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Nanotechnology approaches for antibacterial drug delivery: Preparation and microbiological evaluation of fusogenic liposomes carrying fusidic acid

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ABSTRACT

Many antibacterial drugs have some difficulty passing through the bacterial cell membrane, especially if they have a high molecular weight or large spatial structure. Consequently, intrinsic resistance is shown by some bacterial strains. Reduced cell membrane permeability is one of the mechanisms of resistance known for fusidic acid (FUS), a bacteriostatic steroidal compound with activity limited to Gram-positive bacteria. Moreover, the lipophilic character of FUS has been shown to cause drug retention inside the bilayers of cell membranes, preventing its diffusion towards target sites inside the cytoplasm. Targeting antimicrobial agents by means of liposomes may be a valid strategy in the treatment of infections refractory to conventional routes of antimicrobial treatment. On this basis, loading of FUS in fusogenic liposomes (FLs) was planned in this study. Fusogenic small unilamellar vesicles loaded with FUS were produced to evaluate their influence on improving the cell penetration and antibacterial activity of the antibiotic. The produced carriers were technologically characterised and were subjected to an in vitro microbiological assay against several strains of Gram-negative and Gram-positive bacteria. The experimental results showed that encapsulating FUS in a liposomal carrier can improve antimicrobial efficacy and reduce the effective concentration required, probably through putative mechanisms of increased diffusion through the bacterial cell membrane. In fact, whilst free FUS was active only on the tested Gram-positive strains, incubation of FUS-loaded FLs exhibited growth inhibitory activity both against Gram-positive and Gram-negative strains. The lowest MICs were obtained against *Staphylococcus epidermidis* (<0.15 pg/mL) and *Acinetobacter baumannii* (37.5 pg/mL) clinical strains.

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1. Introduction

Various species of bacteria are considered to be intrinsically resistant to antibiotics owing to the limited permeability of their cell membrane [1]. In addition, in some instances, and especially under the biological 'pressure' of hospital environments, some strains of antibiotic-susceptible bacteria can become resistant due to the above phenomenon.

Because of the overwhelming clinical significance of acquired bacterial resistance, over the last few years innovative technological research has been focusing on the possibility of changing the pharmacokinetic profile of known antibiotics through their association with colloidal (nano-sized) drug delivery systems.

Among the carriers proposed and used for the controlled or targeted delivery of antibacterial drugs, liposomes are probably the most investigated systems [2]. In parallel with many practical drawbacks shown by these nanocarriers, such as their limited physical stability, liposomes possess some important technological features such as good biocompatibility and the possibility of encapsulating both active hydrophilic and hydrophobic compounds [3].

Many different liposome compositions, types and production technologies have been investigated with the aim of either improving the therapeutic potential of antibiotics or ameliorating their

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pharmacokinetic or toxicity profile or, in most cases, enlarging their spectrum of action against resistant or insensitive micro-organisms [2,4].

An interesting class of phospholipid-based vesicles is known as fusogenic liposomes (FLs). They are a particular class of phospholipid vesicles which includes lipids that, in a biological environment, go through a phase transition under specific chemical conditions such as an acidic pH or the presence of cations. Because of their composition, the bilayers of FLs are able to interact, in their liquid-crystalline phase, with cell membranes, promoting reciprocal mixing and destabilisation of the membrane and therefore the release of the encapsulated cargo inside the cytoplasm.

Different types of FLs have been proposed over the last few years, comprising either viral material or natural or synthetic lipids to achieve the required fusogenic properties [5–8]. Among the various studied systems, FLs based on 1,2-dioleoylphosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) have shown a high degree of cell association. Vidal and Hoekstra [9] observed that the presence of a phosphatidylethanolamine derivative, in combination with other phospholipids such as phosphatidylcholine, is essential for the fusion process. An explanation of the mechanism of fusion can be found in the cited paper and in other published articles. Compared with liposomes containing only DOPE, vesicles containing DOPE and CHEMS have a high ability to promote intracellular release of the carried molecules, even those with a high molecular weight, and in a non-pH-sensitive manner.

Although fusion between phospholipid vesicles and biomembranes has mainly been observed with eukaryotic cells, we recently demonstrated that bacterial cells are also able to fuse with this kind of vesicle. For instance, FLs carrying vancomycin (VAN) were able to drive the penetration of the antibiotic inside Gram-negative bacterial cells, inhibiting the growth of bacterial strains usually resistant to the free antibiotic [10–12]. As proof of concept of this technological strategy, when VAN was carried by conventional (not fusogenic) liposomes, no inhibitory activity was observed [10,11]. These studies also showed that the fusogenic liposomal formulations did not cause a cytotoxic effect on bacterial cells [10].

As evidenced by microscopy experiments [10], the mechanism postulated was an interaction and/or fusion of FLs with the outer membrane that surrounds the wall of Gram-negative bacterial cells and that possesses a structural analogy with the plasmatic membrane of eukaryotic cells [13].

With the aim of testing the potentiality of the FL strategy with other antibacterial drugs, this paper reports a preliminary study regarding the production, characterisation and in vitro microbiological evaluation of fusogenic vesicles loaded with fusidic acid (FUS).

FUS is a bacteriostatic antibiotic with a steroidal structure. Its spectrum of activity is quite narrow and includes Gram-positive cocci, *Staphylococcus aureus* and *Staphylococcus epidermidis*, including meticillin-resistant *S. aureus* (MRSA) strains. The relative minimum inhibitory concentrations (MICs) are 0.12–1.0 μg/mL for MRSA and 0.25 μg/mL for *S. epidermidis*. Other Gram-positive cocci are much less susceptible, with MICs of ca. 4–6 μg/mL [14].

FUS behaves as an inhibitor of bacterial protein synthesis by preventing the polymerisation of terminal amino acids owing to inhibition of the elongation factor-G (EF-G)–GDP complex that allows the translocation of tRNA within the 50S subunit of ribosomes. FUS is used almost exclusively as an antistaphylococcal agent, with the exception of meningeal and urinary infections. The clinical indications of this antibiotic mainly concern skin, bone, joint, lung and blood infections (septicaemia), always in combination with a second antistaphylococcal antibiotic to prevent bacterial resistance. In vitro, cell resistance appears easily, but this can also occur in vivo, especially if the drug is used on large wounds. In dermatology, FUS is used as a topical formulation to treat infections caused by *Corynebacterium*, acne with pustules and for the treatment of skin staphylococcal diseases. More recently, it has been used as monotherapy in the treatment of acute pseudomembranous colitis caused by *Clostridium difficile*, against which FUS acts as an inhibitor of L-selectin [15].

Resistance to FUS is determined by a number of mechanisms. The best described are alterations in EF-G and impaired drug permeability [16]. Most *Escherichia coli* are known to be intrinsically resistant to FUS owing to cell wall impermeability. Alterations in permeability as well as enzymatic inactivation by group I chloramphenicol acetyl transferase were also associated with FUS resistance in a few isolates of *Staphylococcus* spp. and Enterobacteriaceae without any evidence of other resistance determinants [16,17].

Chopra [18] studied the phenomenon of resistance to FUS in some *S. aureus* strains and hypothesised a reduction in the permeability of this antibiotic owing to plasmids incorporated in bacterial cells. This plasmid-mediated resistance, which has also been evidenced in *E. coli* [19], might be related to alterations in cell wall/membrane permeability.

Recent studies by Holopainen and colleagues [20,21] have linked the high lipophilic character of FUS to its antibacterial activity profile. In fact, experiments using various model biomembranes showed that FUS is able to interact strongly with the phospholipid bilayers and in particular with the negatively charged lipids [20], remaining embedded in the membrane and forming lateral domains. This behaviour ultimately hinders drug diffusion into the cytoplasm and thus its biological effects at the target site.

Based on the above considerations and our previous results [10,11], fusogenic small unilamellar vesicles (SUVs) were loaded with FUS and were tested in vitro against different bacterial strains to assess whether the proposed delivery strategy is able to enlarge the spectrum of activity of this antibiotic towards naturally insensitive bacteria.

2. Materials and methods

2.1. Chemicals

DOPE and 1,2-dipalmitoylphosphatidylcholine (DPPC) were purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). CHEMS and cholesterol (CHOL) were purchased from Sigma-Aldrich Chimica s.r.l. (Milan, Italy). FUS (purity >99%, HPLC) was a kind gift of Leo Pharma A/S (Ballerup, Denmark). Diethyl ether was purchased from Merck (Darmstadt, Germany). All other chemicals were commercial products of analytical grade or higher. All materials were used as supplied without purification or modification.

2.2. Liposome preparation and characterisation

Multilamellar liposomal vesicles (MLVs) were first prepared by the reverse-phase evaporation technique [22]. Briefly, 10 mg of lipids (DOPE/DPPC/CHEMS in a 4:2:4 molar ratio or DPPC/CHOL in a 7:3 molar ratio) were dissolved in a round-bottomed glass tube with 3 mL of diethyl ether. Then, 1 mL of phosphate-buffered saline (PBS) (pH 7.4) containing 5 mg of FUS was added and the mixture was vortex-mixed for ca. 15 min to obtain an initial water-in-oil emulsion. Plain (unloaded) liposomes were produced analogously without the addition of drug in the buffer solution. The organic solvent was then removed under rotary evaporation in vacuo to induce a phase inversion that produced an oil-in-water secondary emulsion. The water-bath temperature during the whole process was kept constant at 50 °C, i.e. a value higher than the phase transition

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