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Mechanisms of action and *in vivo* antibacterial efficacy assessment of five novel hybrid peptides derived from Indolicidin and Ranalexin against *Streptococcus pneumoniae*

Hassan Mahmood Jindal¹, Keivan Zandi², Kien Chai Ong³, Rukumani Devi Velaythan¹, Sara Maisha¹, Chandramathi Samudi Raju¹, Shamala Devi Sekaran^{Corresp. 1}

¹ Department of Medical Microbiology, Department of Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

² School of Medicine, Emory University, Atlanta, United States

³ Department of Molecular Medicine Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Corresponding Author: Shamala Devi Sekaran

Email address: shamalamy@yahoo.com

Background. Antimicrobial peptides (AMPs) are of great potential as novel antibiotics for the treatment of broad spectrum of pathogenic microorganisms including resistant bacteria. In this study, the mechanisms of action and the therapeutic efficacy of the hybrid peptides were examined.

Methods. TEM, SEM and ATP efflux assay were used to evaluate the effect of hybrid peptides on the integrity of the pneumococcal cell wall/membrane. DNA retardation assay was assessed to measure the impact of hybrid peptides on the migration of genomic DNA through the agarose gel. *In vitro* synergistic effect was checked using the chequerboard assay. ICR male mice were used to evaluate the *in vivo* toxicity and antibacterial activity of the hybrid peptides in a standalone form and in combination with ceftriaxone.

Results. The results obtained from TEM and SEM indicated that the hybrid peptides caused significant morphological alterations in *Streptococcus pneumoniae* and disrupting the integrity of the cell wall/membrane. The rapid release of ATP from pneumococcal cells after one hour of incubation proposing that the antibacterial action for the hybrid peptides is based on membrane permeabilization and damage. The DNA retardation assay revealed that at 62.5µg/ml all the hybrid peptides were capable of binding and preventing the pneumococcal genomic DNA from migrating through the agarose gel. *In vitro* synergy was observed when pneumococcal cells treated with combinations of hybrid peptides with each other and with conventional drugs erythromycin and ceftriaxone. The *in vivo* therapeutic efficacy results revealed that the hybrid peptide RN7-IN8 at 20mg/kg could improve the survival rate of pneumococcal bacteremia infected mice, as 50% of the infected mice were survived up to 7 days post-infection. *In vivo* antibacterial efficacy of the hybrid peptide RN7-IN8 was significantly improved when combined with the standard antibiotic ceftriaxone at (20mg/kg +20mg/kg) as 100% of the infected mice survived up to seven days post-infection.

Discussion. Our results suggest that attacking and breaching the cell wall/membrane is most probably the principal mechanism for the hybrid peptides. In addition, the hybrid peptides could possess another mechanism of action by inhibiting intracellular functions such as DNA synthesis. AMPs could play a great role in combating antibiotic resistance as they can reduce the therapeutic concentrations of standard drugs.

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Authors: Hassan Mahmood Jindal¹, Keivan Zandi², Ong Kien Chai³, Rukumani Devi Velayuthan¹, Sara Maisha¹, Chandramathi Samudi Raju¹, and Shamala Devi Sekaran^{1*}

Affiliations:

¹ Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.

² Department of Pediatrics, School of Medicine, Emory University, 201 Dowman Dr, Atlanta, GA 30322, USA.

³ Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.

Corresponding Author: *Prof. Dr. Shamala Devi Sekaran

E-mail:shamalamy@yahoo.com

Keywords: Antibiotic-resistance, *Streptococcus pneumoniae*, antimicrobial peptides, hybrid peptides, synergistic effect.

Abstract

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1. Introduction

pneumococcus remains one of the major human pathogens in both children and adult (Jacobs, 2004; Cao et al., 2007). Formerly known as *Diplococcus pneumoniae*, *S. pneumoniae* is Gram-positive, α hemolytic and encapsulated bacteria capable of causing both invasive and non-invasive diseases (Moschioni et al., 2012). To date, more than 93 different *S. pneumoniae* serotypes have been recognized based on the immunochemical differences in their capsules. However, few of these serotypes are linked to more than 80% of pneumococcal disease (Lin et al., 2010; Jauneikaite et al., 2012). Globally, this pathogen is responsible for 1.6 million deaths each year with 0.7 to 1 million in children below five years especially in Asian and African countries (Bravo, 2009). According to a study conducted by O'Brien and his team (the Pneumococcal Global Burden of Disease Study Team), it was stated that 5 out of ten countries with the highest number of deaths in children below five years old caused by pneumococcal infections were in Asia (O'Brien et al., 2009).

Since the last three decades, there has been an enormous increase in the incidence of antibiotic-resistant pneumococci due to the extensive use of inappropriate antimicrobials (Zhou et al., 2012; Cornick & Bentley, 2012). The rise of resistant pneumococcal strains to various antibacterial drugs has been documented internationally in several surveillance studies. In the

United States, a total of 18,911 pneumococcal isolates collected from 1998 to 2011 showed that 18.9% were resistant to amoxicillin/clavulanate, 14.8% were resistant to penicillin and 11.7% to ceftriaxone (Jones et al., 2013). Results obtained from 20 European sentinel health centers as part of the SENTRY Program showed that 7% of pneumococci isolates were penicillin – resistant and 21% were intermediate. Among those strains resistant to penicillin, 35% and 55% of them exhibited resistance to clindamycin and erythromycin, respectively (Fluit et al., 1999). Data collected from multi-country studies indicated that the rates of antibacterial resistance are the highest in Asian countries. Pneumococcal strains collected from China, Vietnam, South Korea, Thailand, Hong Kong and Taiwan have the greatest antibacterial-resistance share among Asian countries (Hung et al., 2013). One of the advantageous alternatives to today's antibiotics is antimicrobial peptides (AMPs) (Deslouches et al., 2013; Sánchez-Vásquez et al., 2013). AMPs synthesized by almost all living beings as the first line of defense in their immune system against microbial infection. Many aspects favor AMPs over traditional antibiotics: a broad range of antimicrobial activity against pathogenic microorganisms (including viruses, parasites, bacteria, and fungi), microbial pathogens are less efficient in developing resistance against AMPs as killing take place in a short contact time and AMPs can act in a synergistic manner with traditional antibiotics (Yeaman & Yount, 2003; Torcato et al., 2013; Xi et al., 2013).

It is well known that AMPs principally act by disrupting bacterial membrane integrity. AMPs' membrane destructive properties come from their cationic and hydrophobic composition, which give them the ability to target the negatively charged bacterial membranes and subsequently impact their integrity (Yeaman & Yount, 2003; Wimley, 2010). Besides their capacity to permeabilize bacterial membrane, AMPs have also been acknowledged to achieve their antimicrobial activities by inhibiting the synthesis of intracellular components such as DNA and

protein (Sahl et al., 2005; Straus & Hancock, 2006). In our previous study, we designed 13 novel antimicrobial peptides based on two naturally occurring templates indolicidin and ranalexin (Jindal et al., 2015). Of which, five hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7 and RN7-IN6) presented the most potent antimicrobial activity (7.81-15.62 µg/ml) against 30 pneumococcal clinical isolates. Also, none of the hybrid peptides revealed any cytotoxic effects against human cells at their MIC levels (Jindal et al., 2015). In the present study, we report the mechanisms of action, the *in vitro* synergism effect and the *in vivo* therapeutic efficacy of the hybrid peptides against *Streptococcus pneumoniae*.

2. Materials and Methods

Bacterial culture and assay medium

S. pneumoniae clinical strains used in this study were obtained from University of Malaya Medical Centre (UMMC). Columbia agar with 5% sheep blood was used to culture the bacteria. mueller-Hinton broth (MHB) was used for synergism assay and prepared as described in the guidelines of Clinical and Laboratory Standard Institute (Clinical and Laboratory Standards Institute, 2012).

Transmission electron microscopy (TEM)

Bacteria were prepared for TEM according to the guidelines of the Electron Microscopy Unit at the Faculty of Medicine, University of Malaya. *S. pneumoniae* cultures were grown overnight on Columbia agar with 5% sheep blood and suspended in CAMHB at 10⁸ CFU/ml. Pneumococcal suspensions were incubated with hybrid peptides at 8 × 10⁷ CFU/ml for one hour at 37°C under 5% CO₂. Cells in CAMHB were used as an untreated control. After one hour of incubation,

the pneumococcal cells were washed three times with 10 mM phosphate buffer saline at pH 7.3 and fixed overnight in 4% (v/v) glutaraldehyde. All samples were washed for two times with cacodylate buffer, incubated for 2hr in osmium tetroxide buffer (OsO₄ 1: 1 cacodylate), washed two times with cacodylate buffer and then overnight incubated in cacodylate buffer. All The samples were washed with distilled water for two times and incubated for 10 min with uranyl acetate. After incubation with uranyl, all samples were then washed twice with distilled water and dehydrated in an ascending series of ethanol: 35% (10 min), 50% (10 min), 70% (10 min), 95% (15 min), and three times in 100% ethanol (15 min). After dehydration, Samples were incubated with propylene oxide (15 min), propylene oxide 151 Epon (1hr), propylene oxide 153 Epon (2hr) and finally overnight incubated with Epon. All the samples were embedded in agar 100 resin at 37°C for 5hr and maintained at 60°C until viewing. Reichert Ultramicrotome copper grids 3.05 mm (300 square mesh) (Agar Scientific) were used to prepare Ultrathin sections. Ethanol-based uranyl acetate and lead citrate were used to stain the samples for 5 minutes. Transmission electron microscope (Leo Libra 120) was used to capture the images.

Scanning electron microscopy (SEM)

S. pneumoniae with a starting inoculum of 1×10^8 cells/mL in CAMHB medium was exposed to $8 \times$ MIC of respective peptides and incubated for 1hr at 37°C for under 5% CO₂. After incubation, 20 μ L of the untreated and treated cell suspensions were transferred onto the membrane filters and processed as described by the standard guidelines provided by Electron Microscopy Unit, Faculty of Medicine, University of Malaya. Briefly, the bacterial samples were overnight fixed with 4% glutaraldehyde at 4°C and then washed twice with sodium cacodylate buffer for 10 minutes each. In the second fixation, 1% osmium tetroxide was used to fix the samples for 1 hour

at 4°C and then washed two times with distilled water for 10 minutes each. All the samples were then dehydrated through a serially graded ethanol (30%, 50%, 70%, 80%, 90%, 95% and twice in 100%) for 15 minutes each, followed by dehydration in ethanol:acetone mixtures (3:1, 1:1 and 1:3) for 15 minutes each and three rounds of pure acetone for 20 minutes each. The samples were then dried for 1 hour and kept in a desiccator before the examination. The samples were then mounted on stubs, coated with gold in sputter coater and viewed under the FEI-Quanta 650 Scanning Electron Microscope.

ATP efflux assay

The amount of ATP released from pneumococcal cells incubated with hybrid peptides was measured as described previously with slight modification (Tanida et al., 2006) using an ATP determination kit (Molecular Probes, USA) based on the luciferin/luciferase method according to the manufacturer's instructions. Briefly, pneumococcal cells (1×10^7 CFU/mL) were incubated with hybrid peptides at $8 \times$ MIC for 1, 2 and 3hr in Eppendorf tubes. The samples were then centrifuged at 5000 rpm for 5 min, and ATP efflux was subsequently estimated using an ATP standard curve. Values were obtained from three independent experiments. Ceftriaxone and erythromycin were used as positive controls.

Gel retardation assay

This assay was carried out as described previously with minor modifications (Li et al., 2013). Genomic DNA was isolated from pneumococcal cells using DNeasy Blood & Tissue Kit (Qiagen). The optical density ratio of 260 and 280nm ($OD_{260}/OD_{280} = 1.83$) was used to measure the purity of the DNA. Genomic DNA (250 ng) was incubated with hybrid peptides at various

concentrations in 12µl at room temperature for 10 min. 2µl of loading buffer were added to the mixture and the migration of DNA through 1% agarose gel was evaluated by electrophoresis in 1× Tris borate–EDTA buffer (45mM Tris–borate and 1mM EDTA at pH8.0) and spotted by the fluorescence of gel stain (Gel Red, BIOTIUM).

Synergistic effect

Pneumococcal cells were overnight cultured using Columbia agar with 5% sheep blood at 37 °C under 5% CO₂ and then resuspended in cation-supplemented Mueller-Hinton broth and adjusted to 5×10⁵ CFU/ml following CLSI guidelines. Combinations of hybrid AMPs with each other and with standard drugs (ceftriaxone and erythromycin) were assessed for their synergistic effects by the chequerboard titration method described previously with minor modification (Bajaksouzian et al., 1996). Briefly, each column of the plate contained 50µl of two-fold serial dilutions of drug B starting at 4×MIC and 50µl of a fixed 0.25×MIC of drug A. 100µl of bacterial suspension (5×10⁵ CFU/ml) were then added to each well and the plates were incubated for 24hr at 37 °C under 5% CO₂. The fractional inhibitory concentration (FIC) index of each combination was calculated according to the following formula: FIC index = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). MIC A in combination and MIC B in combination represent the MICs of drug A and B tested in combination. MIC A alone and MIC B alone represent the MICs of drug A and B in standalone. FIC index values were interpreted as follows: Two drugs have synergy if $FIC \leq 0.5$, additive or indifference if $0.5 < FIC \leq 4.0$ and antagonism if $FIC > 4.0$.

***In vivo* assessment**

Mice and environmental conditions

In this study, 4-week old male, pathogen free ICR (CD-1) mice were purchased from InVivos (Singapore) and used the entire animal study to assess the *in vivo* toxicity and antibacterial efficacy of the novel peptides as these animals by far are the most commonly used model for the study of pneumococcal disease (Chiavolini, Pozzi & Ricci, 2008). Mice were kept in ventilated polycarbonate cages (12hr light/dark cycle, 20±2 °C and 55% relative humidity). All mice were familiarized for 7 days before any experimental procedure and were given unlimited pellets and water *ad libitum*. All animal experimentations were conducted according to the guidelines approved by Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC), University of Malaya (ethics Reference no. : 2013-07-15/MMBTR/SDS).

***In vivo* toxicity**

In order to evaluate the possible toxic effects correlated to peptides administered in mice. Four hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 were chosen for *in vivo* toxicity assessment due to their promising *in vitro* antibacterial activity (Jindal et al., 2015). Mice were separated into 4 groups (each with 4 mice) and were injected with respective peptides at 1 hr, 12 hr, and 24 hr (three-dose regimen) via IP, SC, and IN administration routes. The hybrid peptides were firstly administered at high doses (100mg/kg for IP route, 100mg/kg for SC routes and 20mg/kg for IN route). Any abnormal behavior was recorded and survival of mice was noted as well. In the case of adverse effects such as high physical stress, highly lethargic, physical inactiveness, and/or death were detected, lower graded doses were given. All the administered

mice were monitored for 7 days or until death occurs. At day seven post administration, all animals were sacrificed and blood and organs were collected. Untreated mice were used as a control group.

***In vivo* antipneumococcal activity**

Two pneumococcal infection models developed previously in our lab (Le et al., 2015) were used to assess the therapeutic efficacy of peptides *in vivo*. The systemic infection model was used to mimic pneumococcal bacteremia in humans and the pneumococcal pneumonia model was used to mimic pneumococcal pneumonia in humans. A highly virulent strain was used in both models. The bacterial isolate was grown overnight on Columbia agar with 5% sheep blood at 37 °C under 5% CO₂. The bacterial suspension was adjusted to OD₆₂₅ 0.08-0.1 ($1 \sim 2 \times 10^8$ CFU/ml). Mice tested for lethal systemic infection were inoculated with 1.5×10^2 CFU/mouse (100µl) via IP route. Mice used to assess the Pneumococcal pneumonia model were inoculated with pneumococcal cells of 5×10^3 CFU/mouse (50µl) via the intrathoracic route. Both of the infection models caused 100% death within 2 to 4 days post-infection.

After 1hr of inoculation, Mice receiving treatment were randomized and divided into six groups. RN7-IN10 and RN7-IN8 were tested at three different doses for each (5mg/kg, 10mg/kg and 20mg/kg) using a group of 10 mice. Graded doses of ceftriaxone (5mg/kg, 10mg/kg, 20gm/kg, 40mg/kg and 80mg/kg) were also tested to assess the *in vivo* antibacterial activity of this antibiotic. Mice injected with PBS only were served as uninfected control. Mice injected with bacterial inoculum were used as untreated control group and given sterile distilled water only. Survival of mice was documented for seven days or until death. After seven days, the experiment was ended, the blood and homogenates of the five major organs (kidney, brain, spleen, liver and lung) of the

survived mice were plated on Columbia agar with 5% sheep blood to detect the presence of pneumococcal cells.

***In vivo* synergy assessment of peptide/peptide and peptide/ceftriaxone**

After evaluating the *in vivo* antibacterial activity of the hybrids in standalone mode, the *in vivo* efficiency of the hybrid peptides in combination with each other and with the standard antibiotic ceftriaxone was carried out. Graded doses of peptide and ceftriaxone were chosen and prepared at 2X the desired concentration separately in 1 ml tubes, and the volume was 0.1 ml. Immediately before injection, both drugs were combined giving the final desired concentration at a volume of 0.2 ml. The synergetic effect was then performed in infection models (n = 10).

Anesthesia and necropsy

Mice used to evaluate the *in vivo* toxicity and antibacterial activity of the hybrid peptides using the subcutaneous (SC) and intranasal (IN) administration routes were anesthetized using a combination of standard dose of xylazine (ilium xylazil-20, 10 mg/kg) and ketamine (Narketan®-10, 100 mg/kg) through intraperitoneal (IP) injection. After 7 days of treatment, the *in vivo* toxicity and antibacterial efficacy experiments were ended and survived mice were anesthetized. Blood samples for Hematological and biochemical analysis were collected via cardiac puncture using a 25G syringe (BD bioscience, USA). Whole blood for hematological analysis was collected in 500 µl dipotassium EDTA microtainer tubes (BD Bioscience, USA). About 500 µl of blood collected in an eppendorf tube and centrifuged at 8000 rpm for 5 min and then the serum was transferred into a new 1.5 ml tube for biochemistry analysis. The mice were then euthanized by cervical

dislocation, dissected and the following organs were collected for histopathology evaluation (lung, kidney, brain, liver and spleen).

Hematological and biochemical analysis

For whole blood analysis, the parameters were number of red cells (RBC), number of white cells (WBC), lymphocytes, monocytes, eosinophil, granulocytes, haemoglobin (Hgb), mean corpuscular volume (MCV), hematocrit (HCT), platelet Counts (PLT), Mean corpuscular haemoglobin (MCH) and corpuscular haemoglobin concentration (MCHC). For biochemistry analysis, the parameters were alanine transaminase (ALT), creatinine, alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin and urea.

Histopathological examination

The following organs were collected from all the dissected mice: lung, kidney, brain, spleen, and liver. All tissues were fixed in 10% (v/v) buffered formalin and processed for paraffin embedding. Hematoxylin–eosin (HE) was used to stain the histological sections at the histopathology laboratory, Veterinary Laboratory Service Unit, University Putra Malaysia (UPM).

Statistical analysis

GraphPad Prism 5 was used to perform the Statistical analysis. The results were expressed as mean \pm standard deviation. Two-way ANOVA with Bonferroni post-test was used to analyze the significance of difference between treated groups and control in ATP assay. One-way ANOVA with *post-hoc* Dunnett-t test was used to assess the statistical difference between the blood haematogram and blood serum biochemistry parameters of the treated and the untreated control

groups in the *in vivo* toxicity assay. Kaplan-Meier analysis with log-rank test (Mantel-Cox) was used to generate the survival curve for each treated group versus untreated control for both *in vivo* antibacterial activity and *in vivo* synergy assays.

3. Results

Effects of hybrid peptides on cell morphology and membrane permeability

TEM and SEM studies were performed to observe the damaging effect of the hybrid peptides on the pneumococcal cell wall/membrane. The images obtained clearly indicated that all the hybrid peptides were capable of disrupting the integrity of bacterial membranes. As shown in Fig. 1A, the untreated cells appeared with complete cell wall and plasma membrane and therefore preserved the normal integral shape of *S. pneumoniae*. The pneumococcal capsular polysaccharide appeared as a thin layer sheltering the whole cell and the cytoplasm of the cell was compactly packed and occupied the entire space (Fig. 1A, arrow a). Incubation of pneumococcal cells with hybrid peptides has led to a dramatic effect on the morphology of bacterial surface. After 1hr of incubation, the hybrid peptides were able to breach the intact of the cell wall and/or plasma membrane causing membrane breakage and loss of fragments (Fig. 1B-F, arrow b). Additionally, our TEM results have revealed that treatment with hybrid peptides has led to the leakage of the cytoplasmic components to the outer environment through the disruption of the cell wall. As a result, huge halos were detected in the inner space of all these treated cells leading to cell collapse and death (Fig. 1B-F, arrow c). Moreover, the TEM results also revealed partial disconnection of the cell wall from the cell membrane in pneumococci treated with hybrid peptides especially those treated with RN7-IN9, RN7-IN8, RN7-IN7 and RN7-IN6 (Fig. 1C-F, arrow d).


Scanning electron microscopy (SEM) was conducted to understand the impact of the hybrid peptides on the morphology of *S. pneumoniae*. As presented in Fig 2, the hybrid peptides were able to induce significant morphological alterations to pneumococcal cells. The untreated *S. pneumoniae* displayed normal and smooth surface (Fig. 2A, arrow a), whereas *S. pneumoniae* treated with hybrid peptides at $8 \times \text{MIC}$ appeared with a rough and injured surface (Fig. 2B-F, arrow b). This result indicates that hybrid peptides could disrupt and damage the integrity of cell wall/membrane or breach the membrane, which was in agreement with the result of TEM.

In order to evaluate the permeability of membrane and leakage of intracellular components upon treatment with hybrid peptides, the level of ATP in the supernatant following contact of the pneumococcal cells with hybrid peptides was determined using the ATP release assay. After 1h of treatment with hybrid peptide, the levels of ATP released after 1hr of treatment with RN7-IN10 and RN7-IN9 were the highest among the five hybrid peptides tested (54.5 ± 4.7 and 42.27 ± 9.2 pM respectively) (Fig. 3). The ATP efflux steadily decreased, and the ATP release reached 25.42 ± 3.51 and 22.9 ± 3.22 pM after 3h of treatment with RN7-IN10 and RN7-IN9 (Fig. 3). On the other hand, the quantities of ATP released from pneumococcal cells upon incubation with RN7-IN8, RN7-IN7 and RN7-IN6 after 1hr were 39.03 ± 0.2 , 22.35 ± 0.9 and 14.8 ± 0.35 . The levels of ATP release from pneumococcal cells treated with RN7-IN8, RN7-IN7 and RN7-IN6 were 20.54 ± 1.03 , 13.02 ± 2.26 and 11.47 ± 0.32 pM respectively after 3h of treatment (Fig. 3). However, all the hybrid peptides showed better capacity in efflux ATP from pneumococcal cells in comparison with standard antibacterial drugs ceftriaxone and erythromycin. The efflux levels of ATP by ceftriaxone and erythromycin treated cells after 1hr of incubation were 5.24 ± 1.43 pM and 0.49 ± 0.004 pM, respectively (Fig. 3).

DNA retardation activity

To clarify the influence of the hybrid peptides on pneumococcal genomic DNA, the retardation of DNA by the hybrid peptides at various concentration was assessed by analyzing electrophoretic movement of pneumococci DNA bands through the agarose gel (1%, w/v). Our results clearly presented that all five hybrid peptides were capable of inhibiting DNA migration through the gel at a concentration of 62.5µg/ml (Fig. 4A-E). On the other hand, standard drugs ceftriaxone and erythromycin could not prevent the migration of DNA band through the agarose gel up to a concentration of 500µg/ml (Fig. 4F & 4G).

In vitro synergistic effects of peptide/peptide and peptide/antibiotic combinations

The *in vitro* antibacterial activity of peptide/peptide and peptide/antibiotic combinations were evaluated using the chequerboard dilution assay. Our results reveal that combinations of hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7, and RN7-IN6) with each other showed synergistic effects with FIC  less than 0.5 (Table 1) Regardless of the susceptibility of pneumococcal isolates toward standard drugs. Likewise, combinations of standard drugs ceftriaxone and erythromycin with all five hybrid peptides presented synergistic effects with FIC index of ≤ 0.5 against both isolates of *S. pneumoniae* regardless of antibiotics their susceptibility to antibiotics (Table 1). These results indicate that all the hybrid peptides were able to enhance the antibacterial activity of both standard drugs ceftriaxone and erythromycin.

In vivo toxicity of hybrid peptides

The *in vivo* toxicity of four hybrid peptides namely RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 was evaluated following a three dose regimen with the mice at 1hr, 12hr and 24hr using

three different administration routes. The results revealed that for mice treated with all hybrid peptides via subcutaneous (SC) injection at maximum dose (100mg/kg), no animal death or hypersensitivity reactions were observed up to seven days post-treatment. Minor differences were noted for mice given RN7-IN10 via SC which displayed significantly lower granulocytes ($p = 0.0172$) and ALP ($p = 0.0037$) (Table S1, highlighted in yellow), while Mice treated with RN7-IN6 displayed significantly higher platelet counts ($p = 0.0487$) in comparison with the control group (Table S1, highlighted in blue). Histopathological studies were performed with the lung, brain, liver, spleen and kidney of control and treated animals. No histological abnormalities were detected in the organs of any group as all the tissue sections were normal and did not display differences with the control group (Fig. S1). Similarly, no abnormal physical behavior was noted upon given the mice hybrid peptides via the intranasal (IN) route. However, treatment with RN7-IN9 displayed significantly higher granulocytes ($p = 0.0172$) and ALP ($p = 0.0037$) (Table S2, highlighted in yellow) than the control group. Mice treated with RN7-IN6 displayed significantly lower MCV ($p = 0.001$) (Table S2, highlighted in yellow). Whereas RN7-IN6 treated mice revealed significantly lower percentage of granulocytes ($p = 0.0482$) as compared to the control group (Table S2, highlighted in blue). Histological examination of the organs collected from all the treated and control groups did not expose any histopathological changes (Fig. S2).


In terms of intraperitoneal (IP) administration route, all four hybrid peptides caused death and/or high physical stress when injected at a concentration of 100mg/kg. Therefore, low graded doses were attempted until we reach the maximum dose which showed no sign of stress or abnormal behavior. Hybrid peptides RN7-IN10 and RN7-IN8 did not display any sign of toxicity when injected at 20mg/kg; no death occurred in any of the treated mice up to 7 days post-treatment. RN7-IN9 and RN7-IN6 were non-toxic when injected at 10mg/kg. None of the five major organs

of the treated mice revealed any significant histological abnormality as compared to the untreated control group (Fig. S3). However, mice treated with RN7-IN9 (10 mg/kg) via IP route showed significantly lower lymphocytes ($p = 0.0445$) and lower ALP ($p = 0.0187$) (Table S3, highlighted in yellow), while RN7-IN6 treated mice had lower ALT ($p = 0.0425$) when compared to control group (Table S3, highlighted in blue).

***In vivo* antibacterial efficacy of hybrid peptides**

Two peptides RN7-IN10 and RN7-IN8 that showed the fastest killing kinetics (Jindal et al., 2015) and exhibited less toxic effects *in vivo* were selected to evaluate their *in vivo* antibacterial efficacy via IP route, both hybrid peptides were tested at three different doses (5 mg/kg, 10mg/kg and 20 mg/kg) at three treatment regimens (1hr, 12hr and 24hr post-infection). In the pneumococcal bacteremia model, both RN7-IN10 and RN7-IN8 failed to treat any of the infected mice at 5mg/kg. At a dose of 10 mg/kg, 10% of the infected mice survived after treatment with RN7-IN10 ($p = 0.0018$), whereas 30% of the mice was able to survive after treatment with RN7-IN8 ($p = 0.0002$). However, at a dose of 20mg/kg, 30% of the mice treated with RN7-IN10 survived ($p < 0.0001$), while 50% of the mice survived when treated with hybrid peptide RN7-IN8 ($p = 0.0002$) (Fig. 5). No pneumococci were detected from the blood of the mice that survived and none of the mice showed presentation of illness as compared to the untreated group which was severely ill and inactive. Treatment via SC and IN routes had no impact on infected mice up to seven days post-infection and none of the mice survived.

In addition to our designed peptides, ceftriaxone as a standard drug was used to treat infected mice via IP route at 5mg/kg, 10mg/kg, 20mg/kg, 40mg/kg, and 80mg/kg and the survival was 10%, 30%, 50%, 70% and 90% up to seven days post-infection ($p < 0.0001$) (Fig. 6). In the

396 pneumococcal pneumonia model, mice treated with both peptides via IP, SC and IN routes showed
 397 no survival up to seven days post-infection 

398 Combinations of peptide-peptide were also assessed for their ability to treat infected mice
 399 with pneumococcal bacteremia. Two combinations were used, 5mg/kg + 5mg/kg and 10mg/kg +
 400 10mg/kg to treat mice via IP route at three regimens 1hr, 12hr and 24hr. The results indicate that
 401 the combination of 5mg/kg + 5mg/kg was able to treat 40% of the mice and protected them from
 402 death up to 7 day post-infection ($p = 0.0003$), while increasing the dose to 10mg/kg of each peptide
 403 resulted in 60% of the infected mice surviving the pneumococcal infection ($p < 0.001$) (Fig. 7).

405 ***In vivo* synergy assessment of hybrid peptide RN7-IN8 in combination with ceftriaxone**

406 Among the hybrid peptides RN7-IN10 and RN7-IN8, standalone treatment with RN7-IN8
 407 at 20mg/kg was found to confer significant survivability to mice infected by a highly virulent
 408 pneumococcal clinical isolate via IP route. To assess the synergistic effect of RN7-IN8 in
 409 combination with the standard drug ceftriaxone (CTX), three different doses of RN7-IN8 (5mg/kg,
 410 10mg/kg and 20mg/kg) and ceftriaxone (5mg/kg, 10mg/kg and 20mg/kg) were tested using the
 411 same bacteremia infection model in three treatment formulation: RN7-IN8₅ – CTX₅ (5mg/kg of
 412 RN7-IN8 and 5mg/kg of CTX), RN7-IN8₁₀ – CTX₁₀ (10mg/kg of RN7-IN8 and 10mg/kg of CTX)
 413 and RN7-IN8₂₀ – CTX₂₀ (20mg/kg of RN7-IN8 and 20mg/kg of CTX). Using groups of 10 mice,
 414 the combinations of RN7-IN8 and ceftriaxone RN7-IN8₅ – CTX₅ (5mg/kg of RN7-IN8 and 5mg/kg
 415 of CTX), RN7-IN8₁₀ – CTX₁₀ (10mg/kg of RN7-IN8 and 10mg/kg of CTX) and RN7-IN8₂₀ –
 416 CTX₂₀ (20mg/kg of RN7-IN8 and 20mg/kg of CTX) displayed 70%, 80% and 100% survival in
 417 mice infected with highly virulent pneumococcal strain up to seven days post-infection ($p <$
 418 0.0001) (Fig. 8). Our results displayed that that treatment using combinations of peptide-antibiotics

conferred higher survival rate than peptide and antibiotic in their stand-alone form. In addition, all treated mice survived from infection appeared physically active and none of them showed sign of abnormal behavior.

Histopathological evaluation

All the histopathological examinations of the mice infected with bacteremia model with and without treatment are presented in Fig. 9 and Fig. 10. Out of five major organs examined, the lung and spleen of the infected animals were the most severely affected. A number of histopathological changes were observed in the lung of the infected mice. As compared to the uninfected control group, the lung of the infected and untreated group exhibited extensive vascular congestion with foci consolidation. Heavy permeation of the red blood cells into the alveolar spaces strongly denotes pulmonary hemorrhage (Fig. 9A, arrow a). The greatly congested lung appeared with little alveolar spaces (Fig. 9A, arrow b). This is in contrast to the uninfected group, where the normal lung displayed greatly aerated alveolar spaces with a thin layer of the alveolar wall. Severe tissue injury was also noticed in the spleen of the infected group (Fig. 10A). Unlike the normal spleen which showed normal red and white pulps (Fig. 10B, arrow b), the infected spleen demonstrated depleted splenocytes with no white matter/germinal center (Fig. 10A, arrow a). No significant histopathological lesions were observed in other organs such as brain, liver, kidney and heart.

For the respective treatments of infected mice including hybrid peptide RN7-IN8 at 20mg/kg, combination of hybrid peptides RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg) and combination of RN7-IN8 and ceftriaxone (5mg/kg + 5mg/kg, 10mg/kg + 10mg/kg and 20mg/kg + 20mg/kg), it was noticed that although lesions, inflammatory events and the degree of tissues

damage can be found in the organs, however, the degree and severity of the damage were extensively lesser than the infected control group. Unlike the lung of the untreated mice which exhibited severe inflammation and the alveolar spaces were about 90% congested (Fig. 9A), all the lungs harvested from the treated mice revealed only low level of congestion and minor thickening of the alveolar wall even though these histological changes were still noticeable in the mice (Fig. 9C-G). Treatment of infected mice with combination of hybrid peptide RN7-IN8 and ceftriaxone at three different dosages (5mg/kg - 5mg, 10mg/kg - 10mg and 20mg/kg - 20mg/kg) showed gradually decrease in the degree of congestion and damage (Fig. 9E-G). Lungs harvested from mice treated with combination of RN7-IN8 and ceftriaxone at 20mg/kg + 20mg/kg (Fig. 9G) which presented 100% mice survival, were similar to that harvested from the uninfected control mice (Fig. 9B) in degree of normality. Likewise, all the spleens of treated mice displayed no or minimum damage, the white and red pulps where clearly observed (Fig. 10C-G) as compared to the infected one (Fig. 10A). No significant tissue damage was observed in brain, kidney and liver in both treated and untreated mice.

4. Discussion

According to WHO, diseases induced by a pneumococcal infection continue to cause the most deaths among vaccine-preventable diseases (WHO, 2014). A previous study showed that pneumococcal resistance to antibiotics (erythromycin penicillin, fluoroquinolones) had led to 32,398 additional outpatient visits and 19,336 additional hospitalizations. The incremental cost of antibacterial drug resistance accounted for \$91 million (4%) of direct medical fees and \$233 million (5%) of total fees including work and productivity losses (Reynolds et al., 2014). Although Pneumococcal conjugate vaccine PCV served as a great tool against antibiotic resistance by *S.*

465 *pneumoniae* and helped decrease the frequency of vaccine serotypes, there has been a considerable
 466 rise in the disease induced by non-vaccine serotypes (Reynolds et al., 2014). Hence, newer classes
 467 of antibacterial agents to overcome this serious issue is a top priority worldwide. The mechanisms
 468 of actions, *in vivo* toxicity and antibacterial efficiency of five hybrid peptides previously designed
 469 in our lab based on two templates indolicidin and ranalexin were investigated in this study. TEM
 470 and SEM were used to evaluate the morphological alterations caused by hybrid peptides. Results
 471 obtained from TEM gave strong evidence that targeting the bacterial cell wall/plasma membrane
 472 seems to be the main antibacterial mechanism used by hybrid peptides. Unlike the untreated cells,
 473 pneumococcal cells treated with hybrid peptides faced dramatic morphological changes. The
 474 breakage and fragments loss of the bacterial cell wall/membrane is probably a result of the strong
 475 interaction between the negatively charged membrane and the hybrid peptides due to their positive
 476 charge and high hydrophobic content (Jindal et al., 2015). Also, the detachment of the cytoplasmic
 477 membrane from cell wall was observed in pneumococcal cells upon treatment with hybrid peptides
 478 is a possible indication of the capability of hybrid peptides to interpolate themselves between
 479 pyrophosphate-linked cell-wall anchors and the cell membrane. Subsequently, this act would
 480 pullout the isoprenyl anchor chains away from the cell membrane and weaken cell-wall adhesion.
 481 The results obtained by TEM were similar to that reported on the mechanism of nisin against *B.*
 482 *subtilis* (Hyde et al., 2006) and *E. faecalis* (Tong et al., 2014). Likewise, chicken CATH-2 was
 483 able to disrupt and detach the plasma membrane from the cell wall of *C. albicans* (Ordonez et al.,
 484 2014). Another possible explanation is that the breakage of the cell wall will allow the insertion of
 485 water from the medium between the two membranes and detach them (López-Expósito, Amigo &
 486 Recio, 2008). Likewise, SEM studies showed the effects on the bacterial surface. Changes
 487 detected were swelling and aggregation in contrast to the smooth surface of untreated bacterial

cells. Besides, the numerous fragments observed in *S. pneumoniae* cultures treated with hybrid peptides point to a cell wall breakage and cell lysis. Membrane disruption could be associated with leakage of ions and metabolites, depolarization and eventually cell death. Adenosine triphosphate (ATP) is one of most significant molecules for all living cells as it used as an intracellular source of energy for many biological processes (Mempin et al., 2013). In normal conditions, bacterial membranes are impervious to ATP and other intracellular constituents as membrane destabilization might lead to the release of normally impervious substances. Therefore, ATP has been used as a tool to measure the integrity of living cells. Since most of the ATP is found within the cells, any cell injury will result in a prompt reduction in the cytoplasmic ATP. Our results revealed that ATP efflux was not increased by incubation of pneumococcal cells with standard drugs ceftriaxone and erythromycin but was increased by hybrid peptides. the ATP efflux results suggest that our positively charged hybrid peptides have strong affinity to bind to the negatively charged bacterial membrane, disrupting its integrity and allowing a significant amount of ATP to be released to the surrounding environment. However, a reduction in the amount of ATP released to the medium was noticed after 1hr of incubation; this might be due to the rapid degradation of ATP by enzymes released to the medium as a result of membrane damage which subsequently leads to rapid cell death. Such results were also observed when *Candida albicans* was treated with CATH-2 peptide, the measurable ATP after 5min of incubation were higher than the levels of ATP after 1hr of incubation (Ordóñez et al., 2014). Also, the synthetic peptide Tet052 was capable of causing a significant leakage of ATP from *S. aureus* after 30min of treatment (Hilpert et al., 2009). Altogether, results from TEM, SEM and ATP release assay point out that the hybrid peptides destabilize the cell envelope of the pneumococcal cells. Hence it can be hypothesized that the disturbance of the bacterial surface must activate an autolytic and/or cell death mechanism.

However, pore forming and membrane damage do not preclude hybrid peptides for other mechanisms of action due to the fact that antimicrobial peptides could act as membrane-disruptive when present at high concentration but shift to attack intracellular components when present at low concentration, or both (Friedrich et al., 2000, 2001).

It is well known that the cell membrane is not the only target for antimicrobial peptides. AMPs may also attack other cell components such as DNA, RNA and proteins (Li et al., 2013). For instance, the antimicrobial peptide buforin II showed the ability to translocate to the inner leaflet of the plasma membrane and target the DNA after breaching the membranes resulting in rapid cell death (Park, Kim & Kim, 1998). The results from the DNA retardation assay clearly displayed that all the hybrid peptides were capable of binding to DNA efficiently and preventing it from moving down through the agarose gel. These results suggest that hybrid peptides could possess another mechanism of bacterial killing by inhibiting intracellular functions via interference with DNA function. Peptides interaction with DNA can prevent or hinder gene expression which is an efficient tactic to suppress and inhibit normal enzyme and receptor synthesis, disrupt the components required for the life cycle of the bacterial cell and consequently cell death.

Combinations of antimicrobial agents are often used to combat multi-drug resistant isolates (Novy et al., 2011). Several studies have reported synergistic effects of combinations of AMPs with standard antibiotics. The hybrid peptide LHP7 revealed a synergistic effect against a clinical isolate of methicilin-resistant *S. aureus* MRSA when combined with ampicillin (Xi et al., 2013). Similarly, a combination of the A3 peptide with chloramphenicol showed synergistic action against *S. aureus*, *E. coli*, and *P. aeruginosa* (Park, Kim & Hahm, 2004). In the present study, we utilized the checkerboard MIC technique to assess drug-drug interaction. Our results revealed that all the five hybrid peptides exhibited synergistic effects against pneumococcal clinical isolate

when combined with each other and with conventional drugs erythromycin and ceftriaxone. One possible explanation of the synergistic effects of peptides-drugs combinations is that the hybrid peptides may increase permeability by interacting with the bacterial cell wall/membrane, allowing conventional drugs to easier act on their targets. Previous reports have shown that β -lactam antibiotics like ceftriaxone exert higher antimicrobial activity when combined with membranolytic peptides such as nisin, as these AMPs cause changes in cell morphology by forming pores allowing antibiotics to enhance their act and produce a greater damage within the cell wall (Singh, Prabha & Rishi, 2013; Tong et al., 2014). Another possible mechanism of synergistic combinations is that antimicrobial peptides alter the efflux pump systems allowing intracellular antibiotics such as macrolides to act more efficiently on their intracellular targets (Ruhr & Sahl, 1985; Soren et al., 2015).

Toward evaluating the *in vivo* therapeutic efficacy of peptides in living organisms, two hybrid peptides RN7-IN10 and RN7-IN8 were selected to treat mice models infected with highly virulent pneumococcal strain. Among all the doses assessed, RN7-IN8 showed an interesting *in vivo* therapeutic efficacy. At low dose (20mg/kg) RN7-IN8 resulted in 50% survival of the infected mice with bacteremia model up to seven days after infection ($p < 0.001$) as compared to the untreated mice. Combinations of hybrid peptides tested for the synergistic effect resulted in increased the survival rates of infected mice. These findings are in agreement with our *in vitro* synergism results.

The use of two or more antibacterial drugs in combination therapy are an alternative strategy to enhance treatment outcome in a clinical setting (Caballero & Rello, 2011). This is especially valuable in patients with severe pneumococcal infections. For instance, combination antibiotic therapy with both β -lactam and macrolide had a significantly lower case – mortality rate

in comparison with those given single antibiotic therapy (Mufson & Stanek, 2006). Broad-spectrum cephalosporins such as ceftriaxone are important antibiotics in the management of invasive diseases induced by penicillin-resistant pneumococci. However, the rate of pneumococcal strains resistant to ceftriaxone has been increased significantly (Chiu et al., 2007). Hence, RN7-IN8 which showed significant therapeutic efficacy to the infected mice in its standalone form was further assessed for *in vivo* therapeutic synergism in combination with ceftriaxone. Combination of RN7-IN8 and ceftriaxone resulted in a synergistic effect when tested *in vivo* using mice infected with pneumococcal bacteremia model. The survival rates in mice treated with this combination at varying dosages were dramatically increased as compared to the sum of the survival rates of standalone treatment. These findings are in agreement with our *in vitro* synergism results which demonstrated that hybrid peptides and ceftriaxone can act synergistically and kill pneumococci rapidly. Unlike untreated mice that died within four days after infection, 100% mice treated with combination of RN7-IN8 and ceftriaxone (20mg – 20mg) survived at day seven post-infection. Using combination of these two drugs at a low dose (20mg/kg) showed better survival rate than use ceftriaxone alone at high dose (80mg/kg). Thus, giving another advantage to the hybrid peptide RN7-IN8 to reduce the risk of developing resistance by the bacterial pathogen. All the mice treated with RN7-IN8 and the combinations with CTX did not show sign of sickness or abnormal behavior. iotics

5. Conclusion

In summary, our hybrid peptides showed promising *in vitro* and *in vivo* antibacterial activity against *S. pneumoniae*. The results of the *in vitro* and *in vivo* synergism tests clearly presented that the hybrids are not only potent antimicrobials in their standalone form but also when

combined with standard antibiotics, suggesting that these peptides can be used as supporting compounds to reduce the therapeutic dose of antibiotics and thus reducing potential resistance. Although RN7-IN8 showed promising therapeutic outcome, there are some limitations in its efficacy. Primarily, the peptide had no effect in the pneumonia model where the bacterial inoculum was administered directly into the thoracic cavity to infect the lungs while the peptide was given at distant sites indicating that the peptide could not diffuse effectively to the site of infection. This could possibly be due to the degradation by blood or cellular components. On the other hand, the effectiveness of the peptide in the bacteremia model is probably due to both infection and treatment being carried out in the same site. Hence, AMPs have a huge potential to play a crucial role in the combat of resistant bacteria either as standalone therapeutics or in combination with other drugs.

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Transparency declarations

None to declare.

Competing Financial Interests

The authors declare no competing interests.

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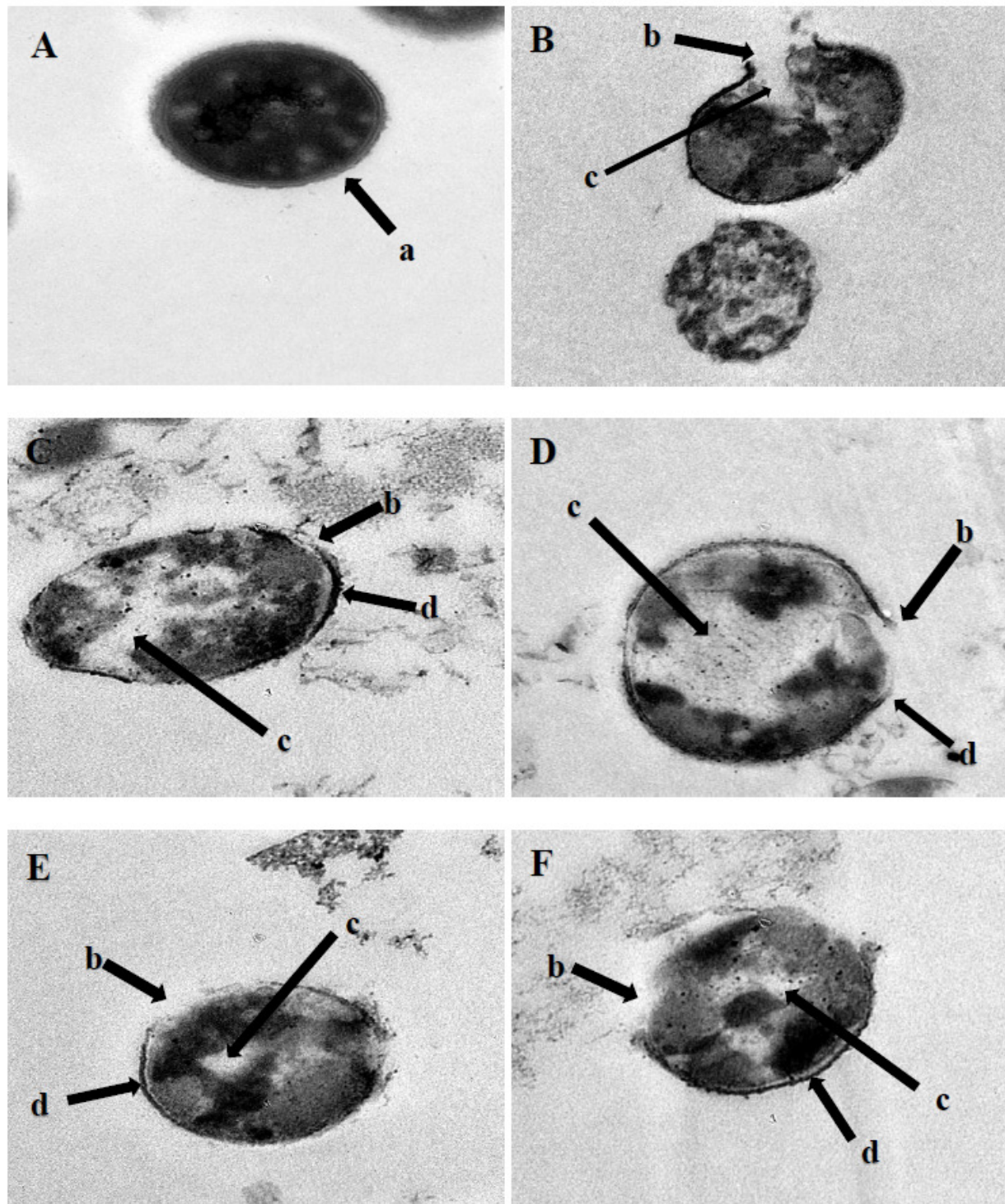


Fig 1. Transmission electron micrographs of *S. pneumoniae* after treatment with hybrid peptides. (A) Control cells without treatment appeared with normal shape (Fig1A, arrow a). Fig B – F display the damage of pneumococcal cells after 1hr incubation in presence of (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, (E) RN7-IN7 and (F) RN7-IN6. (Arrow b) Breakage and loss of cell wall/membrane fragments. (Arrow c) Leakage of cytoplasm and halos formation. (Arrow d) detachment of cytoplasmic membrane from pneumococcal cell wall.

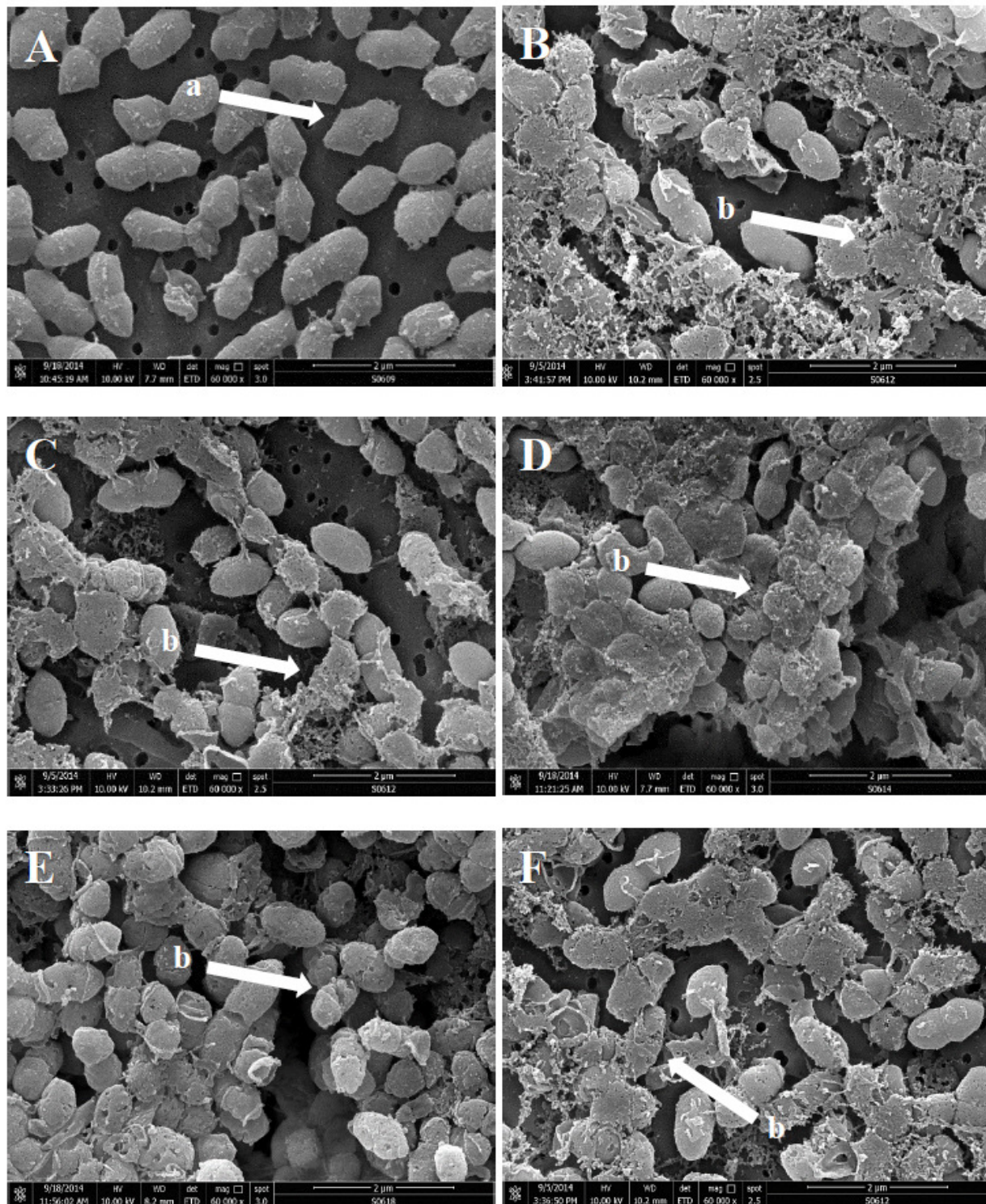


Fig 2. Scanning electron micrographs of *S. pneumoniae* after treatment with hybrid peptides. (A) Control cells without treatment appeared with normal shape and smooth surface (arrow a). Fig B – F show the severe morphological changes and surface disruption (arrow b) of pneumococcal cells following 1hr incubation in presence of (B) RN7-IN10, (C) RN7-IN9, (D) RN7-IN8, (E) RN7-IN7, and (F) RN7-IN6.

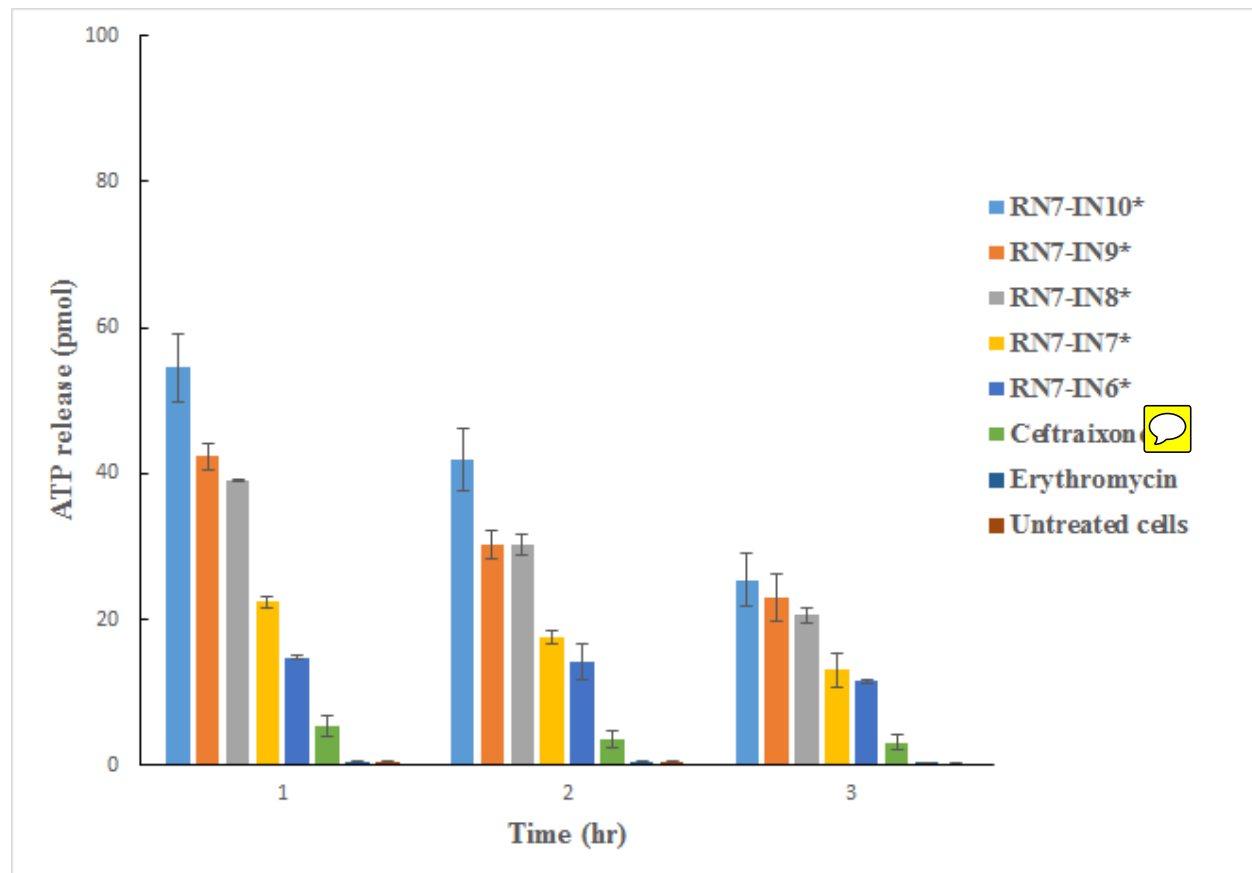


Fig 3. The influence of peptides on ATP release. All Hybrid peptides presented stronger ATP efflux activity than erythromycin and ceftriaxone. Two-way ANOVA with Bonferroni post-test was used to perform the statistical analysis. An asterisk (*) adjacent to peptide name directs statistical significance ($P < 0.0001$).

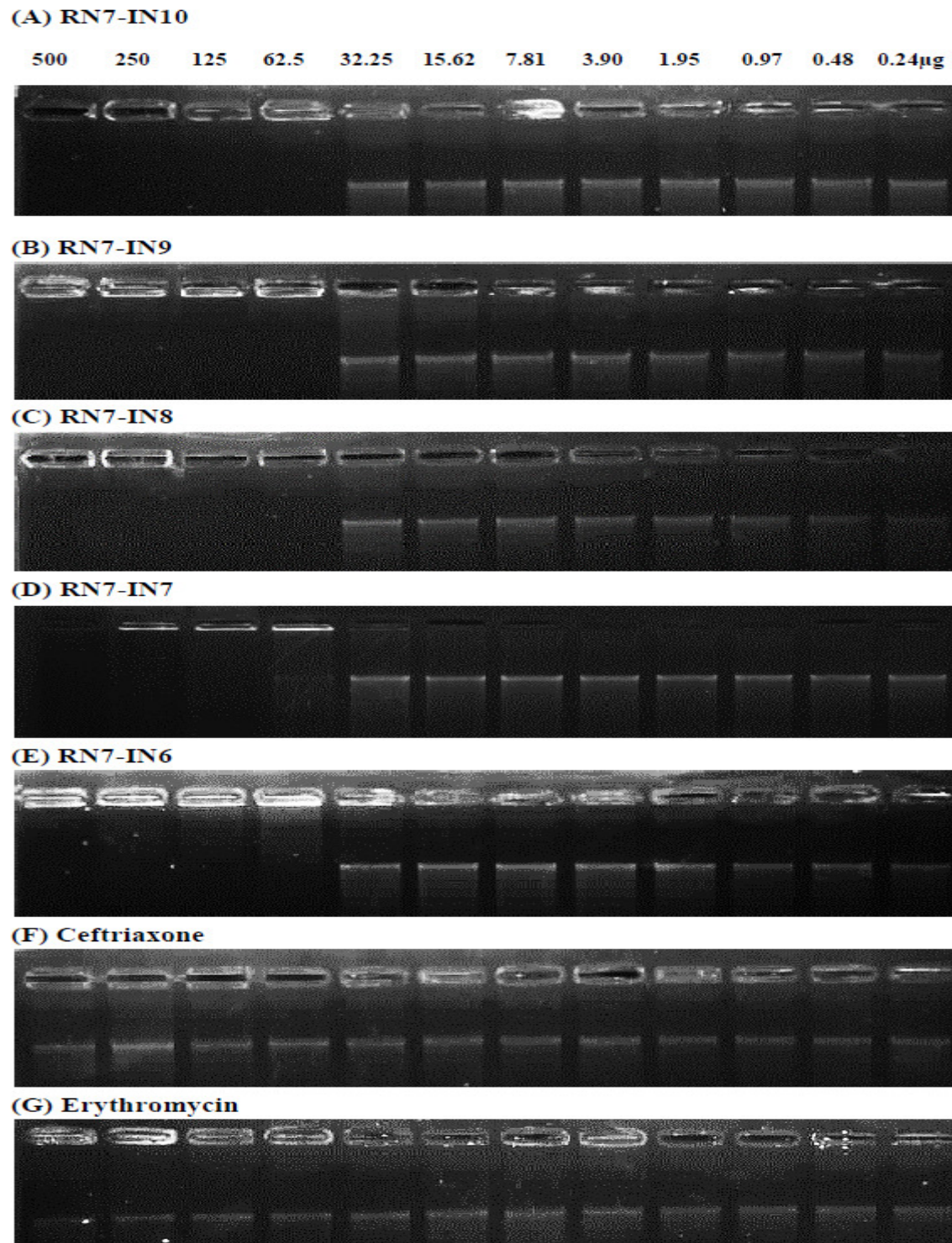


Fig 4. The impact of the hybrid peptides on the migration of genomic DNA. All hybrid peptides prevented the migration of the DNA through the gel at 62.5 μ g/ml. RN7-IN10 (A), RN7-IN9 (B), RN7-IN8 (C), RN7-IN7 (D), and RN7-IN6 (E). While ceftriaxone (F) and erythromycin (G) failed to stop the migration of genomic DNA up to a concentration of 500 μ g/ml.

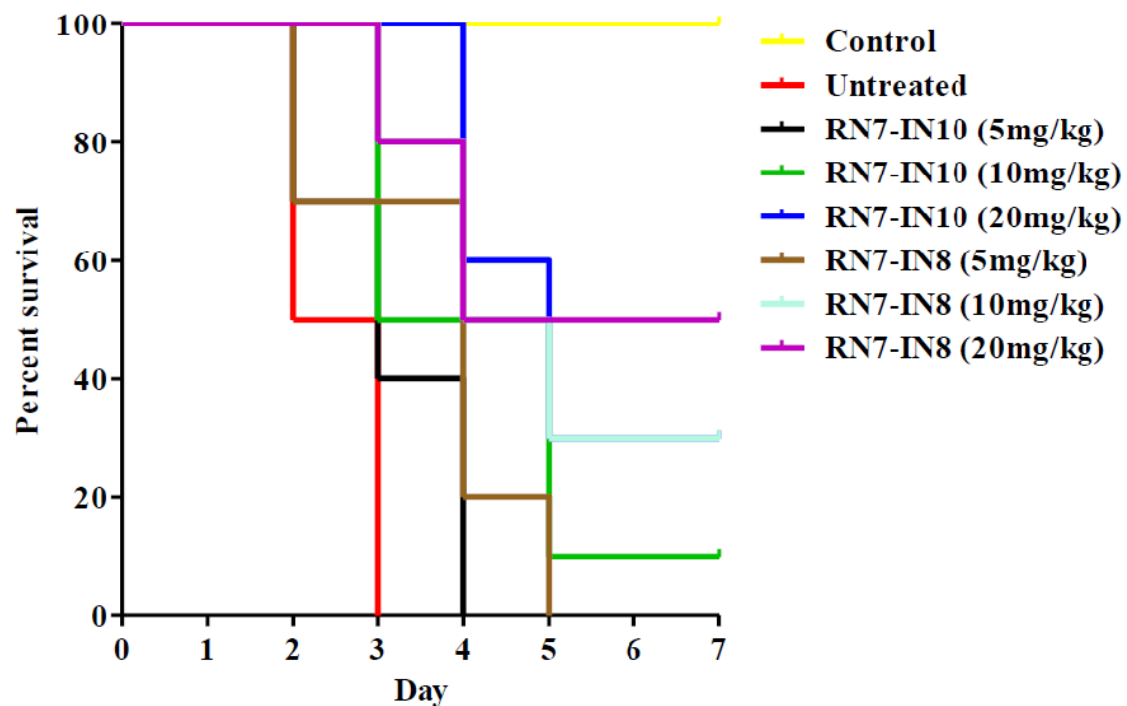


Fig 5. Survival curve of infected mice treated with RN7-IN10 and RN7-IN8. Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical for all treated groups and the untreated control using. Treatment with RN7-IN8 at 20mg/kg displayed the highest survival rate of 50% up to 7 days post-infection ($p < 0.001$).

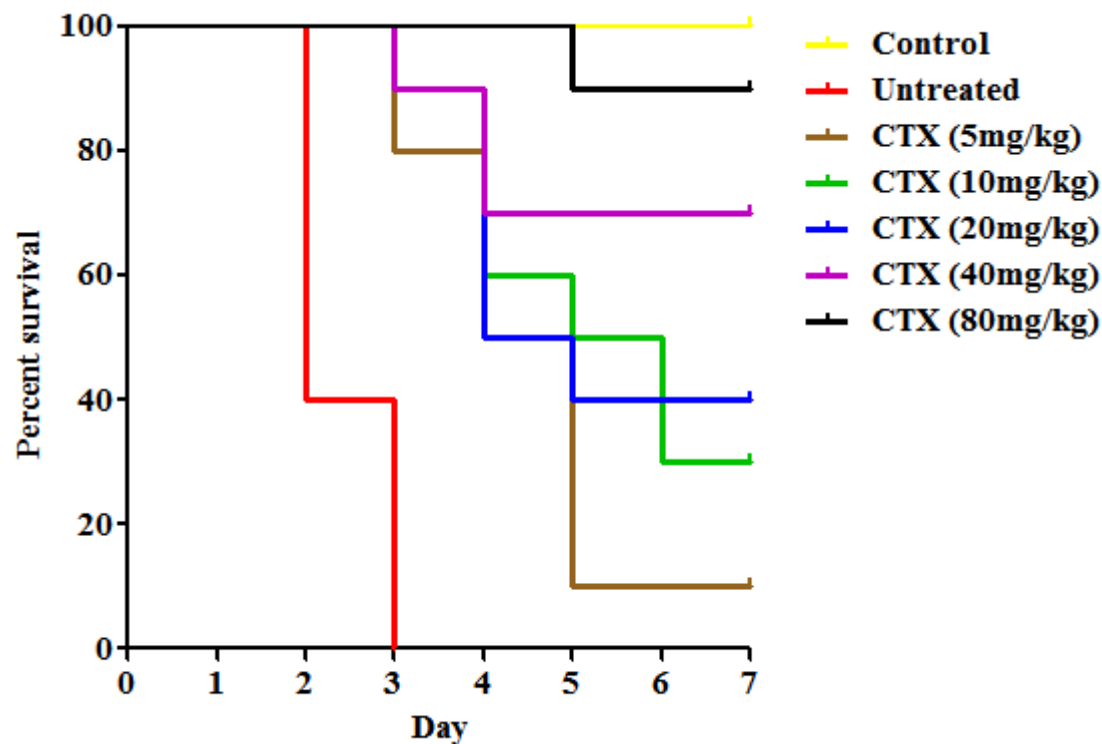


Fig 6. Survival curve of infected mice treated with ceftriaxone (CTX). Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group and the untreated control.

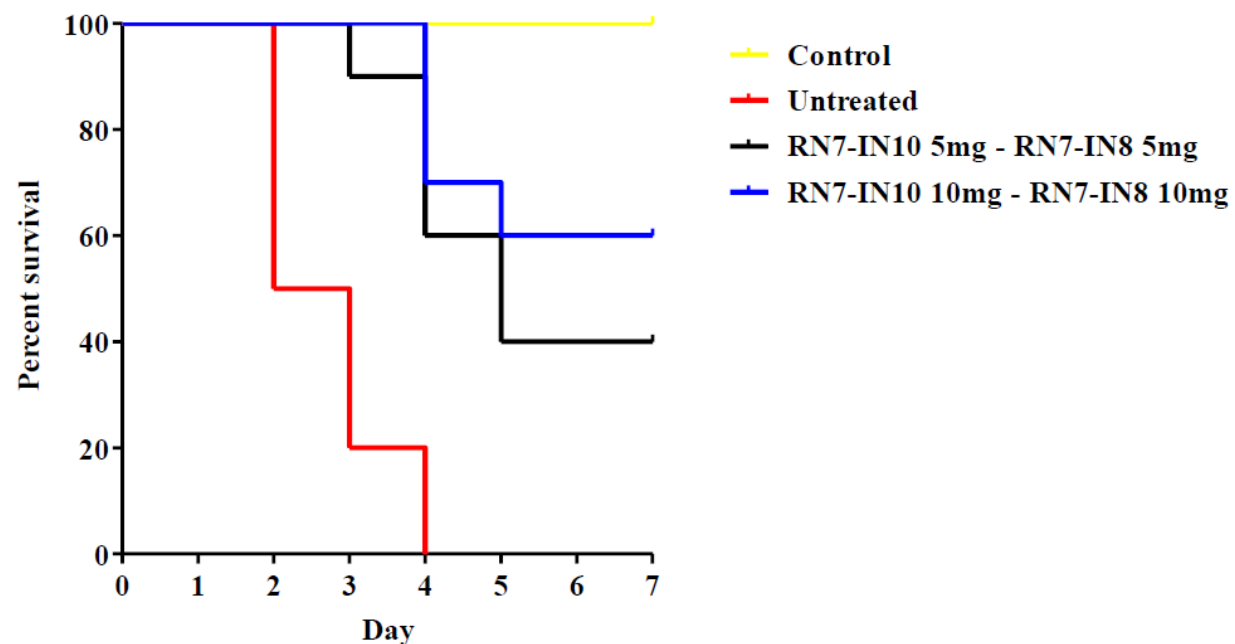


Fig 7. Survival curve of infected mice treated with combinations of RN7-IN10 and RN7-IN8. Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group versus the untreated control.

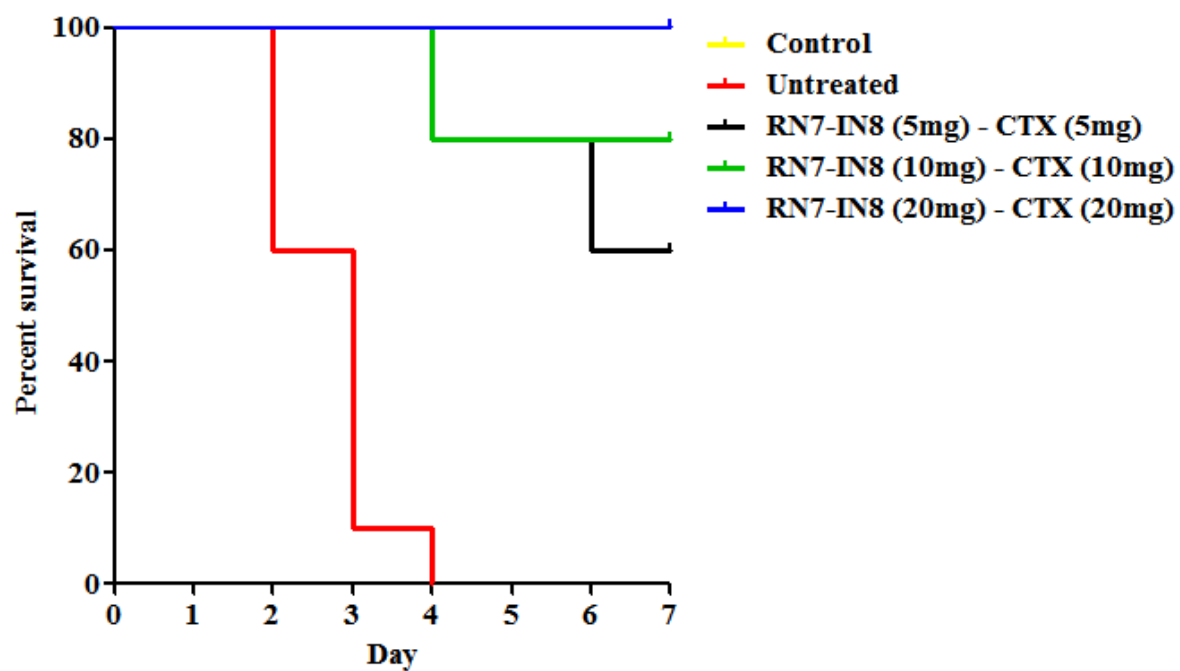


Fig 8. Survival curve of infected mice treated with combinations of RN7-IN8 and ceftriaxone (CTX). Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group and the untreated control.

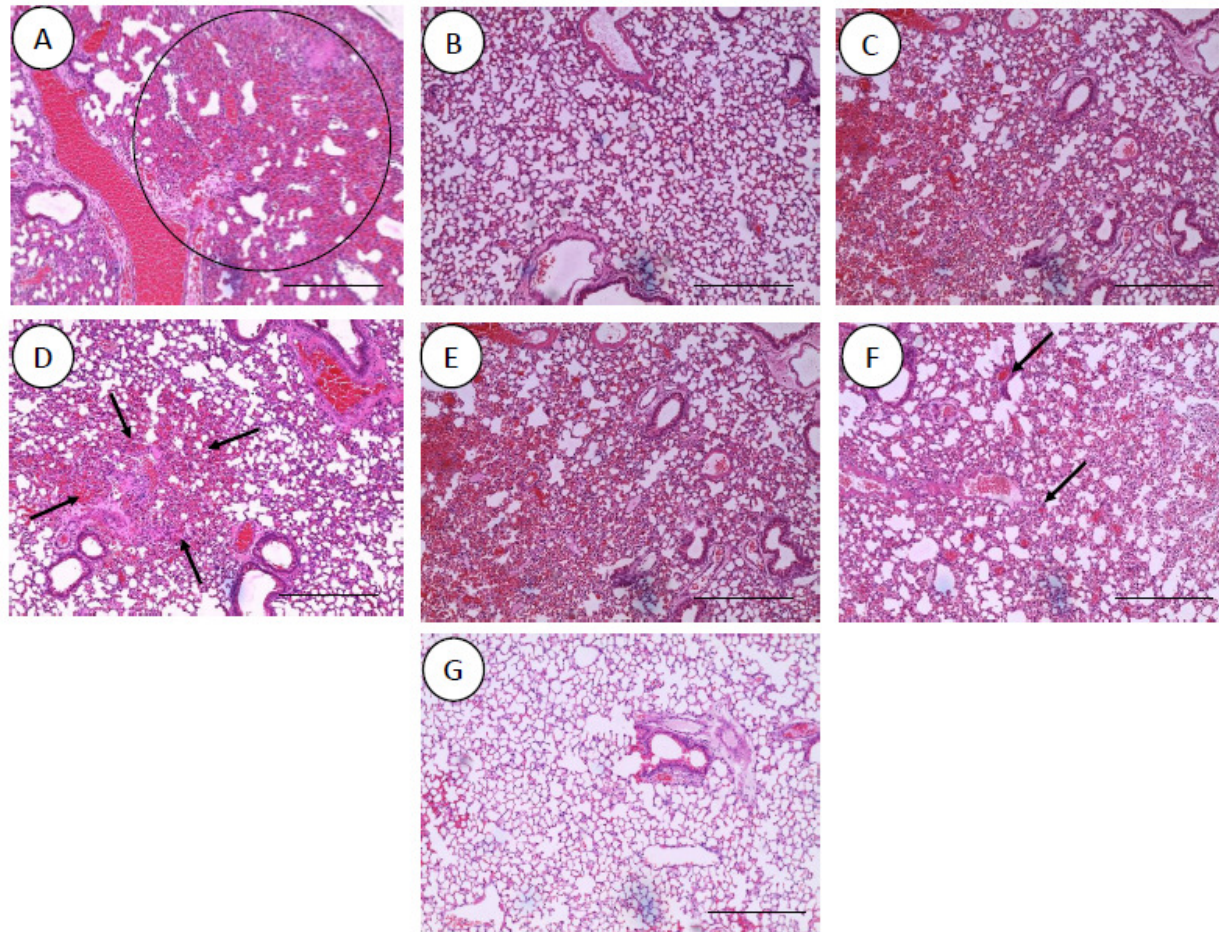


Fig 9. Histology of lungs harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated with combination of RN7-IN8 and CTX (5mg/kg + 5mg/kg), (F) mice treated with combination of RN7-IN8 and CTX (10mg/kg + 10mg/kg), (G) mice treated with combination of RN7-IN8 and CTX (20mg/kg + 20mg/kg). Hematoxylin and eosin stain. Bar indicates 500 μ M.

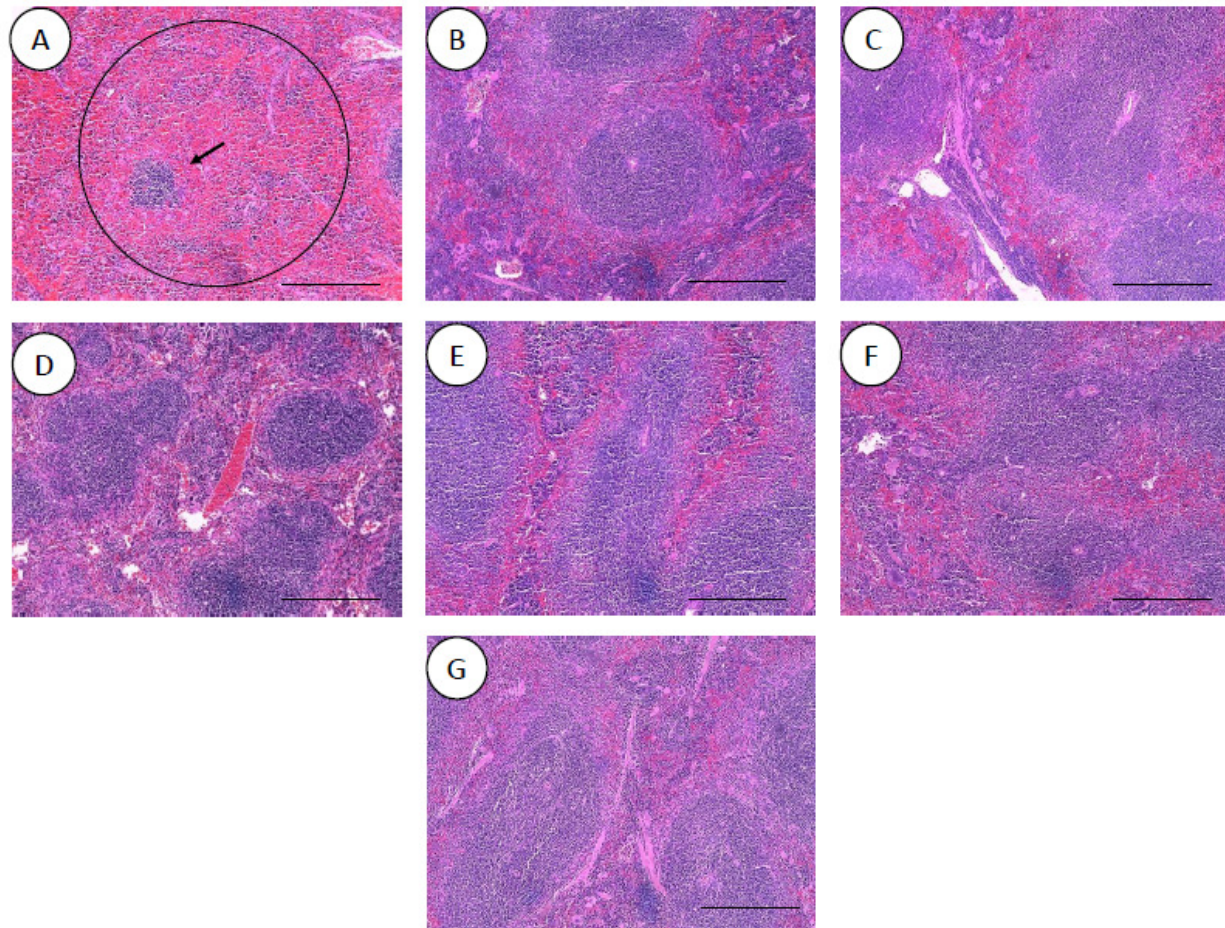


Fig 10. Histology of spleens harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated with combination of RN7-IN8 and CTX (5mg/kg + 5mg/kg), (F) mice treated with combination of RN7-IN8 and CTX (10mg/kg + 10mg/kg), (G) mice treated with combination of RN7-IN8 and CTX (20mg/kg + 20mg/kg). Hematoxylin and eosin stain. Bar indicates 500 μ M.

Table 1: FIC index of various combinations of hybrid peptides with each other and with standard antibiotics against susceptible and resistant *S. pneumoniae*.

Combination		Susceptible <i>S. pneumoniae</i>		Resistant <i>S. pneumoniae</i>	
Drug A	Drug B	FIC index ^a	Interpretation	FIC index ^a	Interpretation
RN7-IN10	RN7-IN9	0.50	Synergism	0.37	Synergism
	RN7-IN8	0.28	Synergism	0.28	Synergism
	RN7-IN7	0.37	Synergism	0.50	Synergism
	RN7-IN6	0.26	Synergism	0.31	Synergism
	Ceftriaxone	0.37	Synergism	0.31	Synergism
	Erythromycin	0.26	Synergism	0.28	Synergism
RN7-IN9	RN7-IN8	0.37	Synergism	0.50	Synergism
	RN7-IN7	0.50	Synergism	0.50	Synergism
	RN7-IN6	0.28	Synergism	0.37	Synergism
	Ceftriaxone	0.31	Synergism	0.37	Synergism
	Erythromycin	0.28	Synergism	0.26	Synergism
RN7-IN8	RN7-IN7	0.50	Synergism	0.50	Synergism
	RN7-IN6	0.31	Synergism	0.37	Synergism
	Ceftriaxone	0.31	Synergism	0.37	Synergism
	Erythromycin	0.28	Synergism	0.26	Synergism
RN7-IN7	RN7-IN6	0.50	Synergism	0.50	Synergism
	Ceftriaxone	0.50	Synergism	0.50	Synergism
	Erythromycin	0.37	Synergism	0.37	Synergism
RN7-IN6	Ceftriaxone	0.37	Synergism	0.31	Synergism
	Erythromycin	0.28	Synergism	0.28	Synergism

^aFIC index ≤ 0.5 represents synergy; $> 0.5 - \leq 4.0$ represents indifference; > 4.0 represents antagonism.

Highlighted in bold: peptide-antibiotic combination with synergistic effect