Journal of Controlled Release 279 (2018) 316–325

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Clearance of intracellular *Klebsiella pneumoniae* infection using gentamicin-loaded nanoparticles

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ARTICLE INFO

Keywords:
Gentamicin
PLGA
Nanoparticles
Klebsiella pneumoniae
Intracellular infection
Macrophage
Inflammation
Pyroptosis

ABSTRACT

Klebsiella pneumoniae is a foremost gram-negative pathogen that can induce life-threatening nosocomial pulmonary infections. Although it can be phagocytosed successfully by lung resident macrophages, this pathogen remains viable within vacuolar compartments, resulting in chronic infection and limiting therapeutic treatment with antibiotics. In this study, we aimed to generate and evaluate a cell-penetrant antibiotic poly(lactide-*co*-glycolide) (PLGA)-based formulation that could successfully treat intracellular *K. pneumoniae* infection. Screening of formulation conditions allowed the generation of high drug loaded nanoparticles through a water-in-oil-in-water approach. We demonstrated the therapeutic usefulness of these gentamicin-loaded nanoparticles (GNPs), showing their ability to improve survival and provide extended prophylactic protection towards *K. pneumoniae* using a *Galleria mellonella* infection model. We subsequently showed that the GNPs could be phagocytosed by *K. pneumoniae* infected macrophages, and significantly reduce the viability of the intracellular bacteria without further stimulation of pro-inflammatory or pro-apoptotic effects on the macrophages. Taken together, these results clearly show the potential to use antibiotic loaded NPs to treat intracellular *K. pneumoniae* infection, reducing bacterial viability without concomitant stimulation of inflammatory or pyroptotic pathways in the treated cells.

1. Introduction

Klebsiella pneumoniae is identified by the World Health Organisation to be of major concern for human health [1]. It is considered one of the most important gram-negative pathogens in nosocomial infection, frequently inducing severe pulmonary infections, particularly in elderly patients with impaired immunological defenses [2,3]. However, it can also infect other parts of the body including the urinary tract, lower biliary tract and surgical wound sites [4–6]. As with other bacterial pathogens, continual use of key therapies is driving the growing prevalence of serious antibiotic resistant strains, leading to increased concerns about present and future treatment options [7].

Recently, it has been reported that *K. pneumoniae* survives intracellularly after phagocytosis into macrophages by limiting the fusion of lysosomes with the *Klebsiella* containing vacuole (KCV), creating an intracellular reservoir of infection [8]. Clinical treatment of this intracellular pool of infection is challenging; for example, aminoglycosides, which can successfully treat extracellular *K. pneumoniae* infections, have poor cellular penetration, unsatisfactory subcellular distribution and consequently have sub-optimal activity towards intracellular infection [9–13]. Taken together, it is clear that both new drugs and novel delivery strategies are needed to treat this persistent and frequently lethal intracellular pathogen.

In this current study, we have examined the ability to enhance the delivery of a clinically relevant antibiotic into *K. pneumoniae* infected macrophages using an alternative nanoformulation approach. Nanoparticle formulations are growing in popularity as potential solutions to improve the pharmacokinetics of active pharmaceutical ingredients (API), alleviate systemic toxicities through drug encapsulation, and also provide controlled or sustained release properties to extend therapeutic windows [14–18].

Nanoparticle drug systems for the treatment of *K. pneumoniae* have

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https://doi.org/10.1016/j.jconrel.2018.04.040
Received 8 January 2018; Received in revised form 16 April 2018; Accepted 20 April 2018
Available online 25 April 2018
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been investigated previously. These include the application of metal nanoparticles such as gold or silver which has inherent anti-microbial properties [19]. Antibiotic loaded nanoparticle systems have also been examined including ceftazidime-loaded liposomes or gentamicin-loaded chitosan/fucoidan nanoparticles to enhance pulmonary delivery of the antibiotics [15,20]. However to date, studies have not addressed the specific treatment of the intracellular reservoir of infection. Herein we have aimed to develop a polymeric based nanoparticle with high drug loading that would be taken up into infected macrophages by phagocytosis to deliver the antibiotic at the site of intracellular infection; providing passive targeting of the antibiotic into the intracellular compartments of the cells to elicit enhanced antimicrobial effects. We demonstrate the potential effectiveness of this approach for the delivery of gentamicin towards intracellular *K. pneumoniae* infection.

2. Materials and methods

2.1. Materials

All general chemicals and reagents were supplied by Sigma-Aldrich UK, unless otherwise specified. Gentamicin sulphate was purchased from Discovery Fine Chemicals, UK. Water used for formulations was double distilled, HPLC grade water.

2.2. Formulation of gentamicin-loaded PLGA nanoparticles (GNPs)

A water-in-oil-in-water (w/o/w) formulation strategy was adopted for the development of the gentamicin-loaded nanoparticles. Briefly, gentamicin was dissolved in 0.5 ml of 1% aqueous polyvinyl alcohol (PVA) solution (*w/v*, in 0.95% MES buffer, pH 7), followed by drop-wise addition of 2 ml of dichloromethane (DCM) containing 20 mg of PLGA (LG 50:50, 502H, 7000–17,000 Da) *via* a 25G needle. For rhodamine-labelled nanoparticles (RNPs), 10% (*w*/w) rhodamine-B conjugated PLGA (AV011, PolySciTech, Akina, USA) was blended with PLGA (502H). The primary emulsion was obtained by sonication at 60 watts for 12 cycles in pulse mode (3 s on, 2 s off) while stirring at 1000 rpm (MS-53 M multiposition stirrer, JEIO TECH, Korea). Subsequently, 10 ml of aqueous PVA solution was poured into the primary emulsion and sonicated for another 18 pulse sonication cycles as before. The nanoparticle suspension was stirred for 4 h to evaporate DCM and then washed twice by centrifugation and resuspension cycles (at 20,000*g*, 20 min, 4 °C) in phosphate buffered saline (PBS). Various modifications to this formulation approach were assessed during process optimization including varying the concentrations of PLGA and PVA, different aqueous phases in the emulsion step and the pH of external aqueous phase as discussed in the results section.

2.3. Characterisation of GNPs

Triplicate NP batches were diluted in PBS and characterised by Zetasizer (Nano ZS, Malvern Instruments Ltd., UK) measurements, recording mean particle size (Z_{ave}), polydispersity index (PDI) and zeta potential. For Scanning Electron Microscopy (SEM), small droplets (10 µl, 3 mg/ml) of the final formulation of GNP and associated blank nanoparticle (BNP) controls were dried and sputter-coated with gold on aluminum stubs and visualized (Jeol 6500 field emission gun, Japan).

2.4. Quantification of drug loading in GNPs

Drug loading was calculated by analysis of residual gentamicin in the supernatants obtained after nanoparticle precipitation. Calibration curves were prepared using known concentrations of gentamicin dissolved in the supernatant of BNPs. In this way, any interference was accounted for at each concentration. Based on a protocol of aminoglycoside detection [21], 50 µl of gentamicin solution was added into a 96-well plate, followed by addition of 50 µl of a mixture of reagent A (1 ml of 80 mg/ml O-phthaldialdehyde in 95% ethanol) and reagent B (200 µl of 0.4 M boric acid pH 9.7, 400 µl of 2-Mercaptoethanol and 200 µl of diethyl ether). The fluorescence was measured at 360/460 nm using a fluorometer (FLUO star Optima, BMG Labtech).

The release of gentamicin from the GNPs was assessed using 2 ml of GNPs (10 mg/ml) in PBS buffer (at either pH 5 or pH 7), which was injected into a Slide-A-Lyzer® Dialysis Cassette 7000 MW (Thermo Scientific, UK). For comparison to free gentamicin diffusion, 2 ml of gentamicin sulfate (2 mg/ml) in PBS buffer (at either pH 5 or pH 7) was injected into separate cassettes. The cassettes were then placed into a 30 ml reservoir of PBS buffer (at either pH 5 or pH 7) in an incubator at 37 °C, with shaking at 120 rpm (SI50 Orbital Incubator, Stuart Scientific, UK). At pre-determined time points, 1 ml samples were removed from the reservoir and replaced with 1 ml fresh PBS buffer. The gentamicin content in samples was quantified by comparison to standards containing known amounts of gentamicin in PBS buffer (at either pH 5 or pH 7).

2.5. Bacterial strains and growth conditions

K. pneumoniae (stain 43816) was cultured on LB (Luria-Bertani) agar plates for 18 h at 37 °C. LB broth was inoculated with one bacteria colony and incubated overnight at 37 °C while shaking at 180 rpm (SI50 Orbital Incubator, Stuart Scientific, UK). The bacteria were harvested by centrifugation (at 2500*g*, 20 min, 24 °C), and then diluted to a defined optical density (A_{600}). The colony forming units (CFU) were determined by plating serial bacterial dilutions prepared in PBS onto agar plates and visible colonies were counted following overnight incubation at 37 °C.

2.6. In vitro antimicrobial activity

For planktonic *K. pneumoniae* assay, broth microdilution tests were performed to determine the minimum inhibitory concentration (MIC) of GNPs against *K. pneumoniae*. After overnight growth and 2.5 h refreshing incubation, the *K. pneumoniae* suspension was adjusted to an optical density of 1.0 (A_{600}) and diluted in LB broth to give a starting inoculum of 5000 CFU/ml. A 100 µl volume of serially diluted free gentamicin, GNPs and BNPs in LB broth were added to a 96-well plate containing 100 µl of diluted *K. pneumoniae*. Concentrations of both free and nanoencapsulated gentamicin were equalised in all studies. Polymer concentrations were also equalised when comparing BNPs and GNPs. The plates were incubated overnight at 37 °C with shaking at 120 rpm. A_{600} was measured and background absorbance from the negatively controlled wells (the absorbance of LB broth or nanoparticles only) was subtracted from all wells before analysis. The lowest concentration at which mean A_{600} was zero was designated the MIC. The minimum bactericidal concentration (MBC) was determined by the absence of growth on LB agar plates of 100 µl mixtures from each challenged well after stipulated incubation times.

Biofilm susceptibility assays were performed using the MBEC® Assay (Innovotech, Edmonton, Alberta, Canada). *K. pneumoniae* (150 µl/well, 5×10^5 CFU/ml in LB broth) was added to an MBEC plate and incubated for 24 h (37 °C, 120 rpm) to allow biofilm formation on the pegs. Biofilms pegs were immersed twice for 2 min in sterile PBS to remove loosely adhered bacteria. Then the pegs were challenged with a range of concentrations of either free gentamicin (0–400 µg/ml) or nanoparticle formulations in 200 µl LB broth for another 24 h (37 °C, 120 rpm). The challenge plate was then measured for biofilm derived MIC, and the lid pegs were rinsed again and then placed in a new plate containing fresh LB broth (recovery plate). Biofilms were disrupted by sonication for 10 min, and then incubated for a further 24 h. The minimum biofilm eradication concentration (MBEC) was designated as the lowest concentration in the recovery plate at which there was no observable growth.

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