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Liposomal mupirocin holds promise for systemic treatment of invasive *Staphylococcus aureus* infections

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ABSTRACT

Staphylococcus aureus is a major cause of severe invasive infections. The increasing incidence of infections caused by antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA), calls for exploration of new approaches to treat these infections. Mupirocin is an antibiotic with a unique mode of action that is active against MRSA, but its clinical use is restricted to topical administration because of its limited plasma stability and rapid

degradation to inactive metabolites. Mupirocin was identified by a machine learning approach to be suitable for nano-liposome encapsulation. The computational predictions were verified experimentally and PEGylated nano-liposomal formulation of mupirocin (Nano-mupirocin) was developed. The aim of this study was to investigate the efficacy of this formulation when administered parenterally for the treatment of *S. aureus* invasive infections. Nano-mupirocin exhibited prolonged half-life of active antibiotic and displayed superior antimicrobial activity against *S. aureus* than free mupirocin in the presence of plasma. Parenteral application of Nano-mupirocin in a murine model of *S. aureus* bloodstream infection resulted in improved antibiotic distribution to infected organs and in a superior therapeutic efficacy than the free drug. Parenterally administered Nano-mupirocin was also more active against MRSA than free mupirocin in a neutropenic murine lung infection model. In addition, Nano-mupirocin was very efficiently taken up by *S. aureus*-infected macrophages via phagocytosis leading to enhanced delivery of mupirocin in the intracellular niche and to a more efficient elimination of intracellular staphylococci. The outcome of this study highlights the potential of Nano-mupirocin for the treatment of invasive MRSA infections and support the further clinical development of this effective therapeutic approach.

Key words: Mupirocin, nanoliposomes, Nano-mupirocin, *Staphylococcus aureus,* invasive infections, parenteral administration

1. INTRODUCTION

Mupirocin, previously known as pseudomonic acid A, is an isoleucyl–adenylate analog naturally produced by *Pseudomonas fluorescens* that inhibits bacterial protein synthesis by preventing the attachment of isoleucine to its cognate tRNA [1]. Because this mechanism of action is unique and not found in other marketed antibiotics, cross-resistance between mupirocin and other classes of antibiotics has not been reported. Mupirocin is

bactericidal against *Staphylococcus aureus*, including beta-lactamase-producing and methicillin-resistant *S. aureus* (MRSA) strains [2] as well as vancomycin resistant strains (A. Cern, Y. Barenholz, unpublished data). Following parenteral administration, mupirocin is rapidly metabolized to monic acid, which has no antimicrobial activity and is mainly excreted in the urine [3]. It is for this reason as well as for its high affinity to bind plasma proteins [2] that the clinical use of mupirocin is limited to topical applications, including treatment of skin infection as well as nasal decolonization of *S. aureus* carriers [4, 5].

Mupirocin was identified by a computational approach to be suitable for liposomal drug development in terms of high loading and stability [6-8]. Prediction models were constructed using a machine learning approach and used for screening large molecular databases consisting of more than 10,000 molecules [6-8]. Mupirocin was found in the group of high-scored molecules for suitability to be formulated for nano-liposomal delivery [8]. Only 2.4% of approved drugs that were used in the screening reached this high level of suitability for being formulated as a nano-liposomal drug [8]. This prediction was proved and verified experimentally [7]. The obtained PEGylated nano-liposomal formulation exhibited high efficiency of active-remote loading of mupirocin and high physical stability, however, it showed rapid release of the liposomal drug in the presence of serum (82% release after 1 h of incubation). This was most probably caused by the high protein binding affinity of mupirocin to serum proteins functioning as a sink to the migration of mupirocin out of the liposomes [9]. The formulation was therefore further optimized to contain 15% w/w hydroxypropyl-beta-cyclodextrin (HPCD) in the intraliposomal aqueous phase, which was found to slow the release of mupirocin in the presence of serum. Using this approach, the release in serum was reduced to 22% following 1 h of incubation and 72% release following 24 h of incubation [9]. The optimized high-loaded stable formulation of liposomal mupirocin in nano-liposomes formulation containing HPCD having significantly slower pattern of release in serum (termed Nano-mupirocin) showed superior pharmacokinetic profile than the parenterally administered free mupirocin in several mammal species (mice, rats, rabbits) [10]. In an effort to increase the preclinical data to advance the clinical development of Nano-

mupirocin, the efficacy of parenterally administered Nano-mupirocin for the treatment of *S. aureus* invasive infections was investigated in the present study using *in vivo* murine infection models as well as *in vitro* systems to better understand the underlying mechanism of action (MoA).

S. aureus is a highly prevalent human pathogen that can cause severe and life-threatening invasive infections such as bacteremia, endocarditis, osteomyelitis and device-associated infections in both hospital and community settings [11]. *S. aureus* is also a pathogen of great concern because of the high prevalence of antibiotic-resistant strains such as MRSA, causing infections generally associated with high mortality, morbidity and health care costs [12-14]. Therapeutic options for serious infections caused by MRSA are limited and vancomycin remains the standard empirical treatment for these infections [15]. Because the penetration of vancomycin in some tissues is rather poor [16, 17], recommendations have been made in recent years to increase the dose of vancomycin administration (between 15 mg/kg and 20 mg/kg) for the treatment of serious staphylococcal infections such as bacteremia, endocarditis, osteomyelitis and pneumonia [18, 19]. However, an association between these vancomycin concentrations and risk of nephrotoxicity has been reported by several studies [20-22]. The efficacy of vancomycin for treatment of MRSA infections is further threatened by emerging vancomycin-intermediate and vancomycin-resistant MRSA strains [23]. Therefore, alternative treatment options for severe *S. aureus* infections, in particular for those caused by MRSA strains, are urgently required. In this study, we demonstrated the therapeutic efficacy of Nano-mupirocin in reducing bacterial loads in infected organs as well as for improving killing of intracellular *S. aureus* in phagocytic cells. Nano-mupirocin therefore holds promise for the treatment of invasive MRSA infections.

2. MATERIALS AND METHODS

2.1. Nanoliposomes preparation

Nanoliposomes were prepared as previously described [24]. Briefly, hydrogenated soy phosphatidylcholine (HSPC), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (mPEG DSPE) (Lipoid GmbH) were mixed at a ratio of a 3:1:1, respectively. Liposomes were mechanically hydrated by

stirring at 65°C with 200 mM calcium acetate pH 5.5 containing 15% (w/w) hydroxy-propyl beta cyclodextrin (HPCD) (Roquette Frères). The liposomal dispersions were downsized by stepwise extrusion by the Northern Lipids extruder (Burnaby) using polycarbonate filter membranes and dialyzed against a 10% sucrose solution. Remote loading was performed by incubating the liposome dispersion with a solution of mupirocin (Teva Pharmaceutical Works) in 200 mM phosphate buffer, pH 6.3, for 10 min at 65°C. Nanoliposomes size was in the range of 74-85 nm and PdI< 0.05. Nanoliposomal mupirocin concentration was in the range of 5.0-5.7 mg/ml. The intraliposomal cargo components are summarized in table 1. Calcium content was determined by Inductively coupled plasma optical emission spectrometry (ICP-OES) using PlasmaQuant® PQ9000 Elite (Analytik Jena). Intraliposomal volume (5.94%) was calculated based on the calcium concentrations and then used to determine the intraliposomal concentrations of mupirocin and HPCD.

The pH of the drug-product dispersion was 6.3. The intra-liposome pH of the drug-product was 7.7 after mupirocin loading and 8.4 before drug remote loading. Intra-liposome pH determination was performed using a method adapted from that described for liposomes exhibiting trans-membrane ammonium gradient used for remote loading of amphipathic weak bases in which pH determination is based on the distribution of radioactive methylamine between liposomes and medium [25]. In our study, the intra-liposome pH of liposomes exhibiting trans membrane acetate gradient used for the loading of amphipathic weak acids was determined according to the distribution of radioactive benzoic acid between liposomes and medium.

Table 1. Intraliposomal cargo components

	Concentration (mg/ml)	Concentration (mM)
Mupirocin	84-96	168-192
HPCD	150	108
Calcium ions	7.7	192

The fluorescent lissamine-rhodamine B-labeled Nano-mupirocin liposomes (LRB-nanoliposomes) were prepared by incubating loaded nanoliposomes with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (LRB-PE) (Avanti polar Lipids) dissolved in ethanol at a final molar ratio of 0.2% for 5 min at 37°C and then overnight at 4°C. Note that LRB-PE is labeled in the polar head group of the phospholipid and therefore is retained within the nanoliposomes.

2.2. Bacteria

The *S. aureus* strains 6850 [26], the GFP-expressing SH1000 [27] and the MRSA strains USA300 [28] and UNT141-3 (strain collection UNT Health Science Centre, Texas, USA) were used in this study. Bacteria were grown to Mid-Log phase in brain heart infusion medium (BHI, Roth) at 37°C with shaking (120 rpm), collected by centrifugation, washed with sterile PBS, and diluted to the required concentration. The number of viable bacteria was determined by tenfold serial dilution and colony count by plating on blood agar.

2.3. MIC and MBC determination

The minimal inhibitory concentration (MIC) of Nano-mupirocin and of free mupirocin for the different *S. aureus* strain was determined using the broth dilution method [29], ranging in concentrations from 0.15625 µg/ml to 10 µg/ml. MIC was defined as the lowest concentration of Nano-mupirocin or of free mupirocin that prevented growth determined by optical density at 600nm after 24 h incubation at 37°C.

The minimum bactericidal concentration (MBC) was determined by adding a *S. aureus* bacterial suspension (1-5 x 10^6/ml) to a 96-well microtiter plate containing serial dilutions of f Nano-mupirocin or of free mupirocin. Viable bacteria were determined after 24 h incubation at 37°C by plating. The MBC was defined as the lowest concentration that induced approximately 4 $\log_{10}$ reduction of the original inoculum.

2.4. Bactericidal assay in the presence of plasma

S. aureus bacteria (5 x 10^6/ml) were incubated with serial dilutions of mouse plasma (1:1, 1:2, 1:5) in the presence of Nano-mupirocin or of free mupirocin at a concentration of either 50 µg/ml or 5 µg/ml for a period of 4 h. Bacteria incubated with blank liposomes were used as control. The number of viable *S. aureus* was determined by plating serial dilutions on blood agar plates.

In some experiments, *S. aureus* bacteria were collected by centrifugation after 4 h incubation in the above-mentioned conditions, disrupted and the intracellular concentration of mupirocin was determined by HPLC.

2.5. Mice and infection models

A previously described infection model of metastatic bloodstream infection was used in this study [30]. In this model, specific-pathogen-free 9-10 weeks old C57BL/6 female mice of approximately 20 g of bodyweight (Envigo) were inoculated with 10^6 CFU of *S. aureus* strain 6850 in 100 µl of PBS via a lateral tail vein. For therapeutic treatment, mice were randomly distributed into 3 groups at day 3 of infection and treated with 50 mg/kg of either Nano-mupirocin or free mupirocin or with empty nanoliposomes (blank liposomes). Treatment was administered intravenously at day 3 and intraperitoneally at days 4, 5, 6 and 7 of infection. Mice were sacrificed at day 8 of infection by CO_2 asphyxiation and bacteria were enumerated in liver, kidneys and tibia by preparing homogenates in PBS and plating tenfold serial dilutions on blood agar. Animal studies were performed in strict accordance with the German animal welfare regulations and the recommendations of the Society for Laboratory Animal Science (GV-Solas). All experiments were approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany (Permit N. 33.19-42502-04-15/2025).

For the neutropenic lung infection model, female CD-1 (ICR) mice, 5-6 weeks old (app. 20 g body weight) were rendered neutropenic by intraperitoneal injection of cyclophosphamide at 150 and 100 mg/kg at days -4 and -1 prior to infection. Mice were anesthetized with ketamine (40 mg/kg) and xylazine (6 mg/kg) in 0.15 ml PBS injected

intraperitoneally and inoculated intranasally with 0.05 ml of a suspension containing app. 5 x 10^7 CFU of *S. aureus* strain UNT141-3 (MRSA). At 2 h post-inoculation, groups of mice (n=5) were treated intravenously with either Nano-mupirocin (50 mg/kg or 75 mg/kg), free mupirocin (50 mg/kg or 75 mg/kg), or blank nanoliposomes. Mice treated with vancomycin 100 mg/kg administered subcutaneously at 2 h post-inoculation (n=5) or 50 mg/kg administered intravenously at 2h and 14h post-infection (n=5) were used as control. Mice were euthanized at 24 h of infection by CO_2 inhalation, and bacteria were enumerated in lungs by preparing homogenates in PBS and plating tenfold serial dilutions on Mannitol Salts Agar (MSA) and Brain-heart infusion (BHI) agar with 0.5% activated charcoal. These studies were approved by the Institutional Animal Care and Use Committee (IACUC) (Permit N. IACUC-2016-0042).

2.6. Determination of mupirocin concentration in organ homogenates and in bacteria by HPLC

The organ homogenates that were used for bacteria counts were also used for the determination of mupirocin concentration. The organ homogenates were diluted five-fold with acetonitrile and, after vigorous vortex and centrifugation, the upper phase was dried under vacuum (Eppendorf Concentrator 5301) and reconstituted with methanol. Recovery of mupirocin from the homogenates was determined by HPLC equipped with a UV detector (YL9100, YL Instruments, South Korea) and the concentration calculated by spiking homogenates that received blank liposomes with mupirocin solution. The concentrations obtained in the homogenates were normalized to the tissue weight.

To determine mupirocin concentration within bacterial cells, bacteria were pelleted by centrifugation, washed with PBS, diluted five-fold with acetonitrile and treated as described above for the tissue homogenates. The isocratic elution program was used and the mobile phase was 50 mM sodium phosphate monobasic (adjusted to pH 6.3): acetonitrile (75:25 % v/v) at the flow rate of 1.0 ml/min. The UV detection was performed at a wavelength of 229

nm and the injection volume was 20 µl. The column used was a Luna C18 column, 5 µm, 4.6 mm × 150 mm (Phenomenex, Torrance, CA, USA).

2.7. IL-6 determination

The concentration of IL-6 in serum was determined by ELISA according to the manufacturer's recommendations (BD Biosciences).

2.8. *In vivo* infection of peritoneal macrophages

For flow cytometry analysis, C57BL/6 mice were injected intraperitoneally with 100 µl of a suspension containing 10^7 CFU of GFP-expressing *S. aureus* strain SH1000 and 100 µl of either blank or LRB-labeled Nano-mupirocin. Mice were sacrificed by CO_2 asphyxiation 2 h after injection and subjected to peritoneal lavage by filling the peritoneal cavity with 5 ml of cold RPMI 1640 tissue culture medium (BioWhittaker). The medium was retrieved from the peritoneal cavity, centrifuged at 650 x *g* for 5 min, resuspended in RPMI medium, stained with APC-labeled antibodies against the macrophage marker F4/80 or against the neutrophil marker Gr-1 (Biolegend) and analyzed by flow cytometry using a BD LSR II running FACSDiva (BD Biosciences) and with FlowJo software.

For determination of bacterial killing by peritoneal macrophages, mice were injected intraperitoneally with 100 µl a suspension containing 10^7 CFU of *S. aureus* strain SH1000 and 100 µl of either Nano-mupirocin (50 mg/kg) or blank nanoliposomes. Mice were sacrificed by CO_2 asphyxiation 2 h after injection and subjected to peritoneal lavage. Peritoneal cells were collected by centrifugation, treated with lysostaphin (5 µg/ml) for 5 min to eliminate non-ingested extracellular staphylococci, seeded into tissue culture microtiter plates and incubated for 3 h at 37°C, 5% CO_2. Non-adherent cells were then removed by washing. Adherent macrophages were lysed with ddH_2O containing 0,1% Tween 20 (Sigma) and plated on blood agar to determine the number of viable bacteria.

2.9. *In vitro* infection of immortalized macrophages and immunofluorescence microscopy

For determination of bacterial killing by macrophages *in vitro*, immortalized murine macrophages derived from C57BL/6 mice were infected with *S. aureus* strain SH1000 at a multiplicity of infection (MOI) of 10:1 for 2 h, treated with lysostaphin (5 µg/ml, Sigma) for 5 min to eliminate non-ingested bacteria, washed twice with sterile PBS and further incubated for 2 h 37°C 5% CO_2 in medium containing 5 µg/ml of either Nano-mupirocin or free mupirocin or blank liposomes. Macrophages were then lysed with ddH_2O containing 0.1% Tween 20 (Sigma) and the amount of viable intracellular bacteria was then calculated by platting serial dilutions on blood agar plates.

For immunofluorescence microscopy, immortalized murine macrophages were seeded on glass coverslips and incubated in medium containing LRB-labeled Nano-mupirocin in the presence or absence of 5 µg/ml of cytochalaysin D (Sigma) for 2 h at 37°C, 5% CO_2. Macrophages were then washed, fixed with 4% paraformaldehyde PBS for 15 min at room temperature and coverslips were mounted on glass slides with Moviol.

2.10. Live-cell imaging

For live-cell imaging, 2.5 x 10^5 macrophages were seeded on µ-Slide with 8 wells culture plates (ibidi) and incubated in RPMI complete media at 37°C, 5% CO_2. Macrophages were infected with GFP-*S. aureus* at a MOI of 10 bacteria per cell and treated with LRB-Nano-mupirocin. Imaging was performed with a confocal laser scanning microscope (SP5, Leica Microsystems) equipped with a 63X/1.4 NA PL APO oil objective and under controlled environmental parameters: 37°C, 5% CO_2 and ~65% humidity. Image stacks with 1 µm slice distance were acquired at various positions within the wells using a three-minute interval (total 4-5 hours). A multi-argon laser (laser line 488 nm) and a 561-nm diode-pumped solid-state (DPSS) laser were used to excite GFP and rhodamine while (emission was collected with photomultiplier detectors and/or hybrid detectors (HyDs) between 500-550 nm (GFP) and 580-650 nm (rhodamine) at a scanning resolution of 1,024 × 1,024 pixels, a scanning frequency of 200 Hz and a line averaging of 2.

2.11. Statistical analysis

Data was analyzed using GraphPad Prism 7.0 (GraphPad software). Results are presented as the mean ± SD. Comparison between groups was performed by the use of one-way ANOVA test. *P* values < 0.05 were considered as significant.

3. RESULTS

3.1. Antimicrobial effect of Nano-mupirocin against *S. aureus*

The MIC and MBC of Nano-mupirocin against methicillin-susceptible *S. aureus* strain 6850 (MSSA) and methicillin-resistant *S. aureus* strain USA300 (MRSA) was determined and compared to those of the free mupirocin. The MIC of Nano-mupirocin for both strains of *S. aureus* was similar to that of free mupirocin (>0.15 µg/ml) (Supplementary Table S1). The MBC of Nano-mupirocin against MSSA and MRSA was also similar to the MBC of free

mupirocin for both MSSA and MRSA (2.5 µg/ml) (Supplementary Table S1). These results indicate that mupirocin loaded into nanoliposomes is bioavailable and exhibits a bacteriostatic and bactericidal effect comparable to that of free mupirocin.

To demonstrate that the bactericidal effect of Nano-mupirocin against *S. aureus* was not due to a spontaneous release of the antibiotic, we compared the bactericidal effect of Nano-mupirocin against *S. aureus* with that of the free mupirocin in the presence plasma. If mupirocin is prematurely released from the nanoliposome formulation, the extent of the antimicrobial activity will be similar to that exhibited by the free drug. The results show that Nano-mupirocin was significantly more efficient than free mupirocin at killing *S. aureus* at 1:1 and 1:2 plasma dilutions at a concentration of 50 µg/ml (Fig. 1A) as well as at concentration of 5 µg /ml (Fig. 1B). No differences in antimicrobial activity were observed between Nano-mupirocin and the free antibiotic at a higher plasma dilution (1:5) (Fig. 1A and 1B). Furthermore, the concentration of mupirocin within bacterial cells was greater after incubation with Nano-mupirocin than after incubation with free mupirocin in the presence of plasma (Fig. 1C). Taken together, these results indicate that nanoliposomes protect mupirocin from plasma inactivation resulting in prolonged half-life of active antibiotic. The results also suggest that mupirocin may be released from nanoliposomes in a controlled manner in order to be able to penetrate into the bacterial cell and exert its bactericidal effect. The mechanistic details underlying this process remains to be elucidated.

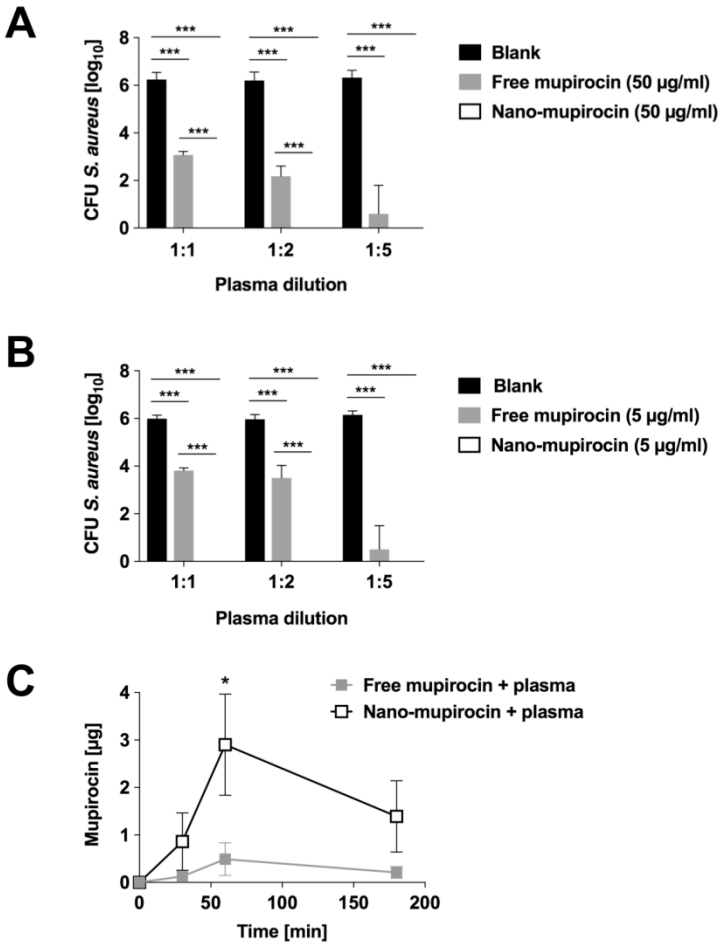

Fig.1. Enhanced killing of *S. aureus* by Nano-mupirocin respect to the free antibiotic in the presence of plasma. (A) Numbers of viable bacteria recovered after 4 h of incubation in different plasma concentrations and in the presence of either blank liposomes (black bars), free mupirocin (50 µg/ml) (grey bars) or Nano-mupirocin (50 µg/ml) (white bars). (B) Numbers of viable bacteria recovered after 4 h of incubation in different plasma concentrations and in the presence of either blank liposomes (black bars), free mupirocin (5 µg/ml) (grey bars) or Nano-mupirocin (5 µg/ml) (white bars). Each bar represents the mean ± SD of three experiments. (C) Time-dependent accumulation of mupirocin within *S. aureus* bacterial cells incubated with either Nano-mupirocin (white symbols) or free mupirocin (grey symbols). Each symbol represents the mean±SD of three experiments. *, $p < 0.05$; ***, $p < 0.001$.

3.2. Superior efficacy of Nano-mupirocin respect to free mupirocin after parenteral administration in a murine model of *S. aureus* bloodstream infection

A previously described murine model of metastatic bloodstream infection that reflects many clinical features of the disease in humans [30] was used to evaluate the efficacy of parenterally administered Nano-mupirocin for the treatment of invasive *S. aureus* infection. Mice were intravenously infected with *S. aureus* strain 6850 and treated with 50 mg/kg of

either Nano-mupirocin or free mupirocin for 5 days, starting at day 3 of infection and following the administration regime depicted in Fig. 2A. The antibiotic concentration and treatment scheme was selected based on pharmacokinetic data published in a previous report [24]. The concentration of mupirocin as well as the bacterial burdens were determined in the kidneys and tibia of infected mice at day 8 of infection. Results displayed in Fig. 2B show that mupirocin was only detectable by HPLC in the infected organs when the antibiotic was administered with the nanoliposome formulation but not when administered as free drug. Accordingly, treatment with Nano-mupirocin was significantly more effective at reducing the bacterial loads in kidneys (Fig. 2C) and tibia (Fig. 2D) than free mupirocin. Furthermore, signs of morbidity like body weight loss (Fig. 2E) and systemic inflammation shown by the serum levels of IL-6 (Fig. 2F) were also significantly lower in mice treated with Nano-mupirocin than in those treated with blank liposomes or free mupirocin. Thus, parenterally administered Nano-mupirocin resulted in a more effective delivery of active mupirocin to the sites of infections, superior bacterial killing and more efficient control of infection than parenterally administered free mupirocin.

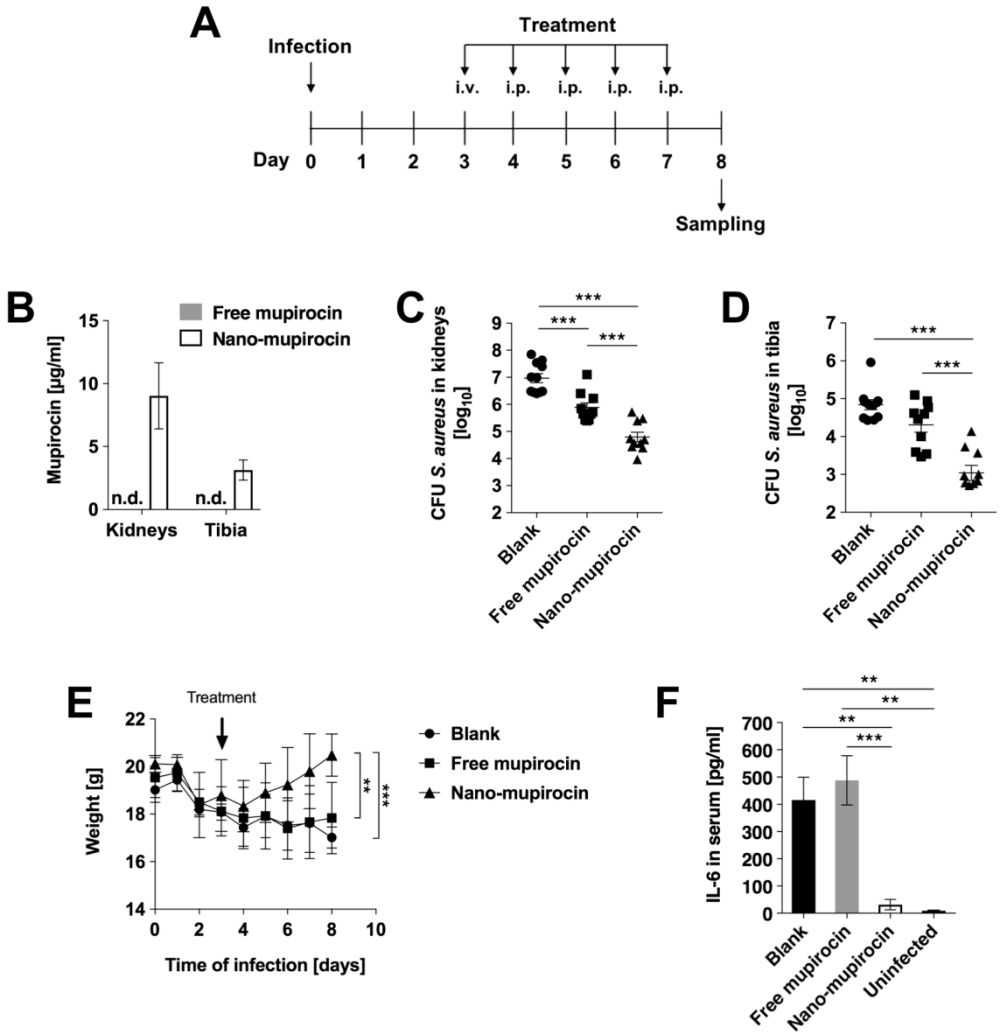

Fig.2. Superior therapeutic efficacy Nano-mupirocin over the free drug after parenteral administration in a murine model of *S. aureus* bloodstream infection. (A) Schematic illustration of therapeutic regimen. C57BL/6 mice were infected intravenously with 10^6 CFU of *S. aureus* strain 6850 and treated with either Nano-mupirocin (50 mg/kg), free mupirocin (50 mg/kg) or empty nanoliposomes (blank liposomes) intravenously at day 3 and intraperitoneally at day 4, 5, 6 and 7 of infection. Mice were sacrificed at day 8 of infection and mupirocin concentration and bacterial loads were determined in kidneys and tibia. (B) Mupirocin concentration in kidneys and tibia of *S. aureus*-infected mice treated with Nano-mupirocin (white bars) Nano-mupirocin (white bars) or with free mupirocin (grey bars) at day 8 of infection. Each bar represents the mean±SD of values pooled from three independent experiments. Bacterial loads in kidneys (C) and tibia (D) of mice treated with either Nano-mupirocin (triangles), free mupirocin (squares) or blank liposomes (circles) at day 8 of infection. Each symbol represents the value for an individual animal (n=10). Data were pooled from three experiments performed independently. Horizontal lines indicate the mean±SD. (E) Changes in body weight with the progression of infection in the different treatment groups. Each symbol represents the mean±SD value of n=5. One representative experiment out of three is shown. (F) Serum concentrations of IL-6 in uninfected or *S. aureus*-infected mice treated with either Nano-mupirocin, free mupirocin or blank liposomes at day 8 of infection. Each bar represents the mean±SD of values pooled from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

3.3. Superior efficacy of Nano-mupirocin in respect to free mupirocin after parenteral administration in a neutropenic murine lung infection model

A neutropenic mouse lung infection model was then used to study the intrinsic superior antimicrobial effect of Nano-mupirocin over free mupirocin without the supplementary antimicrobial activity provided by the innate immune response. Mice were rendered neutropenic by intraperitoneal injections of cyclophosphamide, intranasally inoculated with MRSA and treated with either 50 mg/kg or 75 mg/kg of Nano-mupirocin or free mupirocin according to the scheme depicted in Fig. 3A. Control mice received blank nanoliposomes. Infected mice treated with vancomycin were used for comparison. Treatment with both doses of Nano-mupirocin resulted in significant reduction of MRSA in the lungs of infected mice in respect to blank liposomes treatment and to an extent similar or even greater than that induced by treatment with vancomycin (Fig. 3B). Compared to free mupirocin, administration of Nano-mupirocin resulted in mean bacterial lung titers that were 1.08 - 2.28 $\log_{10}$ CFU lower than the corresponding free mupirocin dose group (Fig. 3B). However, only the results for the 75 mg/kg dose group was determined to be statistically significant.

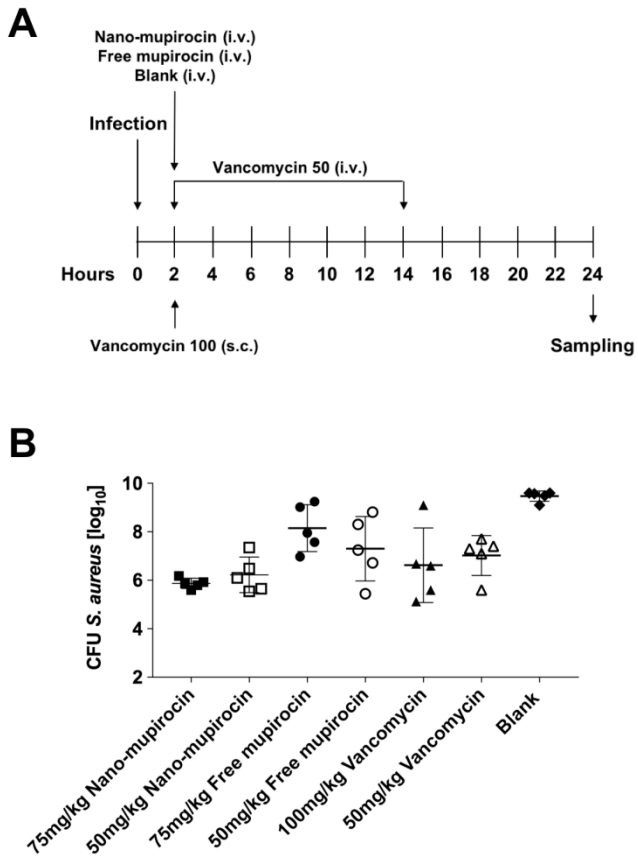

Fig.3. Therapeutic effect of parenterally administered Nano-mupirocin or free mupirocin against MRSA in a neutropenic murine model of lung infection. (A) Schematic illustration of therapeutic regimen in neutropenic mice. CD1 mice were rendered neutropenic and infected intranasally with app. 5 x 10^7 CFU of MRSA strain UNT141-3 and treated with the indicated dose of either Nano-mupirocin, free mupirocin, vancomycin or with blank nanoliposomes. Mice were sacrificed at 24 h of infection and bacteria enumerated in the lungs. (B) Bacterial loads in the lungs of the different groups of mice treated according to scheme depicted in (A) at 24 h of infection. Each symbol represents the value for an individual animal (n=5). Horizontal lines indicate the mean ± SD. Statistical analysis is summarized in Supplementary Table S2.

3.4. Nano-mupirocin liposomes are taken up by phagocytic cells *in vivo* resulting in enhanced intracellular bacterial killing

Although *S. aureus* is considered an extracellular pathogen, it can also invade and survive within a variety of host cells [31, 32], where they establish an infection reservoir. *S. aureus* can also survive the intracellular killing mechanisms of phagocytic cells and use them as a trojan horse for systemic dissemination [33, 34]. Therefore, successful elimination of

intracellular bacteria is a key event for the effective management of *S. aureus* infections. Because standard antibiotics generally fail to accumulate in inhibitory concentrations or are poorly retained inside of host cells, we investigated the capacity of Nano-mupirocin to internalize and deliver antibiotic within phagocytic cells *in vivo* and the consequences for intracellular bacterial viability. For this purpose, GFP-expressing *S. aureus* (green fluorescence) and LRB-labeled Nano-mupirocin (red fluorescence) were intraperitoneally injected into mice and peritoneal cells were harvested by peritoneal lavage 2 h thereafter. Peritoneal cells were stained with APC-labeled antibodies against the granulocyte marker Gr-1 or against the macrophage marker F4/80 and analyzed by flow cytometry. Contour plots depicted in Fig. 4A indicate that approximately 50% of granulocytes were associated with *S. aureus*. Interestingly, analysis of the red fluorescence intensity in the granulocyte populations either harboring or devoid of bacteria indicated that Nano-mupirocin seems to accumulate predominantly in the granulocyte population that harbored *S. aureus* (MFI=510±29 in granulocytes harboring GFP-*S. aureus* and MFI=82±6.7 in granulocytes devoid of bacteria) (Fig. 4A). Within the macrophage population, almost 100% of these cells harbored *S. aureus* and where also associated with high levels of Nano-mupirocin (MFI=869±58) (Fig. 4B).

We next determined the capacity of Nano-mupirocin to target and kill intracellular *S. aureus in vivo*. To this end, mice were infected intraperitoneally with *S. aureus* and with either blank nanoliposomes or Nano-mupirocin, peritoneal macrophages were collected after 2 h by peritoneal lavage and further incubated *in vitro* for 3 h. The amount of intracellularly viable bacteria was then enumerated after macrophage cell lysis. As shown in Fig. 4C, the number of viable bacteria was significantly lower in macrophages from mice treated with Nano-mupirocin than in those treated with blank liposomes. These results highlight the remarkable capacity of Nano-mupirocin to deliver bactericidal concentrations of the cargo antibiotic within the intracellular milieu.

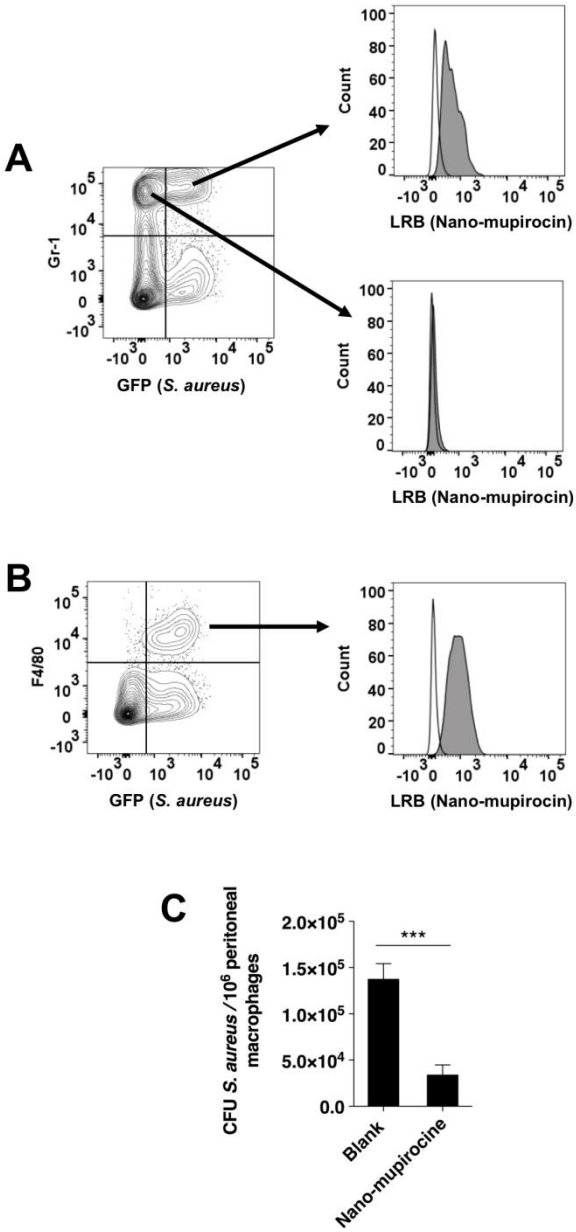

Fig. 4. Uptake of *S. aureus* bacteria and Nano-mupirocin by phagocytic cells *in vivo*. Mice were intraperitoneally injected with GFP-expressing *S. aureus* (green fluorescence) and LRB-nanoliposomes (red fluorescence), peritoneal cells were harvested from the peritoneal cavity of mice 2 h after injection and stained with APC-labeled anti-Gr-1 antibodies to identify granulocytes (A) and with APC-labeled anti-F4/80 antibodies to identify macrophages (B). Contour plots in left panels show the gating of the cell populations harboring (upper right quadrant) or devoid (upper left quadrant) of GFP-*S. aureus*. Histograms on the right panels show the intensity of red fluorescence (LRB-labeled Nano-mupirocin) in each of these populations (solid histograms). Control histogram (open histograms) show background red fluorescence levels in untreated cells. (C) Viable bacterial numbers recovered from *S. aureus*-infected peritoneal macrophages isolated from infected mice treated with either blank liposomes or Nano-mupirocin. Each bar represents the mean ± SD of values pooled from three experiments performed independently (n = 10). ***, $p < 0.001$.

3.5. Visualization of Nano-mupirocin uptake by macrophages in real time using live-cell imaging

To monitor the dynamic interplay between macrophages, Nano-mupirocin and *S. aureus* in more detail, we performed live-cell imaging of murine macrophages cultured *in vitro* and challenged with GFP-*S. aureus* and LRB-labeled Nano-mupirocin. Selected snapshots of the acquired time series depicted in Fig. 5 and the full video sequence (Supplementary video1) demonstrate the remarkable capacity of macrophages to uptake both *S. aureus* (green) and Nano-mupirocin (red).

Because the *in vivo* experiments described in the previous section indicated that Nano-mupirocin preferentially accumulate in phagocytic cells that harbor *S. aureus*, we hypothesized that, in a similar way to *S. aureus,* Nano-mupirocin may be taken up by these cells also via the phagocytic pathway. To test this hypothesis, we incubated murine macrophages *in vitro* with LRB-labeled Nano-mupirocin and determined the extent of nanoliposome internalization in the presence and absence of the phagocytosis inhibitor cytochalasin D. Immunofluorescence photograph depicted in Fig. 5B showed a much lower level of Nano-mupirocin internalized within macrophages following treatment with cytochalasin D. These observations indicate that the uptake of Nano-mupirocin by macrophages was mediated by a phagocytic mechanism.

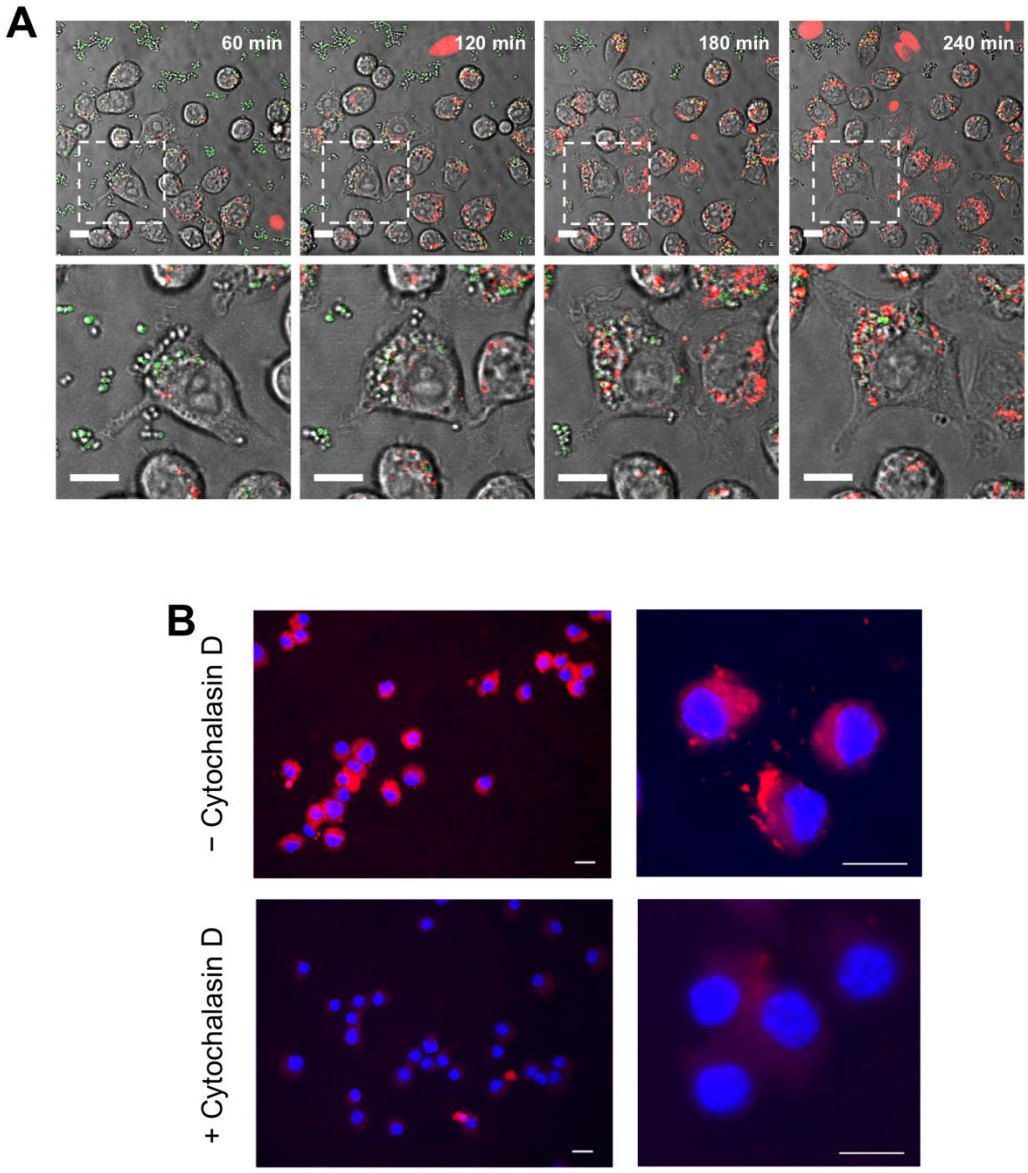

Fig. 5. Live-cell imaging showing the uptake of *S. aureus* and Nano-mupirocin by macrophages *in vitro.* (A) Representative snapshots of macrophages infected with GFP-*S. aureus* (green fluorescence) in the presence of LRB-nanoliposomes (red fluorescence). Macrophages were imaged for approx. 4 h. The time point of the snapshots are indicated in the pictures. Insets show magnifications of one enclosed cell highlighting the accumulation of both bacteria and Nano-mupirocin. Bars represent 10 µm. (B) Immunofluorescence microscopy photographs illustrating the uptake of LRB-labeled Nano-mupirocin by macrophages in the absence (upper panels) or absence (lower panels) of the phagocytosis inhibitor cytochalasin D. Lower magnification photographs are shown in the left panels with bars representing 10 µm and high magnification in the right panels with bars representing 20 µm.

We also evaluated the efficacy of Nano-mupirocin to enhance killing of intracellular *S. aureus* in *in vitro*-cultured macrophages. Similar to the observations in the *in vivo* system, a significantly greater reduction in the numbers of intracellular *S. aureus* was observed in

macrophages incubated with Nano-mupirocin in comparison to those incubated with blank liposomes (Fig. 6). Importantly, the killing of intracellular *S. aureus* was significantly higher after treatment with Nano-mupirocin than after treatment with free mupirocin (Fig. 6). Overall, these results demonstrate the great capacity of Nano-mupirocin to be taken-up by macrophages, delivery the antibiotic cargo into the intracellular milieu and enhance intracellular bacterial killing.

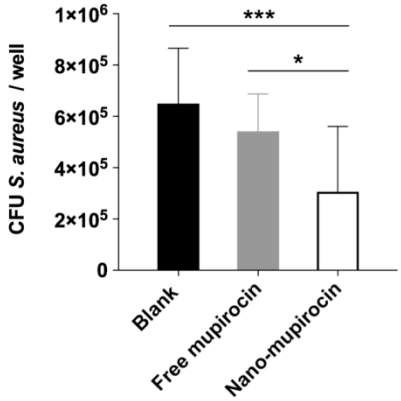

Fig. 6. Enhanced killing of intracellular *S. aureus* by Nano-mupirocin respect to the free antibiotic in an *in vitro* system. Immortalized murine macrophages were infected *in vitro* with *S. aureus* at a multiplicity of infection of 10 bacteria per macrophage for 2 h, treated with lysostaphin to eliminate non-ingested bacteria and further incubated for 2 h in the presence of 5 µg/ml of either Nano-mupirocin (white bar) or free mupirocin (grey bar) or with blank liposomes (black bar). Macrophages were then lysed and the number of viable bacteria determined by plating. Each bar represents the mean ± SD of values pooled from three experiments performed independently (n = 10). ***, $p < 0.001$; *, $p < 0.05$.

4. DISCUSSION

Finding new applications for already approved anti-infective can help to alleviate the problem posed by the lack of development of new antibiotics and the alarming rise in the incidence of antibiotic-resistant bacteria. Mupirocin is an example of a safe drug that, despite its excellent activity against clinical isolates of *S. aureus* including MRSA strains [35], is restricted to topical application because its rapid degradation to an inactive metabolite in the liver and its high plasma binding activity that neutralize drug activity [2, 3]. Formulation of mupirocin in nanoliposomes was shown to overcome these major limitations and enable its administration via the parenteral route [24]. The pharmacokinetic profile of Nano-mupirocin demonstrated orders of magnitude greater exposure and longer half-life than the free drug and was more effective than the free drug in a murine model of *Streptococcus pyogenes* necrotizing fasciitis and in a rabbit model of *S. aureus* endocarditis [24]. In the current study, we demonstrated the suitability of parenterally administered Nano-mupirocin for the treatment of invasive *S. aureus* infections, including MRSA. Encapsulation in nanoliposomes improved mupirocin distribution and guarantied maximal bacterial exposure in the infected organs, resulting in increased therapeutic efficacy when compared to that of the free drug. Our results are of clinical relevance because treatment of invasive *S. aureus* infections can be difficult, in particular of infections caused by MRSA, and usually involves a prolonged antibiotics course. Although vancomycin is the drug of choice for the parenteral treatment of severe MRSA infections, the emergence of staphylococcal strains with reduced susceptibility to this antibiotic [36] highlights the need to expand the clinical arsenal of drugs that can be used to effectively treat these infection and that can help to preserve the activity of reserve antibiotics.

The superior efficacy of Nano-mupirocin over the free drug for the treatment of *S. aureus* invasive infections can be explained, at least in part, by an improved delivery of active mupirocin to the sites of infection and to the enhanced accumulation of mupirocin within the bacterial cell. A major factor responsible for the increased antibiotic delivery may be the barrier effect of nanoliposomes that protects mupirocin from inactivation by plasma

constituents and from rapid metabolic degradation and thus increased the half-life of the circulating antibiotic. Furthermore, Nano-mupirocin is based on a PEGylated nano-liposomal formulation containing hydroxypropyl-β-cyclodextrin (HPCD) in the aqueous phase, which enable the controlled slow release of mupirocin in the presence of serum [9]. In addition, the possibility of a direct interaction between Nano-mupirocin and *S. aureus* bacteria should not be discarded and needs to be addressed in future studies. Besides the protective effect conferred by the nanoliposome formulation, the reported ability of the loaded nanoliposomes to extravasate through blood vessels, taking advantage of the leaky vasculature and reduced lymphatic clearance [37, 38], may also contribute to the improved accumulation or mupirocin into the infected tissue thereby improving its *in vivo* efficacy.

We also demonstrated in this study that delivery of mupirocin as Nano-mupirocin formulation enhanced antibiotic uptake by phagocytic cells. We could show that Nano-mupirocin was very efficiently taken up by professional phagocytic cells via phagocytosis, which is also the main pathway used by phagocytic cells to uptake *S. aureus.* That bacteria and Nano-mupirocin share the same internalization pathway, may explain the observation that Nano-mupirocin is predominantly taken up by those phagocytic cells harboring *S. aureus* and enhanced the capacity of these immune cells to kill the intracellular bacteria. This is an important issue because, although *S. aureus* is considered an extracellular pathogen, it can survive within phagocytic cells and use them as a 'Trojan horse' for disseminating from the site of infection to distant anatomical sites [39]. Furthermore, persistent intracellular *S. aureus* has been associated with chronic or recurrent infections [40]. Therefore, elimination of intracellular *S. aureus* is key for successful treatment. Standard antibiotics generally fail to accumulate in inhibitory concentrations or are poorly retained inside of host cells in the infected tissue. In the current study, we demonstrated that Nano-mupirocin overcome these limitations as it is easily uptake by phagocytic cells, most probably because its particulate structure, and facilitate the delivery of the antibiotic cargo directly into the intracellular compartment where the bacteria is located and, consequently, enhancing intracellular bacterial killing. A similar situation has been observed with liposome-encapsulated

vancomycin. Thus, Pumerantz and colleagues reported that, in contrast to free vancomycin which is unable to accumulate in macrophages at sufficient concentrations to kill intracellular MRSA, liposome-encapsulated vancomycin increased intracellular antibiotic concentration and enhanced bactericidal effect against MRSA [41]. We also provided evidence that internalization of Nano-mupirocin within macrophages favors a phagocytic mechanism, rather than endocytosis or fusion with cell membrane, as demonstrated by the significantly diminished nanoliposomes internalization observed in the presence of the actin polymerization inhibitor cytochalasin D.

5. CONCLUSIONS

This study demonstrated the feasibility of using parenterally administrated Nano-mupirocin for the treatment of invasive *S. aureus* infections. The superior antimicrobial activity of Nano-mupirocin over the free drug was owed not only to the protective effect conferred by the liposomes from metabolic degradation and plasma inactivation but also to the improved delivery of antibiotic to the infected organs and to the intracellular compartment in *S. aureus*-harboring phagocytic cells.

Intranasal application of mupirocin is commonly used for eradication of MRSA carriage in the hospital setting [42, 43] and this decolonization strategy seems to be highly effective at reducing transmission and prevent infection in *S. aureus* carriers [44, 45]. Furthermore, although resistance to mupirocin has been reported in several studies in localized areas, usually in the context of widespread mupirocin use [46, 47], MRSA strains resistant to mupirocin are uncommon indicating that mupirocin is an antibiotic still effective against most MRSA strains. Therefore, resistance will not pose a limitation for the use of Nano-mupirocin to treat MRSA infections. In summary, the results of our study indicate that nanoliposomal formulation of mupirocin changed the pharmaco-dynamic of mupirocin extending its application from a topically-only used antibiotic to an efficacious systemic antibiotic. These data when combined with our “clean” toxicology studies and the long-term

stability of this drug-product (our unpublished data) support the further clinical development of Nano-mupirocin for the treatment of severe MRSA infections.

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Conflict of interest

Ahuva Cern and Yechezkel Barenholz are co-inventors on US patent 10,004,688 entitled Liposomal mupirocin. This patent was licensed to Integra LTD which focus on treatment of gonorrhea infection by intramuscular injection.

Goldmann O, Muesken M, Rohde M, Weiss W and Medina E declare no conflict of interest.

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Medina E: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Supervision, Writing- review & editing.

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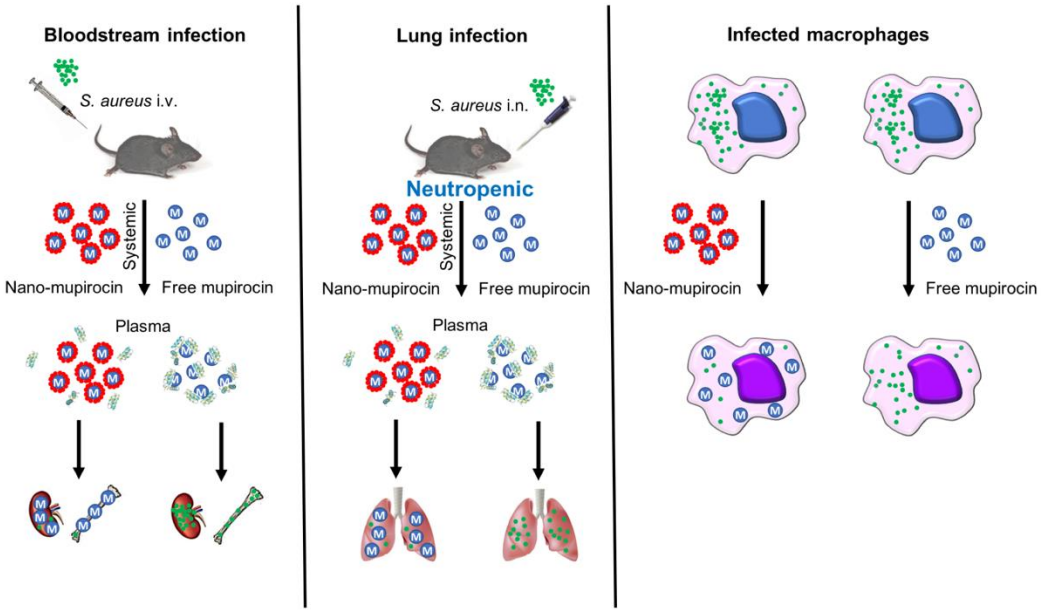
Barenholz Y: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing- review & editing.

Medina E: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Supervision, Writing- review & editing.

Graphical Abstract



"Highlights"

- Mupirocin is limited to topical use due to short half-life and high protein binding
- PEGylated nano-liposomal formulation of mupirocin (Nano-mupirocin) were generated
- Nano-mupirocin effectively kill *S. aureus* MSSA and MRSA in the presence of plasma
- *Nano-mupirocin enhances the intracellular killing* of *S. aureus* by phagocytic cells
- Nano-mupirocin is effective against *S. aureus* after parental administration *in vivo*