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Liposome delivery of ciprofloxacin against intracellular *Francisella tularensis* infection

Jonathan P. Wong[a,*], Huiming Yang[a], Karen L. Blasetti[b], Glen Schnell[a], John Conley[b], Lawrence N. Schofield[a]

[a] *Chemical and Biological Defence Section, Defence R&D Canada-Suffield, Box 4000 Station Main, Medicine Hat, Alberta, Canada T1A 8K6*
[b] *Scilab Inc., Medicine Hat, Alberta, Canada*

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Abstract

The effect of liposome delivery on the controlled release and therapeutic efficacy of ciprofloxacin against intracellular *Francisella tularensis* infection in vivo was evaluated in this study. Ciprofloxacin was encapsulated in small unilamellar vesicles by a remote loading procedure using an ammonium sulfate gradient. This procedure produced uniform sized liposomes (100 nm) with an entrapment rate of 90 ± 3.5%. Following administration of unencapsulated or liposome-encapsulated ciprofloxacin by intravenous injection or aerosol inhalation, levels of ciprofloxacin in sera, lungs, liver and spleen were determined using ^{14}C-ciprofloxacin as radiotracer for ciprofloxacin. Intravenous injection of liposome-encapsulated ciprofloxacin resulted in higher serum levels of drug in serum, as well as increased drug retention in lungs, liver and spleen, compared to that of free encapsulated drug. Aerosol administration of liposome-encapsulated ciprofloxacin by jet nebulization resulted in significantly higher drug levels and prolonged drug retention in the lower respiratory tract compared to the free drug. Aerosol inhalation of liposome-encapsulated ciprofloxacin, given either prophylactically or therapeutically, provided complete protection to mice against a pulmonary lethal infection model of *F. tularensis*. In contrast, ciprofloxacin given in its free form, was ineffective. These results suggest that liposome encapsulation of ciprofloxacin enhances drug delivery to the primary site of infection and results in increasing therapeutic efficacy against *F. tularensis*.

Keywords: Liposome; Delivery; Ciprofloxacin; Efficacy; Tularemia

1. Introduction

Infectious diseases caused by intracellular bacteria present a significant challenge to antibiotic therapy. Antibiotic treatment of these types of infections has been associated with high failure and/or relapse rates [1,2]. Intracellular pathogens, whether obligate or facultative, can hide, reside and multiply within the phagocytic cells of the reticuloendothelial system (RES), and by virtue to their intracellular location, are protected from the actions of the immunological defence cells and of antimicrobial agents [2–5]. The ineffectiveness of conventional antibiotics against intracellular infections may also be attributable to

* Corresponding author. Tel.: +1-403-544-4689; fax: +1-403-544-3388.
E-mail address: jonathan.wong@drdc-rddc.gc.ca (J.P. Wong).

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poor drug penetration, limited drug accumulation in subcellular compartments and/or drug inactivation by acidity in subcellular compartments [3–5]. These factors may explain why some antibiotics are bactericidal against extracellular bacteria in vitro, but are ineffective in killing intracellular forms of the bacteria [4–6].

Ciprofloxacin, a fluoroquinolone, is a potent and broad-spectrum antibiotic, and has good antibacterial activity against most gram-negative bacteria and gram-positive cocci. Ciprofloxacin has been shown to have a superior ability to penetrate most tissues compared to other antibiotics [7–10], accumulates in macrophages [11] and neutrophils [12] and is bactericidal in low pH environment [13]. These attributes contribute partly to ciprofloxacin being the drug-of-choice for the treatment of infectious diseases caused by intracellular pathogens. Furthermore, ciprofloxacin, when orally or intravenously administered, is known to reach such organs as liver, spleen, lungs and lymph nodes [14], which are important infection sites for intracellular bacteria. However, ciprofloxacin does not preferentially accumulate well at these tissues and may therefore not reach high sustain therapeutic levels at these sites.

Liposomes have been shown to be a promising delivery system for a number of antimicrobial drugs including antibiotics [15–18]. Conventional liposomes are readily taken up by phagocytic cells of the RES system, including macrophages. They therefore constitute a valuable delivery vehicle for targeting high therapeutic doses of antibiotics to those intracellular sites where the parasitic bacteria reside. In addition, the sustained release of antibiotics from the liposomes may prolong the half-lives of these drugs in the body.

The objective of this study was to evaluate the effect of liposome encapsulation on drug accumulation in the primary sites of infection for intracellular bacteria and to compare the therapeutic efficacy of ciprofloxacin and liposome-encapsulated ciprofloxacin against a pulmonary infection caused by an intracellular pathogen, *Francisella tularensis*. *F. tularensis* is a facultative intracellular bacterium that can cause tularemia, which can be a potentially fatal human disease if untreated. *F. tularensis* is a potential biological warfare/bioterrorism agent, and medical and public health management of the infectious disease caused by this bacterial agent is important [19]. Infection by *F. tulerensis* involves the RES system and leads to bacterial growth within the lungs, liver and spleen [20]. Tularemia in humans can be treated with antibiotics, including streptomycin, gentamicin and chloramphenicol, but even with prolonged daily therapy, relapse and failure rates can range from 0% to 33% [21]. Tularemia in mice can be treated subcutaneously with ciprofloxacin and doxycycline, but to be effective, antibiotic treatment was given twice daily and given 48 h before infection and continued for 5 days post infection. Even with the prolonged antibiotic treatment in these mice, occurrence of relapse was significant [22]. Successful treatment of tularemia using ciprofloxacin depends on high sustain drug concentrations in tissues and intracellular sites where the bacteria reside and multiply. Due to controlled release and intracellular targeting provided by liposome delivery, liposome-encapsulated ciprofloxacin may represent a promising therapeutic drug for the prevention and treatment of intracellular infections, including tularemia.

2. Materials and methods

2.1. Chemicals

Phosphatidylcholine and cholesterol used for the preparation of liposomes were purchased from Avanti Polar Lipids (Alabaster, AL.). Ciprofloxacin (Bayer of Canada, Etobicoke, Ontario) was purchased through a local pharmacy. ^{14}C-Ciprofloxacin (Bayer, Leverkusen, Germany) was obtained from McGill University (Montreal, Quebec).

2.2. Animals

Six-week-old BALB/c female mice were obtained from the mouse breeding colony at Defence R&D Canada-Suffield (DRDC-Suffield), with breeding pairs purchased from Charles River Canada (St. Constant, Quebec, Canada). The use of animals described in this study was approved by DRDC-Suffield's Animal Care Committee. Care and handling of animals described in this study followed guidelines set out the Canadian Council on Animal Care.

2.3. Bacteria

F. tularensis Live Vaccine Strain (LVS, ATCC 296684, American Type Culture Collection, Rockville, MD) was cultured on cysteine heart agar plates supplemented with 5% defibrinated rabbit blood (Remel Labs, Lenexa, KS) for 4 days in 5% CO_2 as described previously [20]. Individual colonies were then selected for growth in modified Mueller–Hinton broth (Difco Laboratories) supplemented with ferric PP_i and IsoVitaleX (Becton Dickinson, Cackeysville, MD). The broth cultures were incubated at 37 °C for 4–5 days. The cultures were then aliquoted and frozen in 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). For the determination of 50% lethality dose, aliquots were thawed and diluted serially in sterile PBS just prior to administration into animals.

2.4. Preparation of liposome-encapsulated ciprofloxacin

The liposomes used for the encapsulation of ciprofloxacin were prepared by the remote-loading procedure using an ammonium sulfate gradient described previously [23,24]. The liposomes were made from egg phosphatidylcholine and cholesterol in a molar ratio of 1:1. Entrapment rate of ciprofloxacin was determined by mixing a trace amount of ^{14}C-ciprofloxacin with cold unlabeled ciprofloxacin prior to drug loading into the liposomes. Following the drug loading procedure, the radioactivity associated with the liposome pellet and with the washed supernatant (containing free unencapsulated drug) was determined. The entrapment efficiency of ciprofloxacin was defined as the ratio of cpm associated with liposome pellet to total cpm of liposome pellet plus supernatant, as expressed as a percentage. This procedure yielded reproducible liposome-encapsulated ciprofloxacin preparations with high drug entrapment rate of $90 \pm 3.5\%$.

2.5. Pharmacokinetics studies of liposome-encapsulated ciprofloxacin in mice

The effect of liposome encapsulation on the serum and organ levels of ciprofloxacin following intravenous injection and aerosol inhalation was determined in mice using ^{14}C-ciprofloxacin as a radioisotope tracer. In this study, groups of mice were either injected intravenously with 100 μl of free unencapsulated or liposome-encapsulated ciprofloxacin (22 mg/ml of cold unlabelled ciprofloxacin mixed with 2 μCi/ml of ^{14}C-ciprofloxacin). For the administration of liposome-encapsulated or free unencapsulated ciprofloxacin by aerosol inhalation, groups of mice were placed in a 24-port nose-only aerosol exposure chamber (In-Tox Products, Albuquerque, NM). The mice were then exposed to the aerosol particles generated by nebulization using PurRD jet nebulizer containing liposome-encapsulated ciprofloxacin (22 mg/ml). The generation and characterization of aerosol particles, and the drug deposition of liposome-encapsulated or free ciprofloxacin, and the exposure time in mice, had been previously described in details [24]. Drug deposition studies using a similar jet nebulizer and nose-only aerosol exposure chamber for mice had shown that $0.0087 \pm 0.0021\%$ of drug placed in the nebulizer was deposited in the lungs of mice [25]. Based on this % drug deposition, it is estimated that 30 μg of ciprofloxacin (free or encapsulated) was delivered to the lungs of mice for a single 20-min exposure in this study.

At various times post drug administration, groups of animals were anesthetized with ketamine/xylazine (200 mg/kg, intramuscular route), and approximately 1 ml of the blood was then collected by intracardiac puncture. The mice were then sacrificed by cervical dislocation, and the spleen, kidneys, liver, brain and lungs were surgically removed. Urine samples were also collected from the bladder when possible. The individual organs were mixed with 5 ml of PBS (15 ml for liver samples) and minced with scissors, and were homogenized with a Virtis hand-held homogenizer. The amounts of radioactivity present in the tissue, serum and urine samples were used to estimate the drug levels in these samples, or to calculate the drug concentrations in micrograms per milliliter of body fluids or micrograms per gram of wet tissues.

2.6. Protection and treatment studies using a lethal pulmonary F. tularensis infection model

A live vaccine strain of *F. tularensis* was used in the experimental infection of BALB/c mice. Prior to use, the bacteria were grown on Chamberlain synthetic agar medium, pH 6.5 at 37 °C with 5% CO_2. The

bacteria were suspended in saline and were given to mice by the intranasal (IN) route through the nostril (50 μl) using a micropipette, or by intravenous injection (100 μl) via the tail vein. One lethal dose$_{50}$ (LD_{50}) bacterial load corresponds to 100 and 10 colony forming units (CFU) of bacteria for IV and IN administrations, respectively.

For the treatment studies to determine the efficacy of liposome-encapsulated ciprofloxacin against an experimental challenge of 10 LD_{50} of *F. tularensis*, groups of mice were first anesthetized with sodium pentobarbital (50 mg/kg body weight given by intraperitoneal route). When the animals were unconscious, they were intranasally infected with 10 LD_{50} of *F. tularensis* applied gently with a micropipette into the nostrils (50 μl/mouse). At 24 h post infection, the animals were placed in a 24-port nose-only aerosol exposure chamber where the animals were exposed for 20 min to single dose of aerosolized liposome-encapsulated ciprofloxacin or free unencapsulated ciprofloxacin (22 mg/ml of ciprofloxacin). These aerosols were generated with the PurRD raindrop nebulizer and characterized using the aerodynamic particle sizer as described previously [24]. The infected animals were monitored daily for signs of symptoms and for deaths from the infection. At day 14 after infection, the number of mice that survived the otherwise lethal infection was recorded. For the study on the effect of delayed treatment on survival, treatment with the aerosolized free and liposome-encapsulated ciprofloxacin was administered at 24, 48, 72 and 96 h post infection. The survival rates in the treated mice were determined at day 14 as described before. The therapeutic efficacy of liposome-encapsulated ciprofloxacin given intravenously was evaluated against a systemic form of *F. tulerensis* infection. In this experiment, mice were first infected intravenously with 10 LD_{50} of the bacteria. At 24 h post infection, the mice were treated with a single IV injection of 100 μl of free and liposomal drug (22 mg/ml) via the tail vein. The mice were then monitored for survival for 14 days post infection.

For prophylaxis, mice were given a single aerosol exposure dose of ciprofloxacin or liposome-encapsulated ciprofloxacin by either aerosol inhalation as described earlier, or by IV injection through the tail vein (100 μl containing 1 μmol lipid equivalent, 22 mg ciprofloxacin/ml). At 24 h post drug administration, the mice were infected intranasally with 10 LD_{50} of *F. tularensis*. The survival rates of the mice were then determined and recorded at day 14 post infection.

2.7. Bacterial determination of organ homogenates

To determine the bacterial loads in organs of control and treated mice, the lungs, spleens and livers were aseptically harvested. The organs were then homogenized in 5 ml sterile PBS using a hand-held tissue grinder. The supernatants were then plated for growth in cysteine heart agar plates supplemented with 5% defibrinated rabbit's blood. The inoculated plates were incubated at 37 °C for 4 days, and the numbers of colony forming units of *F. tularensis* were determined.

2.8. Statistical analysis

The survival rates of the treatment and non-treatment control groups were compared using two-tailed *t* test (GraphPad Prism, version 2.0; GraphPad Software, San Diego, CA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. The effect of liposome delivery on drug levels in serum and RES organs

Levels of ciprofloxacin in serum and in various organs at 2 h following IV injection of liposome-encapsulated ciprofloxacin or free unencapsulated ciprofloxacin in mice are shown in Fig. 1. Ciprofloxacin level in serum at 2 h post injection was observed to be higher for liposome-encapsulated ciprofloxacin group compared to the free encapsulated ciprofloxacin group ($p < 0.05$). At 12 h post IV injection, there was little accumulation of free ciprofloxacin in the spleen, liver and lungs of mice injected IV with ciprofloxacin (data not shown). In contrast, ciprofloxacin encapsulated within liposomes was efficiently retained in the spleen and liver, and their levels persisted in these organs for long periods of time (levels were still detectable after 12 h post administration, data not shown).

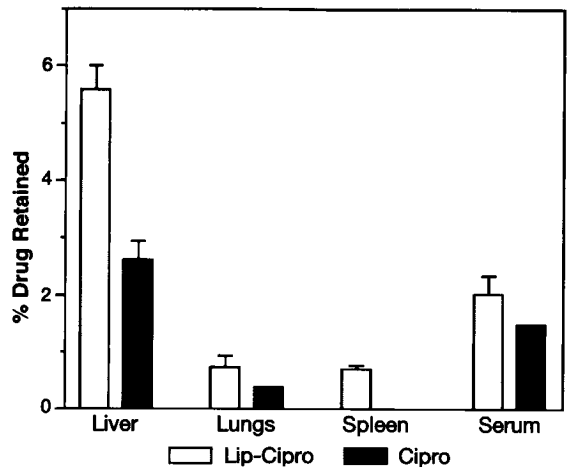

Fig. 1. The serum level and organ distribution of ciprofloxacin at 2 h post intravenous injection of free unencapsulated and liposome-encapsulated ciprofloxacin (100 μl of 22 mg/ml ciprofloxacin, mixed with 2 μCi/ml of ^{14}C-ciprofloxacin).

The levels of ciprofloxacin in the lungs of mice given a single aerosol exposure dose of free or liposome-encapsulated ciprofloxacin are shown in Fig. 2. In mice given ciprofloxacin, the levels of the drug decreased rapidly in the lungs upon administration, and the drug was almost completely eliminated from the lungs by 2 h post administration. The elimination half-life of the free ciprofloxacin in lungs was extrapolated and was estimated to be 1 h. With liposome-encapsulated ciprofloxacin, the drug levels in the lungs were significantly higher at all time points post administration compared to that of the free ciprofloxacin group ($p < 0.05$). The results also showed higher drug levels at 8 h post administration in the liposome group compared to 2 h post administration in the free drug group.

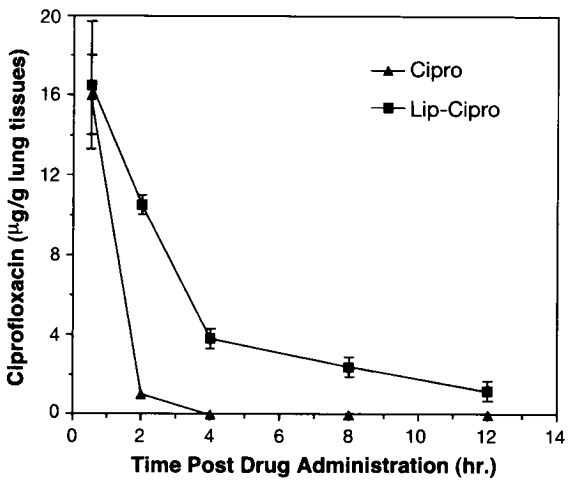

Fig. 2. The lung retention of ciprofloxacin in the lungs following aerosol inhalation of liposome-encapsulated (Lip-Cipro) or free unencapsulated ciprofloxacin (Cipro) through nebulization. Groups of mice were exposed to aerosol particles using a 24-port nose-only exposure chamber.

3.2. The effect of liposome delivery on prophylactic and therapeutic efficacy of ciprofloxacin against F. tularensis infection

The therapeutic efficacy of liposome-encapsulated ciprofloxacin and free unencapsulated drug were compared against a pulmonary infection of *F. tularensis*. Groups of mice were intranasally infected with 10 LD_{50} *F. tularensis*, and at 48 h post infection, they were treated with aerosolized liposome-encapsulated ciprofloxacin or ciprofloxacin. The survival rates in these groups of mice at day 14 post infection were compared (Fig. 3). Untreated control mice began to

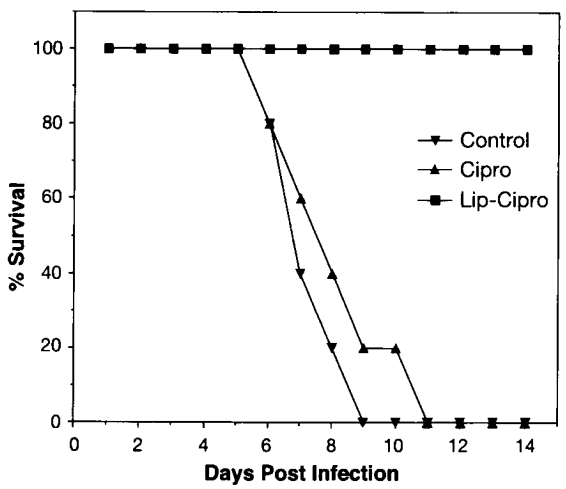

Fig. 3. The therapeutic efficacy of aerosolized liposome-encapsulated ciprofloxacin (Lip-Cipro) and free ciprofloxacin (Cipro) against pulmonary *F. tulerensis* infection in mice. Mice were first intranasally infected with 10 LD_{50} of *F. tularensis*, and at 48 h post infection, they were treated with aerosolized free and liposome-encapsulated ciprofloxacin. The survival of the control and treated mice were monitored for 14 days post infection.

succumb to the infection as early as day 5 post infection and by day 9, all mice in the group were dead. Little or no protection was observed in mice treated with aerosolized free unencapsulated ciprofloxacin. All the mice in the ciprofloxacin treated group died by day 9 post infection. In mice exposed to 20 min of aerosolized liposome-encapsulated ciprofloxacin, all the mice survived ($p<0.01$ vs. control, ciprofloxacin group). These results suggest that liposome-encapsulated ciprofloxacin delivered by aerosol inhalation was highly effective in the treatment of respiratory *F. tularensis* infection in mice.

To test the effectiveness of aerosolized liposome-encapsulated ciprofloxacin in the delayed treatment of highly advanced form of *F. tularensis* infection, groups of infected mice were treated at 48, 72 and 96 h post infection with free and liposome-encapsulated ciprofloxacin as described before. All mice treated with aerosolized liposome-encapsulated ciprofloxacin within 72 h of infection survived the infection (Fig. 4). When the treatment was delayed to 96 h post infection, the survival rate dropped slightly to 80%, but still was statistically significant compared to the control group ($p<0.05$ vs. control). Similarly, mice infected intravenously with 10 LD_{50} *F. tularensis* at 24 h post infection and treated with a single IV dose of liposome-encapsulated ciprofloxacin were completely protected (100%, $p<0.01$ vs. control) (Fig. 5), while free ciprofloxacin was not effective. The single IV of ciprofloxacin represents the single optimal dose because the solubility of ciprofloxacin decreases considerably for drug concentrations exceeding 22 mg/ml.

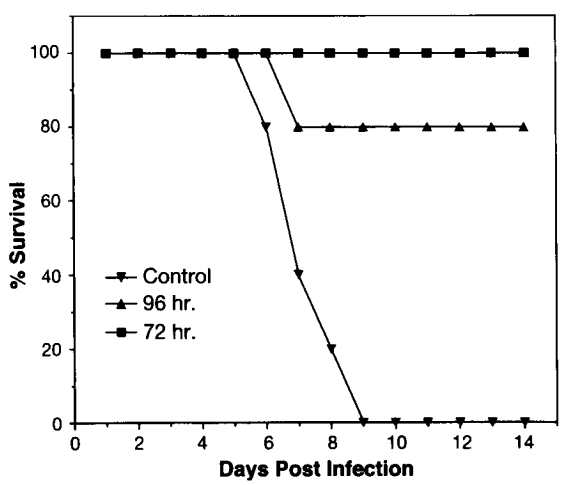

Fig. 4. The effect of delayed treatment by aerosolized liposome-encapsulated ciprofloxacin on the efficacy against pulmonary *F. tularensis* infection in mice. The survival rates and patterns for mice treated at 24 and 48 h post infection are identical to that obtained at treatment given at 72 h post infection.

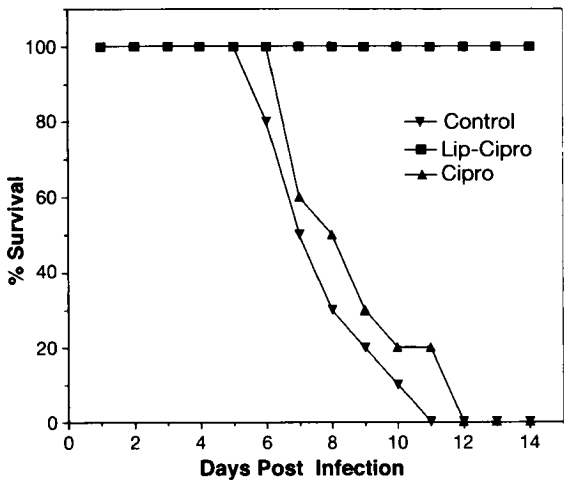

Fig. 5. The therapeutic efficacy of free and liposome-encapsulated ciprofloxacin administered intravenously against a systemic form of tularemia in mice. Mice were infected intravenously with 10 LD_{50} of *F. tularensis* and were treated with a single IV injection of free or liposome-encapsulated ciprofloxacin (22 mg/ml, 100 μl/animal). The survival of the animals was monitored for 14 days post infection.

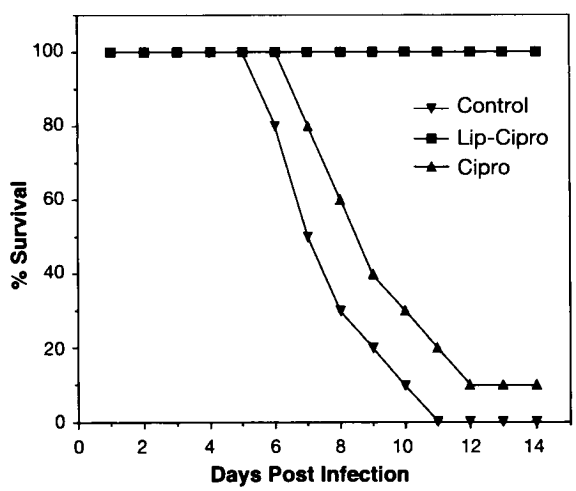

Fig. 6. The prophylactic efficacy of aerosolized liposome-encapsulated ciprofloxacin (Lip-Cipro) and free ciprofloxacin (Cipro) against pulmonary *F. tularensis* infection in mice.

3.3. Prophylactic efficacy of aerosolized liposome-encapsulated ciprofloxacin

To determine whether aerosolized liposome-encapsulated ciprofloxacin could be used prophylactically to protect mice against pulmonary challenge with 10 LD_{50} *F. tularensis,* groups of mice were pretreated with aerosolized liposome-encapsulated ciprofloxacin or free ciprofloxacin at 24 h prior to bacterial challenge (Fig. 6). All mice pretreated with aerosolized liposome-encapsulated ciprofloxacin were completely protected against the bacterial challenge. In contrast, pretreatment with aerosolized ciprofloxacin did not provide significant protection and was ineffective in protecting mice against the bacterial challenge.

4. Discussion

Liposomes have been shown in this study to be an effective drug delivery system for ciprofloxacin, particularly for the treatment of intracellular infections, including those caused by *F. tularensis.* These types of infections are especially challenging and difficult to eradicate, and relapses are common. Unfortunately for the defence community, almost all biological warfare agents of bacterial origin are either intracellular parasites themselves (*Yersinia pestis*, *Brucella* sp., *Coxiella burnetti* and *F. tularensis*) or have intracellular life cycle (*Bacillus anthracis*). This may partly explain why these agents are such effective biological warfare or bioterrorism agents, as they can cause intracellular infections that are difficult to treat.

The respiratory tract remains the most common route to entry for these agents, and the lungs represent the primary sites of infection. In addition, the inhalation or the pulmonary forms of these agents are also the most lethal, as evident from deaths resulting from recent incidences of inhalation anthrax in the US. To develop effective medical countermeasures against these agents, it is important to deliver concentrated doses of antibiotics to the lower respiratory tract so that these agents can be killed before they have the opportunity to spread systemically from the lungs. Therefore, pulmonary delivery of antibiotics, particularly using a liposome delivery system, presents an increasing important and rationale approach for the management of these types of infections.

Oral and intravenous forms of ciprofloxacin have been clinically used to treat respiratory tract infections. Although fluoroquinolones penetrate reasonably well into lung tissues following multiple dosing schedules, drug concentrations of ciprofloxacin in the bronchial mucosa, epithelial lining fluid and alveolar macrophages appear to be considerably lower compared to that of other fluoroquinolones [26].

Aerosol inhalation provides an effective and practical approach to deliver liposome-encapsulated ciprofloxacin to the lungs. When delivered by an appropriate nebulizer, liposome-encapsulated ciprofloxacin has been shown to be stable when aerosolized with no measurable liposome disruption [27]. The aerosol delivery of liposome-encapsulated ciprofloxacin provided very effective prophylaxis and post exposure treatment of pulmonary *F. tularensis* infection in mice, while aerosolized unencapsulated ciprofloxacin, provided little or no protection. The enhanced therapeutic efficacy can be partly attributable to increased lung retention of ciprofloxacin provided by the sustained release from the liposomes, as well as to enhanced intracellular delivery of ciprofloxacin by liposomes. Indeed, our previous results showed the total eradication of intracellular bacteria from the lungs, spleen and liver [24], which serve as the primary infection sites for these agents. When encapsulated in liposomes, ciprofloxacin can be delivered effectively using aerosol inhalation, intravenous, intranasal or intramuscular administrations [24,28]. In all cases, liposome-encapsulated ciprofloxacin was shown to have superior antibacterial activity compared to the unencapsulated free form of the drug [24,28]. Efficacy of liposome-encapsulated ciprofloxacin has been shown against a number of microbial infections, including brucellosis [28], salmonellosis [29], pneumococcal pneumonia [30], *Mycobacterium avium–Mycobacterium intracellulare* complex [18,23] and *Staphyloccoccus aureus* infections [31]. Furthermore, results from this study show that delayed treatment with liposome-encapsulated ciprofloxacin was very effective against an advanced form of pulmonary *F. tularensis* infection. This finding is very important in the medical management of biological warfare infections, as it potentially gives a greater window of therapy for military personnel to be

removed from the operation theater and to be transported to the appropriate field hospitals to receive treatment.

Results from this study, in conjunction from other studies, have shown liposome-encapsulated ciprofloxacin to be a promising antimicrobial agent for the treatment of, as well as prophylaxis against, infections caused by intracellular pathogens. Ciprofloxacin is a potent and broad-spectrum antibacterial agent, displaying good in vitro activity against most biological warfare bacteria. The results shown here suggest that liposome-encapsulated ciprofloxacin is very efficacious at treating of pulmonary tularemia in mice, even when the treatment is significantly delayed for up to 4 days. Aerosol delivery of liposome-encapsulated ciprofloxacin enhances the delivery and drug retention in the lower respiratory. Together, these results may suggest that aerosolized liposome-encapsulated ciprofloxacin is a promising medical countermeasure that could be used against the most lethal (pulmonary or inhaled) form of biological warfare agents.

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