to Korsmeyer-Peppas, the release from GB-Tw SLNs follows zero order kinetics ($n = 1.013$, $R^2 = 0.923$). The zero order release profile from these SLNs may be explained by several factors. The size of SLNs is small, so particle diffusion length may not be reflected in release kinetics. The enormous surface area is reported to contribute to the zero order release profile since the rate of exchange of drug substance with the aqueous phase is high [43]. In contrast, QSI released from all other formulations was better described by Higuchi model despite the minor difference in size compared to GB-Tw SLNs. According to Venkateswarlu and Manjunath, release of clozapine from triglyceride-based SLNs was found to follow Weibul and Higuchi model better than first-order kinetics [50]. Authors suggested that the amorphous clozapine dissolves in lipid, diffuses to the surface and undergoes partitioning between lipid and aqueous phase.

Figure 2

4. Stability on storage:

Plain and loaded SLNs retained their colloidal stability upon storage at 4 °C up to 2 months. Determination of drug content indicated no leakage of the drug over the storage period, Fig. S4 (supp. materials).

5. Mucus interaction:

A very crucial issue for the treatment of CF is the ability of the drug carrier to penetrate the pathologically thickened mucus layer lining the airways and deliver the payload close to the bacteria [51]. Mucoadhesive particles that tend to bind to the superficial mucus layer are likely to be either trapped on the mucus surface or expelled before reaching the targeted area underneath [28]. GB-Tw SLNs were selected for studying SLNs-mucus interaction.

Colloidal stability of SLNs in mucus and absorbance measurements:

The ability of SLNs to retain their size and zeta potential after incubation with mucin was taken as a rough measure for lack of interaction and accordingly ability of mucus penetration. Along 2 h incubation of SLNs with mucin, no distinct increase in size was observed, Fig. 3A, whereas a shift in PDI from 0.2 to 0.4 might be related to the various components in mucin. Mucin is negatively charged, electrostatic interaction with our SLNs of similar charge is seldom to occur. In contrast, cationic nanoparticles would be immediately surrounded by a mucin layer, the thickness of which varies according to the charge density leading to a net null zeta potential and formation of particle agglomerates.

The interaction was further assessed by absorbance measurement. While the absorbance of mucin solution was almost zero, relatively higher values were recorded for SLNs-mucin mixtures indicating particle scattering, Fig. 3B. No remarkable differences between the absorbance of SLNs in water and that in mucin were observed.

3D-time laps imaging by CLSM:

The diffusion of NR-labeled GB-Tw SLNs in stained artificial sputum (green) was tracked as indicated in the z-stacks at different time intervals, Fig. 3C. As can be deduced from the Figure, fluorescent particles can be easily recognized within the ASM 30 min after deposition, the amount of diffused particles increased heavily by time. Using image analysis software '*Image J*', Fig. 3D, the total fluorescent signal of combined 3D-laps was determined; fluorescence was doubled from 15 to 30 min and became 6 times higher at 60 min. Previous attempts to enhance particle penetration of CF sputum has focused on either reducing its barrier properties via mucolytic agents or decreasing particle adhesion to sputum constituents by coating particle surface with non-mucoadhesive polymers namely PEG [29, 30]. It is to be noted that the penetration of SLNs in sputum has never been previously addressed. Besides, the potential of tween-coated SLNs to efficiently penetrate the mucus barrier can be considered an interesting alternative to PEG.

ASM represents CF-patient sputum containing artificial mucin, free DNA and amino acids and was reported as suitable medium for the growth of *P. aeruginosa* [34]. In the current study, ASM was used as a feasible *in vitro* model to investigate possible interaction of our particles with sputum components. However, it should be pointed out that the viscosity of ASM is far below that of CF-patient sputum. Therefore, penetration experiments using CF-patient sputum are ongoing.

Studying SLN interaction with mucus is of ultimate significance and therefore necessitates a precise and reproducible method enabling appropriate particle tracking within mucus. Diffusion chamber, fluorescence recovery after photobleaching and multiple particle tracking studies all are methods reported to study particle transport in CF sputum [52, 53]. However, these methods have some limitations. For instance, although the diffusion-chamber method is conceptually straightforward, it may be sensitive to parameters, such as precise thickness of the mucus layer, uniformity of the mucus distribution across the face of the filters, blockage of the filter pores by mucus, and alterations in mucus properties during preparation [54]. The newly developed technique for studying particle-mucus interaction by CLSM can be regarded as promising and versatile. Results obtained were in accordance with size and absorbance measurements confirming the reliability of our procedures. Yet, various experimental parameters are to be optimized towards a more standardized procedure and more statistically relevant data.

Figure 3

6. Cytotoxicity:

An important aspect to be verified is the safety of our delivery system on the pulmonary epithelium. Despite the high safety threshold of the lipids and emulsifiers used, cytotoxicity testing of plain and loaded SLNs was checked on Calu-3 cells representing the bronchial area (target area). The effect of lipid/emulsifier type, SLNs concentration and presence of the drug was verified. As depicted from Fig. 4 A & B, around 80 - 100 % of Calu-3 cells remained viable after incubation with either plain or QSI-loaded precirol-based SLNs at all concentrations tested and regardless of the emulsifier used. Meanwhile, tween-stabilized GB SLNs showed some concentration dependent toxicity, which was not recognized in the corresponding PVA-stabilized

particles, Fig. 4 C & D. Dry powder of itraconazole, tween 80 and poloxamer 407 in a 1:0.75:0.75 ratio administered to mice by nebulization every 12 h for up to 12 days did not cause inflammation or changes in pulmonary histology and was not associated with pro-inflammatory cytokine production [55]. The observed small effect *in vitro* was therefore considered as not prohibitive for further investigating the efficacy of these nanocarriers. Viability in case of tristearin SLNs was generally > 90 %, Fig. 4 E & F. Similar results were observed in previous studies [22, 56]. In many cases, SLNs proved to be less toxic (10- and 100-fold lower) than polymeric nanoparticles e.g. PLGA and butyl cyanoacrylate nanoparticles, respectively [22, 57]. One of the main advantages of SLNs is that the lipids used are physiological and generally recognized as safe (GRAS) for which metabolic pathways exist. Special consideration is to be given to the toxicity aspects of surfactants. The concentration dependent toxicity of poloxamer 188-stabilized SLNs was reported, whereas poloxamer 407 was found to be much safer [22].

Figure 4

7. Nebulization, deposition pattern and aerodynamic parameters:

For pulmonary application, the colloidal stability of SLNs was verified after nebulization with a commercially available ultrasonic nebulizer (PARI eFlow®) using different mesh sizes (N30 and N35). Fig. 5 A & B shows distinct increase in size and PDI, with negligible alteration in charge. The heat resulting from frictional forces induced by movement of the vibrating piezoelectric crystal might influence the stability of the lipidic particles. SLNs were nebulized with an output rate of 0.31 ± 0.01 ml/min and a residual volume of 15 ± 3.5 %. No clear differences were observed with varying mesh sizes (N30 and N35) presumably as the energy brought into the system to modify droplet size is not high enough to change the SLNs.

The deposition pattern and aerodynamic behavior of nebulized SLNs in the respiratory tract was studied using NGI. Fig. 5C represents the % SLNs deposited in each compartment depending on the droplet size and density. Accordingly, 68 % of the nebulized droplets were in the size range 2 – 8.6 μ m. No real impact of the chamber mesh size (N30 and N35) could be observed. Assessment of the aerodynamic parameters revealed an MMAD of 2.2 μ m, FPF around 85 % and GSD of 2.1, indicating that more than 80 % of aerosolized SLNs were below 5 μ m and they have the potential for reaching the deep lung. Generally speaking, pulmonary drug delivery devices generate particles with an MMAD (3 – 10 μ m) for deposition in the tracheo-bronchial region in order to treat the airways, or in the alveolar region (1 – 3 μ m) for systemic drug absorption. Particles > 5 μ m are generally believed to deposit in upper airways (mouth, trachea and main bronchi) by inertial impaction, whereas 1 - 5 μ m particles are deposited by gravitational settling in the central and distal tract [58]. CF-related pathology was reported to initiate in small airway characterized by

lower intramural pressure, smaller lumen, and reduced airway stability [59]. Soon after birth, initial infection with bacterial pathogens commences and is associated with an intense neutrophilic response localized to the peribronchial and endobronchial spaces. Several studies in toddlers and older children with CF have shown a robust inflammatory response in the airways and submucosal glands with elevated interleukin (IL)-8 and neutrophil elastase [60]. Aerosols and nebulizers often generate a certain spectrum of particles that is more or less likely to target larger versus smaller airways; the deposition is rarely specific enough to only reach one region of the lung. Newer aerosol devices are intended to achieve higher deposition in small airways through improvement in flow at lower lung volumes [59]. As a high fraction of our nebulized system would reach alveolar part, nebulization might not be then the most relevant technique for this application. Thus, preparation of SLNs-embedded microparticles by spray drying with carbohydrate carriers intended for dry powder inhalation (DPI) is currently in progress.

Figure 5

8. Inhibition of the virulence factor pyocyanin:

Pyocyanin, the blue pigment produced by *P. aeruginosa*, is required for full virulence in animal models and has been detected in the sputum of infected patients. It promotes virulence by interfering with numerous cellular processes in the host such as electron transport, cellular respiration, energy metabolism, gene expression, and innate immune responses [61]. Importantly, it has been demonstrated that the concentration of pyocyanin in the lungs of CF patients negatively correlates with lung function [62]. As pyocyanin formation is controlled by the *pqs* QS system in *P. aeruginosa*, its inhibition can be taken as a suitable read out for the anti-virulence activity of *pqs* QSIs in the context of CF-related infections [15].

First, we investigated the effect of the QSI encapsulated GB-Tw SLNs on pyocyanin formation in comparison with the free drug. Therefore, different concentrations of free and nanoencapsulated QSI were incubated with *P. aeruginosa* PA14 and the extent of inhibition of pyocyanin formation relative to control was determined. As shown in Fig. 6A, the formulation did not only maintain the activity of the free QSI, but was superior at all tested concentrations. Interestingly, the activity improvement was most pronounced at lower concentrations. While the free compound exhibited a clear dose-dependent behavior, the loaded particles showed weaker dependence of dose.

Interestingly, all types of QSI-loaded SLNs were superior to the free QSI in pyocyanin inhibition (*significant difference*, Two-way ANOVA, Holm-Sidak method, $p < 0.01$) except for Tri-Tw SLNs, for which the activity of the free QSI was at least maintained (*insignificant difference*, Two-way ANOVA, Holm-Sidak method, $p = 0.549$), Fig. 6B. Preliminary postulations would hence be improved solubility, bacterial uptake or ensuring prolonged release due to the lipidic nanocarriers. This coincides with Bargoni et al who proposed that SLN increased the passive transport of tobramycin following duodenal administration in rats, masking the drug and thus deceiving P-gp efflux pump [63]. It was then crucial to explore whether the co-incubation with the SLN components or the entrapment of QSI in SLNs would be the determinant. Moreover, the effect of plain SLNs of different composition on pyocyanin production remained questionable.

Analysis of the effect of plain SLNs alone revealed a strong inhibition of pyocyanin formation by all types of particles themselves, Fig. 6B. These findings strongly suggest an additive inhibitory potential by the QSI and the SLNs. To further investigate the role of the carrier in improving anti-virulence properties of the QSI, we examined the effect of a mixture of both - the free QSI and plain SLNs - on pyocyanin formation. As can be deduced from Fig. 6B, for all SLN types, there was no significant difference in anti-pyocyanin activity between the encapsulation of QSI in SLNs and the co-incubation of QSI with plain SLNs (*insignificant difference, Two-way ANOVA, Holm-Sidak method, $p = 0.64$*). This implies that other properties of SLNs different from their function as carrier are decisive for improvement of anti-virulence activity.

Most interestingly, as can be clearly deduced from the growth curves of *P. aeruginosa*, Fig. 7A and B, the pyocyanin inhibition by the plain SLNs was not due to killing of the bacteria, an effect that is often associated with nanoparticles [64]. Encouraged by this anti-virulence property of the SLNs, we aimed at elucidating the contribution of the ingredients used for SLN preparation. Therefore, we studied the effect of the different emulsifiers (Tween, Poloxamer, PVA) involved in the formulations at different concentrations including a concentration comparable to the amount of emulsifier present in the respective SLN formulation (C2 = 16-66 $\mu\text{g/ml}$). Indeed, a pronounced anti-pyocyanin activity can be attributed to the emulsifiers, mainly to Tween 80 and Poloxamer 407, demonstrating their role in anti-virulence activity of the SLNs, Fig. 7C.

Figure 6

Additional control experiments have been carried out; the interference of SLNs with the assay procedure, possible adsorption of pyocyanin compound to the SLNs or interference of the particles with optical density measurements have been excluded for the applied concentrations (data not shown). Meanwhile, preliminary experiments proved the colloidal stability of the SLNs in the culture medium used in this assay (Fig. S5, supp. materials).

In brief, the mechanism by which SLNs improved the anti-virulence activity of the QSI is worth to implement further investigations at a molecular level. Possible common postulations such as enhanced solubility and/or uptake of the lipophilic compound in bacteria due to the presence of lipids would not be persuasive. In fact, such results represent a startling discovery; on their own, emulsifiers commonly used in drug delivery can act as potent anti-virulence agents. Unexpected pharmacological activity of excipients should no longer be underestimated. In line with our finding, the recent discovery of the role of N-methylpyrrolidone solvent as an immunomodulator and antimyeloma compound and the famous effect of Cremophor EL on the pharmacokinetics and erythrocyte accumulation of paclitaxel were reported [65, 66].

Figure 7

Conclusions:

Various ultra-small solid lipid nanoparticles (< 100 nm) efficiently loaded with the novel anti-infective QSI could be prepared. Particles ensured prolonged release of the payload over > 8 h in simulated lung fluid and maintained the viability of epithelial cells (Calu-3) at wide concentration range and various compositions. The hydrophilic surface properties allowed for mucus penetration representing the main barrier for efficient CF-therapy. Nebulized SLNs provided high deposition in the bronchial area, the target site. Improved anti-virulence activity was notably observed with nanoencapsulated QSI compared to the free compound. Surprisingly, significant reduction in pyocyanin formation was observed with plain SLNs, which was shown

to be related to the emulsifiers used. These startling findings represent a new perspective of ultimate significance in the area of the nano-based delivery of novel anti-infectives.

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Figure captions:

- Fig. 1** Characterization of SLNs: A) Particle size and PDI of plain and QSI-loaded SLNs (insignificant difference, Mann-Whitney Rank Sum Test, $p = 0.818$), B) DSC heating and cooling curves of pure precirol lipid, free QSI, plain Pre-PVA SLNs and QSI-loaded Pre-PVA SLNs, C) SEM micrograph, and D) TEM micrograph of GB-Tw SLNs.
- Fig. 2** QSI-loaded SLNs: A) Encapsulation Efficiency, B) *In vitro* release of QSI in PBS, C) *In vitro* release of QSI in SLF from different SLNs.
- Fig. 3** SLN-mucus interaction: A) Particle size after incubation of SLNs with mucin at 37 °C, B) Absorbance after incubation of SLNs with mucin at 37 °C, C) Z-stacks representing the penetration of Nile Red-labeled SLNs in stained artificial sputum (green) at different time intervals, D) Total fluorescence of labeled SLNs in different Z-stacks determined using 'Image J' software.
- Fig. 4** Cytotoxicity of plain and QSI-loaded SLNs (MTT assay) on Calu-3 cells: A) Pre-Tw SLNs, B) Pre-PVA SLNs, C) GB-Tw SLNs, D) GB-PVA SLNs, E) Tri-Tw SLNs, F) Tri-P SLNs
- Fig. 5** Colloidal stability of SLNs after nebulization: A) Particle size and PDI before and after nebulization, B) Zeta potential, C) Particle deposition by NGI at flow rate 15 L/min
- Fig. 6** Anti-virulence activity assay: A) Effect of different concentrations of QSI encapsulated in GB-Tw SLNs *versus* free QSI on pyocyanin formation (*significant difference, Student's t-test, *** $p < 0.001$*), B) Effect of QSI encapsulated in SLNs of different compositions *versus* free QSI, plain SLNs, (plain SLNs + free QSI mixture) on pyocyanin formation at a concentration of 125 nM (*significant difference, Two-way ANOVA, Holm-Sidak method, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*), mean values of at least three replicates, error bars represent standard deviation
- Fig. 7** A) and B) Growth curves of *P. aeruginosa* in presence of SLNs of different compositions, C) Anti-virulence activity assay using emulsifiers at different concentrations (C1, C2, C3 are 1.67, 16.67, 66.67 µg/ml, respectively, for tween and poloxamer 407 and 0.67, 66.67, 100 µg/ml for PVA)

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Graphical abstract