How terpene-based nanoparticles, harboring stimuli-responsive linker, overcome poor diffusion of β-lactam antibiotic to treat intracellular infection?

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ABSTRACT SUMMARY
To overcome poor diffusion of antibiotics such as β-lactam, we developed and compared antibacterial activity of new terpene-based nanoparticles (NPs) coupled to Penicillin G (PNG). Squalene-penicillin G with or without a pH-sensitive linker (SqPNG-pH or SqPNG) have been synthesized and characterized. An in vitro comparative efficacy study has been performed and SqPNG-pH NPs have proved to increase the intracellular antibacterial activity on the intracellular pathogen Staphylococcus aureus compared to the free drug.

INTRODUCTION
In the field of antibiotherapy, it is now well established that the poor intracellular penetration of antibiotics reduces the effectiveness of many treatments. During infections, some bacteria such as Staphylococcus aureus are able to hide from host defense and/or action of antibiotics. They survive and replicate into the host cells, thus establishing a cell-niche causing persistent or recurrent infections. Moreover, sub-lethal concentrations of antibiotics provide to bacteria a suitable hostile environment for the emergence of antibiotic resistance. To overcome it, a drug targeting strategy has proved be useful in conveying antibiotics directly into host cells and reaching therapeutically active concentrations. In our laboratory we have developed the “squalenoylation” technology which has already shown remarkable pharmacological activity with drugs like gemcitabine (anti-cancer drug) or other nucleosides analogues (antiviral drugs). This concept is based on a covalent chemical conjugation of a natural and biodegradable lipid, the squalene (or others terpenic moiety), to an active drug in order to prepare stable nanoparticles (NPs).

In this context, we synthesized two prodrugs derived from the bioconjugation of the historical β-lactam antibiotic, Penicillin G (PNG) to the squalene (Sq) moiety with and without a pH sensitive linker (SqPNGpH and SqPNG). All these analogs self-assembled in water to form stable nanoparticles (≈ 145 nm). Moreover, all NPs have shown similar morphology and surface charge, but those with pH-sensitive linker induced a better intracellular antibacterial activity on the facultative intracellular pathogen S. aureus.

EXPERIMENTAL METHODS
SqPNG and SqPNG-pH nanoparticles were obtained using nanoprecipitation procedure. Briefly, 4 mg of compounds were dissolved in 500 μL of ethanol before being added drop-wise under stirring into 1 mL MilliQ water. Then, ethanol was completely evaporated using a Rotavapor® to obtain an aqueous suspension of SqPenG or SqPenGpH NPs.

When needed, the green dye cholesterly Bodipy FLC12 (at 0.5 % molar), was used to prepare florescent-labeled NPs by adding the probe to the organic phase before nanoprecipitation.

The diameter-average of the nanoparticles was determined by dynamic light scattering (DLS), using a Zetasizer Nano 6.12, (Malvern Instrument Ltd, Worcestershire, UK). The zeta potential (ζ) was measured using the same equipment. Nanoparticles’s morphology was observed by cryogenic transmission electron microscopy.

The experiments utilized penicillin-sensitive strain, Staphylococcus aureus (ATCC 55585) which was obtained from the ATCC and growth in Brain Heart Infusion (BHI) media (Invitrogen) at 37°C. Murine macrophage cell line (J774) were chose and cultured in RPMI 1640 (Roswell Park Memorial Institute) medium supplemented with 10% of inactivated fetal bovine serum (FBSd) (Gibco) at 37 °C in humidified atmosphere containing 5% CO2.

Intracellular antimicrobial activity of NPs was determined using the following method. J774 cells were seeded in 24-well plates at a concentration of 2 × 105 cells per well and allowed to adhere overnight. After washing with PBS, cells were pre-treated with NPs or free PNG during 6 h. J774 cells were washed and allowed to engulf S. aureus at a multiplicity of infection (MOI) of 10 for 2 h. To remove the extracellular bacteria, J774 macrophages were then washed again and incubated with gentamicin (50 μg/mL) during 6 or 24 hours. Cells were lysed and viable bacteria plated onto BHI/agar for colony forming unit (CFU) enumeration after 24 h. For confocal microscopy studies, J774 cells were treated then infected in similar conditions explained previously. Then LIVE/DEAD BacLight bacterial viability kit (Molecular Probes) were used to visualize viable/dead bacteria, intracellularly.
Fluorescent-NPs helped to explore and compare macrophage capture and intracellular localization of NPs using confocal microscopy and flow cytometry analysis.

RESULTS AND DISCUSSION

SqPNG and SqPNG-pH compounds showed the capability to spontaneously self-assemble as stable NPs in water. The SqPNG and SqPNG-pH NPs’ average diameter was 140 and 150nm, respectively, with a very low polydispersity. Their Z-potential was about -50 mV. Similar results (size, zeta potential and stability) were obtained when fluorescent probe was added to the formulation process.

Cryo transmission electron microscopy (CryoTEM) analysis of SqPNG-pH NPs revealed a spherical and regular shape (Figure 1).

![Figure 1: Cryogenic transmission electron microscopy of a suspension of SqPNGpH nanoparticles. Scale bars = 100 nm](Image)

The ability of SqPNG and SqPNG-pH NPs to be internalized by macrophages was studied by flow cytometry and confocal microscopy. We observed that both types of NPs were internalized (and not adsorbed on the cells surface) differently by macrophages, depending on the linkage. SqPNG NPs reached a maximum of internalization at 6 h, while fluorescence of SqPNG-pH NPs continued to increase during 24 h.

![Figure 2: Confocal fluorescence microscopic images of viable bacteria infected J774 cells, after 2h of pretreatment with SqPNGpH NPs or free PNG and following by S. aureus infection. Viable S. aureus cells are stained using LIVE/DEAD BacLight kit. In green: viable bacteria and in red: dead bacteria.](Image)

Intracellular survival assay were carried out in infected J774 cell line in order to determine the efficiency of our systems in delivering antibiotic intracellularly. Practically, cells were preincubated with different NP formulations maintaining similar PNG concentrations (20 μg/mL) and then infected with S. aureus bacteria. The control PNG-unloaded NPs as well as free PNG had no antimicrobial activity on intracellular bacteria while SqPNG and SqPNG-pH NPs decrease the number of intracellular bacteria after only 6h. 24h after infection this effect was even more pronounced. However, in both case (6 and 24h post treatment), the presence of pH-sensitive linker improved the antibacterial effect suggesting that PNG was more efficiently released from SqPNG-pH NPs, intracellularly. Moreover, further investigation using the LIVE/DEAD BacLight kit, which allowed to differentiate between viable and dead S. aureus, clearly shown that free PNG did not induce any significant mortality of intracellular S. aureus, unlike SqPNG-pH NPs, which raise the number of red/dead bacteria (Figure 2).

Finally, NPs and bacteria were found to localize in different intracellular compartments suggesting that intracellular released PNG diffused and crossed the endolysosomal membranes in order to reach intracellular bacterial vacuole and kill bacteria.

We further investigated the modification of terpene moieties in order to improve even more the antibacterial activity of penicillin G. Preliminary results shown remarkable antibacterial effect (with 3-log reduction of viability) on intracellular S. aureus.

CONCLUSION

In brief, two bioconjugates of penicillin G with squalene, using either a pH-sensitive (SqPNG-pH) or a pH-insensitive linker (SqPNG), were synthesized and found to spontaneously form stable nanoparticles which were able to enter and release Penicillin G intracellularly.

The SqPNG-pH nanoparticles have proved to induce fast and significant killing of S. aureus-infected J774 cells. This approach opens interesting perspectives to combat invasive bacterial infections such as S. aureus, which represents a worldwide public health concern and a major burden for the dairy industry.

REFERENCES


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