Mechanisms of action and *in vivo* antibacterial efficacy assessment of five novel hybrid peptides derived from Indolicidin and Ranalexin against *Streptococcus pneumoniae* (#18224)

First revision

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Mario Alberto Flores-Valdez / 30 Jul 2017

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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*

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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 signficantly 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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Abstract

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study, the mechanisms of action and the *in vivo* antibacterial 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 used 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 checkerboard 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.

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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. The ability of the hybrid peptides to act synergistically with standard antibiotics, proposes that our novel AMPs could play a great role in combating antibiotic resistance, as they can reduce the therapeutic concentrations of standard drugs.

1. Introduction

Pneumococcus is a major human respiratory pathogen in both children and adults (Jacobs, 2004; Cao et al., 2007). Formerly known as *Diplococcus pneumoniae, S. pneumoniae* is Gram-positive, α hemolytic and encapsulated bacterium 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, a 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, of which 0.7 to 1 million are 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, 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 pneumococcal strains resistant 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% 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). An earlier 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%) in 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. pneumoniae* and helped reduce the frequency of vaccine serotypes, there has been a considerable rise in the disease induced by non-vaccine serotypes (Reynolds et al., 2014). Hence, newer classes of antibacterial agents to overcome this serious issue are a top priority worldwide. One of the advantageous alternatives to today's antibiotics is antimicrobial peptides (AMPs) (Deslouches et al., 2013; Sánchez-Vásquez et al., 2013). AMPs are 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 micro-organisms (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 act principally by disrupting bacterial membrane integrity. AMPs' membrane destructive properties come from their cationic and hydrophobic composition, which gives 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 earlier study, we had designed 13 novel antimicrobial peptides, based on two naturally occurring templates, indolicidin and ranalexin (Jindal et al., 2015). Of these, five hybrid peptides (RN7-IN10, RN7-IN9, RN7-IN8, RN7-IN7 and RN7-IN6) presented the most potent antimicrobial activity against 30 pneumococcal clinical isolates. The MICs of RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 ranged from 7.81 to 15.62μg/ml for each peptide, while the MIC of RN7-IN7 was 62.5 μg/ml. Also, none of the hybrid peptides revealed any cytotoxic effects against human cells at their MIC levels. RN7-IN10 peptide was designed by fusing the first seven amino acids at the N-terminus (FLGGLIK) of ranalexin with the 4^{th} to 13^{th} residual fragment (WKWPWWPWRR) of indolicidin. Likewise, RN7-IN9, RN7-IN8, RN7-IN7 and RN7-IN6 were also designed by trimming the first seven amino acid residues of ranalexin and fusing it with 5^{th} to 13^{th}, 6^{th} to 13^{th}, 7^{th} to 13^{th} and 8^{th} to 13^{th} residual fragments of indolicidin (Jindal et al., 2015) in order to preserve the biological activity of both segments in the newly designed hybrid peptides (Table 1 illustrate the sequences and physicochemical properties of all five hybrid peptides). In this study, we describe the mechanisms of action, the *in vitro* synergism effect and the *in vivo* antibacterial 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 cationally adjusted as described in the guidelines of Clinical and Laboratory Standard Institute (2012).

116 **Transmission electron microscopy (TEM)**

117 Bacteria were prepared for TEM according to the guidelines of the Electron Microscopy
118 Unit at the Faculty of Medicine, University of Malaya. *S. pneumoniae* cultures were grown
119 overnight on Columbia agar with 5% sheep blood and suspended in cationally adjusted Mueller-
120 Hinton broth (CAMHB) at 10^8 CFU/ml. Pneumococcal suspensions were incubated with hybrid
121 peptides RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 at 125µg/ml and with RN7-IN7 at
122 500µg/ml (8 × of their respective MIC) in a 1.5ml eppendorf tube for 1hr at 37°C under 5% CO_2.
123 Cells in cationally adjusted Mueller-Hinton broth (CAMHB) were used as an untreated control.
124 After an hour of incubation, the pneumococcal cells were centrifuged to discard the medium,
125 washed thrice with 10 mM phosphate buffer saline at pH 7.3 and fixed overnight in 4% (v/v)
126 glutaraldehyde. All samples were washed twice with cacodylate buffer, incubated for 2hr in
127 osmium tetroxide buffer (OsO4 1: 1 cacodylate), washed twice with cacodylate buffer and then
128 incubated overnight in cacodylate buffer. All the samples were washed with distilled water twice
129 and incubated for 10 min with uranyl acetate. After this, all samples were then washed twice with
130 distilled water and dehydrated in an ascending series of ethanol: 35% (10 min), 50% (10 min),
131 70% (10 min), 95% (15 min), and thrice in 100% ethanol (15 min). After dehydration, Samples
132 were incubated with propylene oxide (15 min), propylene oxide 1:1 Epon (1hr), propylene oxide
133 1:3 Epon (2hr) and finally incubated overnight with Epon. All the samples were embedded in
134 agar 100 resin at 37°C for 5hr and maintained at 60°C until viewing. Reichert Ultramicrotome
135 copper grids 3.05 mm (300 square mesh) (Agar Scientific) were used to prepare Ultrathin
136 sections. Ethanol-based uranyl acetate and lead citrate were used to stain the samples for 5
137 minutes. Transmission electron microscope (Leo Libra 120) was used to capture the images.

138 **Scanning electron microscopy (SEM)**

S. pneumoniae with a starting inoculum of 1×10^8 cells/mL in CAMHB medium, was treated with hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 at 125µg/ml and with RN7-IN7 at 500µg/ml of respective peptides and incubated for 1hr at 37°C for under 5% CO_2. After incubation, 20 µL of the untreated and treated cell suspensions were transferred onto 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 fixed overnight 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 twice 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 an 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 a slight modification (Tanida et al., 2006). The ATP determination kit (Molecular Probes, USA) was used to measure the amount of ATP released based on the luciferin/luciferase method according to the manufacturer's instructions. Briefly, *S. pneumoniae* were grown overnight on Columbia agar with 5% sheep blood. Bacterial suspensions were spectrophotometrically adjusted to (1×10^7 CFU/mL) and incubated with hybrid peptides RN7-IN10, RN7-IN9, RN7-IN8 and RN7-IN6 at 125µg/ml and with RN7-IN7 at 500µg/ml. The amount of ATP released form the pneumococcal cells were measured at three time points 1, 2 and

3hr. 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). *S. pneumoniae* were grown overnight on Columbia agar with 5% sheep blood. A few bacterial colonies were transferred into a 1.5ml eppendorf tube containing phosphate buffered saline (PBS). The bacterial cells were centrifuged, PBS was discarded and 50 µL of TE buffer containing 0.08g/mL of lysozyme and 150 U/mL of mutanolysin was added to the cells. Genomic DNA was isolated from pneumococcal cells using DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's giudlines. The optical density ratio of 260 and 280nm (OD260/OD280 = 1.83) was used to measure the purity of the DNA. Genomic DNA (250 ng) was incubated with hybrid peptides at various concentrations (0.24-500µg/ml) 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 cultured overnight using Columbia agar with 5% sheep blood at 37 °C under 5% CO_2, resuspended in cation-supplemented Mueller-Hinton broth and adjusted to 5×10^5 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 checkerboard titration method described previously, with minor modification (Bajaksouzian et al., 1996). Briefly, 50µl of eight serial two-fold dilutions of drug B starting at 4×MIC were added to each column of the plate followed by 50µl of a fixed 0.25×MIC of drug A, this yielded 8 peptide-peptide combinations at different ratios. 100µl of bacterial suspension (5×10^5 CFU/ml) were then added to each well and the plates were incubated for 24hr at 37 °C under 5% CO_2. The fractional inhibitory concentration (FIC) index of each combination was calculated according to the following formula:

$$\mathbf{FI} \quad \frac{\textbf{MIC of drug A in combination}}{\textbf{MIC of drug A alone}} + \frac{\textbf{MIC of drug B in combination}}{\textbf{MIC of drug B alone}} \quad \mathbf{CI} =$$

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≤ 0.5, additive or indifference if $0.5 < FIC \leq 4.0$ and antagonism if $FIC > 4.0$. The experiment was done in triplicate.

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 to assess the *in vivo* toxicity and antibacterial efficacy of the novel peptides, as these animals are by far the most commonly used model for the study of pneumococcal disease (Chiavolini, Pozzi & Ricci, 2008). The 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 first 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 case adverse effects such as high physical stress, severe lethargy, physical inactiveness, and/or death were detected, lower graded doses were given. All the administered mice were monitored for 7 days or until death occurred. 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 the models. The bacterial isolate was grown overnight on Columbia agar with 5% sheep blood at 37 °C under 5% CO_2. The bacterial suspension was adjusted to OD_{625} 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 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. Only mice injected with PBS 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 surviving 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 the 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. Just before injection, both the 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 a 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 the surviving 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 heamatological analysis was collected in 500 µl dipotassium EDTA microtainer tubes (BD Bioscience, USA). About 500 µl of blood collected an eppedorff 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

272 embedding. Hematoxylin–eosin (HE) was used to stain the histological sections at the
273 histopathology laboratory, Veterinary Laboratory Service Unit, University Putra Malaysia (UPM).

274 **Statistical analysis**

275 GraphPad Prism 5 was used to perform the Statistical analysis. The results were expressed
276 as mean ± standard deviation. Two-way ANOVA with Bonferroni post-test was used to analyze
277 the significance of the difference between the treated groups and control in ATP assay. One-way
278 ANOVA with *post-hoc* Dunnett-t test was used to assess the statistical difference between the
279 blood haematogram and blood serum biochemistry parameters of the treated and the untreated
280 control groups in the *in vivo* toxicity assay. Kaplan-Meier analysis with log-rank test (Mantel-
281 Cox) was used to generate the survival curve for each treated group versus untreated control, for
282 both *in vivo* antibacterial activity and *in vivo* synergy assays.

283 **3. Results**

284 **Effects of hybrid peptides on cell morphology and membrane permeability**

285 TEM and SEM studies were performed to observe the damaging effect of the hybrid
286 peptides on the pneumococcal cell wall/membrane. The images obtained clearly indicated that all
287 the hybrid peptides were capable of disrupting the integrity of bacterial membranes. As shown in
288 Fig. 1A, the untreated cells appeared with complete cell wall and plasma membrane and therefore
289 preserved the normal integral shape of *S. pneumoniae*. The pneumococcal capsular
290 polysaccharide appeared as a thin layer sheltering the whole cell and the cytoplasm of the cell
291 was compactly packed and occupied the entire space (Fig. 1A, arrow 1). Incubation of
292 pneumococcal cells with hybrid peptides had led to a dramatic effect on the morphology of
293 bacterial surface. After 1hr of incubation, the hybrid peptides were able to breach the intactness
294 of the cell wall and/or plasma membrane, causing membrane breakage and loss of fragments

(Fig. 1B-F, arrow 2). 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 3). 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 4).

Scanning electron microscopy (SEM) was employed 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 1), whereas *S. pneumoniae* treated with hybrid peptides at 8 × MIC appeared with a rough and injured surface (Fig. 2B-F, arrow 2). The numerous fragments observed on the bacterial surface are an indication of cell wall breakage and fragments loss upon treatment with hybrid peptides. 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 the 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±92pM respectively) (Fig. 3). The ATP efflux steadily decreased, and the ATP release reached 25.42±3.51 and 22.9±3.22pM 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±0.32pM 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±1.43pM and 0.49±0.004pM, 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 concentrations was assessed by analyzing electrophoretic movement of pneumococci DNA bands through the agarose gel (1%, w/v). Our results clearly indicated that all the 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, the 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 was 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 FICI of less than 0.5 (Table 2), regardless of the susceptibility of pneumococcal isolates towards standard drugs. Likewise, combinations of standard drugs ceftriaxone and erythromycin with all five hybrid peptides presented synergistic effects with fractional inhibitory concentration (FIC) index of ≤0.5 against both isolates of *S. pneumoniae,*

regardless of their susceptibility to antibiotics (Table 2). These results indicate that all the hybrid peptides were able to enhance the antibacterial activity of both the 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 in the case of mice treated with all hybrid peptides via subcutaneous (SC) injection at the 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 giving the mice hybrid peptides via the intranasal (IN) route. However, treatment with RN7-IN9 displayed significantly lower MCV ($p = 0.001$) (Table S2, highlighted in yellow) than the control group. Mice treated with RN7-IN6 displayed significantly lower percentage of granulocytes ($p = 0.0482$) (Table S2, highlighted in blue), as compared to the control group. Histological examination of the organs collected from all the treated and control groups did not expose any histopathological changes (Fig. S2).

In terms of the 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

366 graded doses were attempted until we reached the maximum dose at which no signs of stress or
367 abnormal behavior were evident. Hybrid peptides RN7-IN10 and RN7-IN8 did not display any
368 sign of toxicity when injected at 20mg/kg; no death occurred in any of the treated mice up to 7
369 days post-treatment. RN7-IN9 and RN7-IN6 were non-toxic when injected at 10mg/kg. None of
370 the five major organs of the treated mice revealed any significant histological abnormality, as
371 compared to the untreated control group (Fig. S3). However, mice treated with RN7-IN9 (10
372 mg/kg) via IP route showed significantly lower lymphocytes ($p = 0.0445$) and lower ALP ($p =$
373 0.0187) (Table S3, highlighted in yellow), while RN7-IN6 treated mice had lower ALT ($p =$
374 0.0425) when compared to the control group (Table S3, highlighted in blue).

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

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

389 In addition to our designed peptides, ceftriaxone, as a standard drug, was used to treat
390 infected mice via IP route at 5mg/kg, 10mg/kg, 20mg/kg, 40mg/kg, and 80mg/kg and the survival
391 was 10%, 30%, 40%, 70% and 90% up to seven days post-infection ($p < 0.0001$) (Fig. 6). In the
392 pneumococcal pneumonia model, none of the mice treated with both peptides via IP, SC and IN
393 routes survived up to seven days post-infection and therefore, this model was excluded from
394 further studies.

395 Combinations of peptide-peptide were also assessed for their ability to treat infected mice
396 with pneumococcal bacteremia. Two combinations were used, 5mg/kg + 5mg/kg and 10mg/kg +
397 10mg/kg to treat mice via IP route at three regimens 1hr, 12hr and 24hr. The results indicate that
398 the combination of 5mg/kg + 5mg/kg was able to treat 40% of the mice and protected them from
399 death up to 7 day post-infection ($p = 0.0003$), while increasing the dose to 10mg/kg of each
400 peptide resulted in 60% of the infected mice surviving the pneumococcal infection ($p < 0.001$)
401 (Fig. 7).

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

403 Among the hybrid peptides RN7-IN10 and RN7-IN8, standalone treatment with RN7-IN8
404 at 20mg/kg was found to confer significant survivability on mice infected by a highly virulent
405 pneumococcal clinical isolate via IP route. To assess the synergistic effect of RN7-IN8 in
406 combination with the standard drug ceftriaxone (CTX), three different doses of RN7-IN8
407 (5mg/kg, 10mg/kg and 20mg/kg) and ceftriaxone (5mg/kg, 10mg/kg and 20mg/kg) were tested,
408 using the same bacteremia infection model in three treatment formulations: $RN7\text{-}IN8_5 - CTX_5$
409 (5mg/kg of RN7-IN8 and 5mg/kg of CTX), $RN7\text{-}IN8_{10} - CTX_{10}$ (10mg/kg of RN7-IN8 and
410 10mg/kg of CTX) and $RN7\text{-}IN8_{20} - CTX_{20}$ (20mg/kg of RN7-IN8 and 20mg/kg of CTX). Using
411 groups of 10 mice, the combinations of RN7-IN8 and ceftriaxone $RN7\text{-}IN8_5 - CTX_5$ (5mg/kg of
412 RN7-IN8 and 5mg/kg of CTX), $RN7\text{-}IN8_{10} - CTX_{10}$ (10mg/kg of RN7-IN8 and 10mg/kg of CTX)
413 and $RN7\text{-}IN8_{20} - CTX_{20}$ (20mg/kg of RN7-IN8 and 20mg/kg of CTX) led to survival rates of

60%, 80% and 100% in mice infected with highly virulent pneumococcal strain up to seven days post-infection ($p < 0.0001$) (Fig. 8). Our results displayed 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 which survived from the infection appeared physically active and none of them showed signs 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 the 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 denoted 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 (Fig. 9B). Severe tissue injuries 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 the 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

438 damage were found in the organs, the degree and severity of the damage were significantly less
439 than the infected control group. Unlike the lung of the untreated mice which exhibited severe
440 inflammation and the alveolar spaces were about 90% congested (Fig. 9A), all the lungs
441 harvested from the treated mice revealed only low level of congestion and minor thickening of
442 the alveolar wall, even though these histological changes were still noticeable in the mice (Fig.
443 9C-G). Treatment of infected mice with a combination of hybrid peptide RN7-IN8 and
444 ceftriaxone at three different dosages (5mg/kg - 5mg, 10mg/kg - 10mg and 20mg/kg - 20mg/kg)
445 showed gradual decrease in the degree of congestion and damage (Fig. 9E-G). Lungs harvested
446 from mice treated with a combination of RN7-IN8 and ceftriaxone at 20mg/kg + 20mg/kg (Fig.
447 9G) which presented 100% mice survival, were similar to those harvested from the uninfected
448 control mice (Fig. 9B) in degree of normality. Likewise, all the spleens of treated mice displayed
449 no or minimum damage, with the white and red pulps being clearly observed (Fig. 10C-G), as
450 compared to the infected one (Fig. 10A). No significant tissue damage was observed in the brain,
451 kidney and liver in both treated and untreated mice.

452 **4. Discussion**

453 We report here the mechanisms of actions, *in vivo* toxicity and antibacterial efficiency of
454 five hybrid peptides designed earlier in our lab, based on two templates, indolicidin and
455 ranalexin. TEM and SEM were used to evaluate the morphological alterations caused by hybrid
456 peptides. Results obtained from TEM displayed strong evidence that targeting the bacterial cell
457 wall/plasma membrane is the main antibacterial mechanism used by hybrid peptides. Unlike the
458 untreated cells, pneumococcal cells treated with hybrid peptides faced dramatic morphological
459 changes. The breakage and fragments loss of the bacterial cell wall/membrane is probably a result
460 of the strong interaction between the negatively charged membrane and the hybrid peptides due
461 to their positive charge and high hydrophobic content (Jindal et al., 2015). Unlike the normal

462 mammalian cell membranes, bacterial membranes are richer in highly electronegative lipids such
463 as phosphatidylserine (PS), cardiolipin (CL) or phosphatidylglycerol (PG). These acidic
464 phospholipids tend to make the bacterial membrane highly negative in charge and thus attract the
465 positively charged antimicrobial peptides to attach to the bacterial membranes and make them
466 preferred by AMPs over mammalian membranes (Ghavami et al., 2008). To the contrary, the
467 membrane of mammalian cells is enriched with zwitterionic phospholipids such as
468 sphingomyelin (SM), phosphatidylethanolamine (PE) or phosphatidylcholine (PC), which are
469 neutral in net charge. These substances prevent the amalgamation of peptide molecules into cell
470 membranes and thus prevent pores formation (Yeaman & Yount, 2003). In addition to their
471 positive charge, these five hybrid peptides have a high content of hydrophobic residues. Peptide
472 hydrophobicity is another critical property that governs the attraction of AMPs toward bacterial
473 membrane, as it directs the level to which an AMP can penetrate into the lipid bilayer (Yeaman &
474 Yount, 2003). The high content of tryptophan (Trp) is another advantage of these hybrids. It is
475 well known that Trp has a significant role in the interaction of antimicrobial peptides with the
476 bacterial membrane, as this amino acid strongly prefer the interfacial regions of lipid bilayers. In
477 certain cases, Trp is considered hydrophobic due to its uncharged sidechain. However, it is
478 observed that Trp residues do not reside in the hydrocarbon region of lipid bilayers and
479 accordingly it is placed towards the more hydrophilic side of the scale (Chan, Prenner & Vogel,
480 2006). Another key factor of this amino acid is its ability to form an extensive π–electron system.
481 Cation–π interaction occurs between the cationic sidechains of the basic amino acids arginine
482 (Arg) or lysine (Lys) and the aromatic sidechains of the aromatic amino acids tryptophan (Trp),
483 tyrosine (Tyr) or phenylalanine (Phe) (Gallivan & Dougherty, 1999). Cation–π interactions are
484 significant for peptide self-association inside membranes and enable deeper insert into
485 membranes by sheltering the cationic side chains (Torcato et al., 2013). The detachment of the
486 cytoplasmic membrane from cell wall observed in pneumococcal cells upon treatment with

487 hybrid peptides, is a possible indication of the capability of hybrid peptides to interpolate
488 themselves between pyrophosphate-linked cell-wall anchors and the cell membrane.
489 Subsequently, this act would pullout the isoprenyl anchor chains away from the cell membrane
490 and weaken cell-wall adhesion. The results obtained by TEM were similar to those reported on
491 the mechanism of nisin against *B. subtilis* (Hyde et al., 2006) and *E. faecalis* (Tong et al., 2014).
492 Likewise, chicken CATH-2 was able to disrupt and detach the plasma membrane from the cell
493 wall of *C. albicans* (Ordonez et al., 2014). Another possible explanation is that the breakage of
494 the cell wall allows the insertion of water from the medium into the space between the two
495 membranes and detach them (López-Expósito, Amigo & Recio, 2008). Likewise, SEM studies
496 showed the damaging effects of hybrid peptides on the bacterial surface. Unlike the untreated
497 cells which appeared with normal and smooth cell surface, pneumococcal cells incubated with
498 hybrid peptides were appeared with swelling and aggregation. Besides, the numerous fragments
499 observed in *S. pneumoniae* cultures treated with hybrid peptides point to a cell wall breakage and
500 cell lysis. Membrane disruption could be associated with leakage of ions and metabolites,
501 depolarization and eventually cell death. Adenosine triphosphate (ATP) is one of most significant
502 molecules for all living cells, as it used as an intracellular source of energy for many biological
503 processes (Mempin et al., 2013). In normal conditions, bacterial membranes are impervious to the
504 efflux of ATP and other intracellular constituents, as membrane destabilization might lead to the
505 release of normally impervious substances. Therefore, ATP has been used as a tool to measure the
506 integrity of living cells. Since most of the ATP is found within the cells, any cell injury will result
507 in a prompt reduction in the cytoplasmic ATP. Our results revealed that the ATP efflux was not
508 increased by the incubation of pneumococcal cells with standard drugs ceftriaxone and
509 erythromycin, but was increased by incubation with hybrid peptides. Although ceftriaxone is a
510 member of the β-lactam family of antibiotics, its ability of releasing intracellular ATP was less
511 than that of the hybrid peptides. This is probably due to the fact that AMPs exert their

antimicrobial activity faster than standard drugs. As we have shown in our previous paper, the hybrid peptides were able to exert their bactericidal activity within one hour of incubation with resistant *S. pneumoniae,* whereas ceftriaxone could not eliminate *S. pneumoniae* up to 240 min of incubation (Jindal et al., 2015). 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 levels of ATP released after 5min of incubation were higher than the levels of ATP after 1hr of incubation (Ordonez 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 the TEM, SEM and ATP release assay indicate 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 in a membrane-disruptive way when present in a high concentration, but shift to attack intracellular components, when present in a 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 or proteins (Li et al., 2013). For instance, the antimicrobial peptide buforin II has the ability to translocate itself 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

illustrated 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. Hsu and co-workers have revealed through their work that the ability of the parent peptide indolicidin to permeabilize bacterial membranes is not the only mechanism of antimicrobial action. Indolicidin is also capable of binding efficiently to DNA and form a complex. The ability of indolicidin to penetrate the cell membrane allows the peptide to translocate itself to the cytoplasm and bind to the negatively charged DNA via its positive charge (Hsu et al., 2005). Moreover, Ghosh and co-workers have identified the central motif (PWWP) of Indolicidin responsible of stabilizing the DNA duplex and thus inhibiting DNA replication and transcription. The two tryptophan residues of the central motif play a significant role in stabilization of the duplex by desolvating the core of the DNA (Ghosh et al., 2014). This motif is conserved in our hybrid peptides and therefore, we hypothesize that the hybrid peptides like their parent indolicidin are most probably able to bind to bacterial DNA and preventing its intracellular function. The interaction of peptides with bacterial DNA can prevent or hinder gene expression, which is an efficient way to suppress and inhibit normal enzyme and receptor synthesis, damaging the intracellular components required for the life cycle of the bacterial cell and thus leads to 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 chequerboard MIC technique to assess peptide-drug interaction. Our results

562 revealed that all the five hybrid peptides exhibited synergistic effects against pneumococcal
563 clinical isolate, when combined with each other and with conventional drugs erythromycin and
564 ceftriaxone. One possible explanation of the synergistic effects of peptides-drug combinations is
565 that the hybrid peptides may increase permeability by interacting with the bacterial cell
566 wall/membrane, making it easier for conventional drugs to act on their targets. Previous reports
567 have shown that β-lactam antibiotics like ceftriaxone exert higher antimicrobial activity, when
568 combined with membranolytic peptides such as nisin, as these AMPs cause changes in cell
569 morphology by forming pores, allowing antibiotics to enhance their action and produce a greater
570 damage within the cell wall (Singh, Prabha & Rishi, 2013; Tong et al., 2014). Another possible
571 mechanism of synergistic combinations is that the antimicrobial peptides alter the efflux pump
572 systems, allowing intracellular antibiotics such as macrolides to act more efficiently on their
573 intracellular targets (Ruhr & Sahl, 1985; Soren et al., 2015).

574 For evaluating the *in vivo* therapeutic efficacy of peptides in living organisms, two hybrid
575 peptides RN7-IN10 and RN7-IN8 were selected to treat mice models infected with a highly
576 virulent pneumococcal strain. Among all the doses assessed, RN7-IN8 showed an interesting *in*
577 *vivo* antibacterial efficacy. At low dose (20mg/kg) RN7-IN8 resulted in 50% survival of the
578 infected mice with bacteremia model, up to seven days after infection ($p < 0.001$), as compared to
579 the untreated mice. Combinations of hybrid peptides tested for the synergistic effect resulted in
580 increased survival rates of infected mice. These findings are in agreement with our *in vitro*
581 synergism results which showed that hybrid peptides are able to enhance the biological activity of
582 each other.

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

rate, in comparison with a 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 increased significantly (Chiu et al., 2007). Hence, RN7-IN8, which showed significant therapeutic efficacy in 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 increased dramatically 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% of mice treated with combination of RN7-IN8 and ceftriaxone (20mg – 20mg) survived at day seven post-infection. Using a combination of these two drugs at a low dose (20mg/kg) showed better survival rate than the use of ceftriaxone alone at a 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 ceftriaxone did not show sign of sickness or abnormal behavior.

5. Conclusion

In sum, 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, thus reducing potential resistance.

Although RN7-IN8 showed promising therapeutic outcome, there are some limitations in its efficacy. Primarily, the peptide had no effect on 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 at the same site. Hence, AMPs have a huge potential to play a crucial role in combating resistant bacteria, either as standalone therapeutics or in combination with other drugs.

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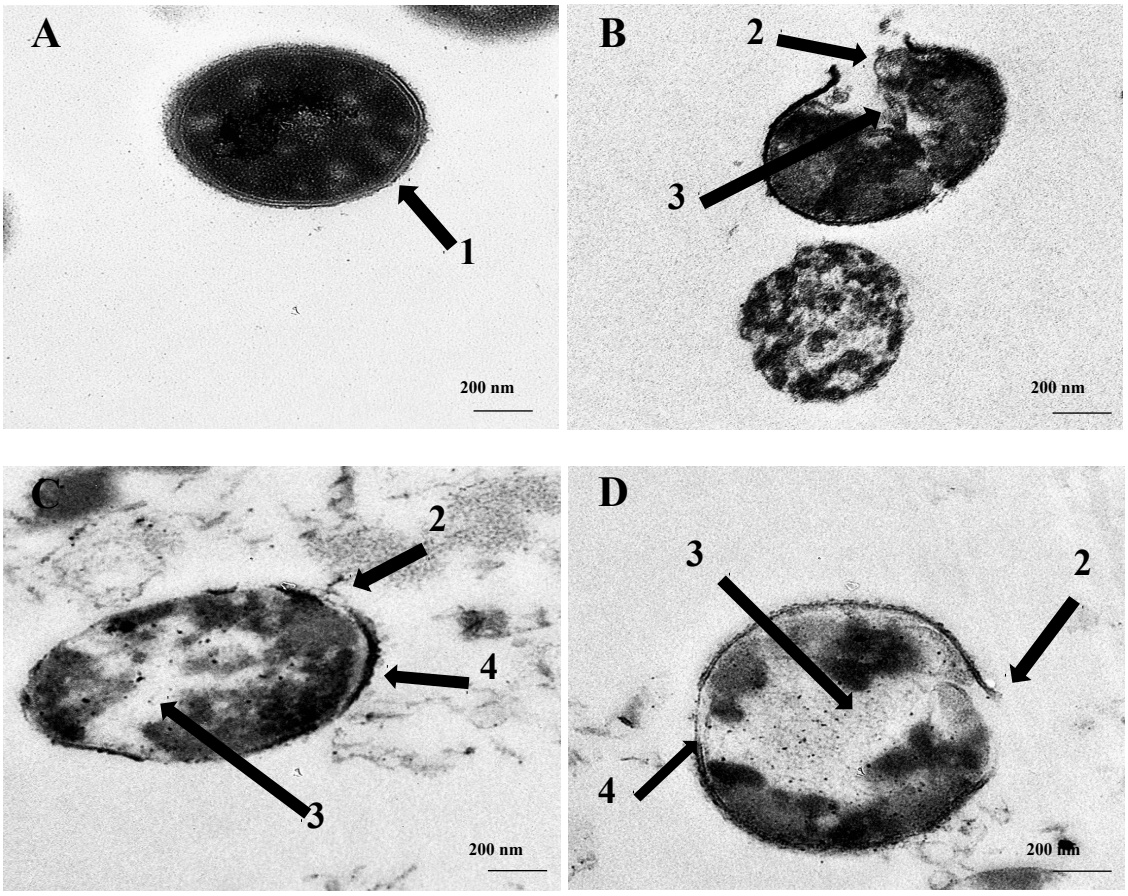
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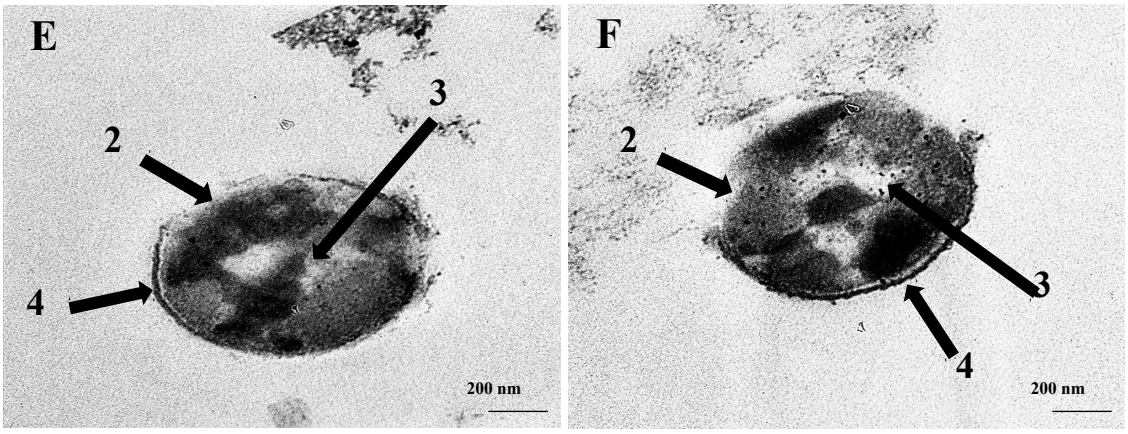
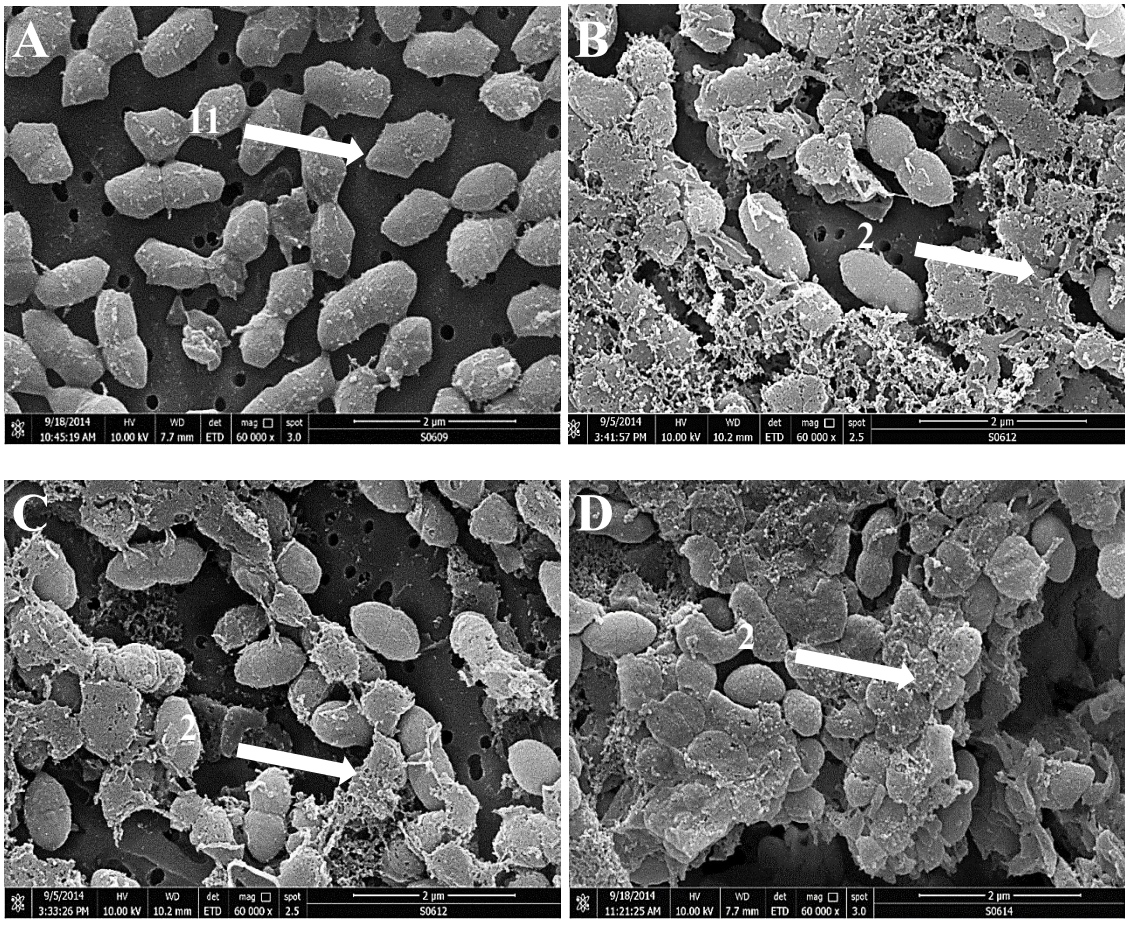


789 **Fig 1. Transmission electron micrographs of *S. pneumoniae* after treatment with hybrid**
790 **peptides.** (A) Control cells without treatment appeared with normal shape (Fig1A, arrow a). Fig
791 B – F display the damage of pneumococcal cells after 1hr incubation in presence of (B) RN7-
792 IN10, (C) RN7-IN9, (D) RN7-IN8, (E) RN7-IN7 and (F) RN7-IN6. (Arrow b) Breakage and loss
793 of cell wall/membrane fragments. (Arrow c) Leakage of cytoplasm and halos formation. (Arrow
794 d) detachment of cytoplasmic membrane from pneumococcal cell wall. Bar indicates 200 nm.



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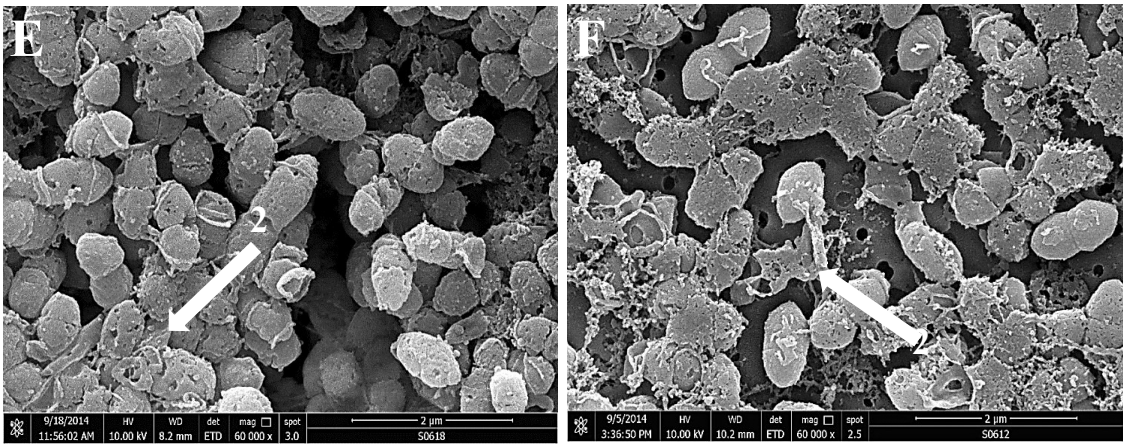
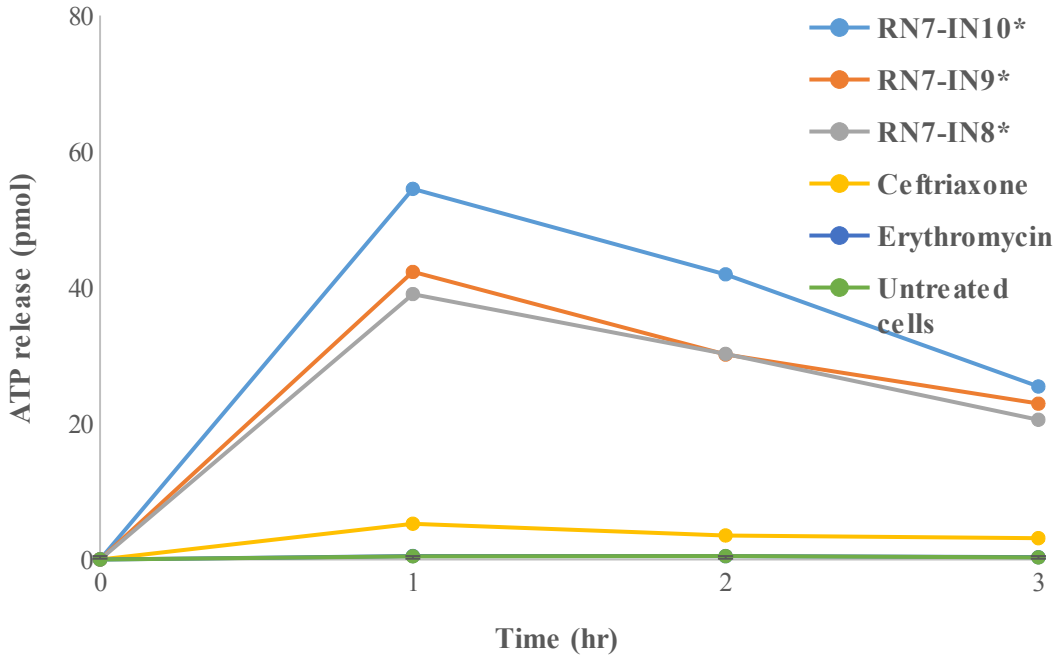


Fig 2. Scanning electron micrographs of *S. pneumoniae* after treatment with hybrid peptides. (A) Control cells without treatment appeared with normal shape and smooth surface (arrow a). Fig B – F show the severe morphological changes and surface disruption (arrow b) of pneumococcal cells following 1hr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



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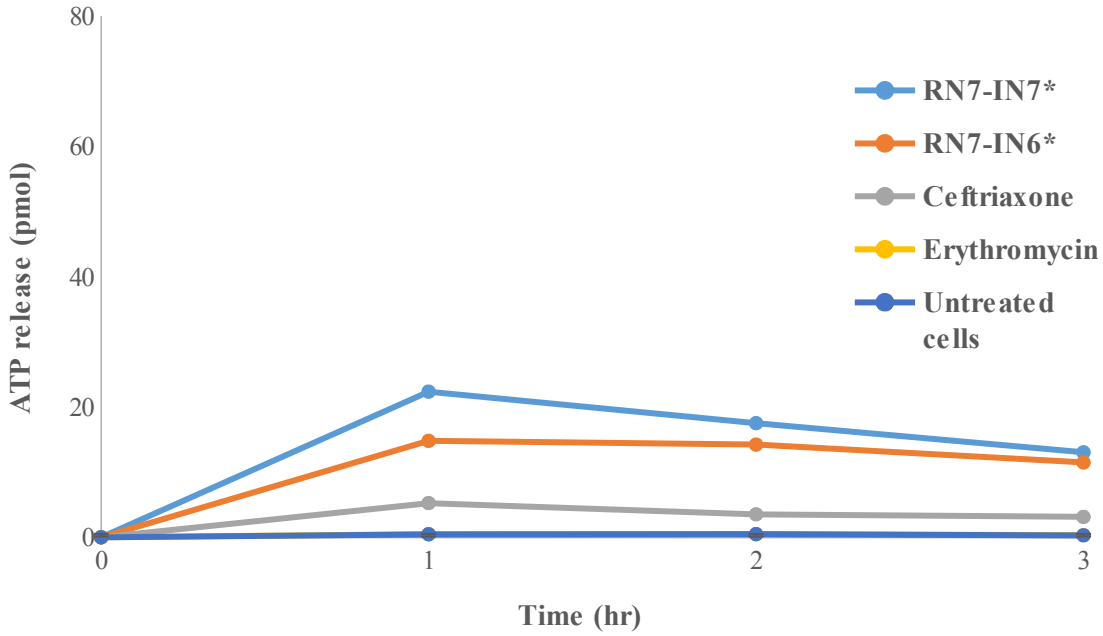

Fig 3. The influence of peptides on ATP release. All Hybrid peptides presented stronger ATP efflux activity than erythromycin and ceftriaxone. Two-way ANOVA with Bonferroni post-test was used to perform the statistical analysis. An asterisk (*) adjacent to peptide name directs statistical significance ($P < 0.0001$). Fig 3A shows the amount of ATP released upon treatment with RN7-IN10, RN7-IN9 and RN7-IN8. Fig 3B shows the amount of ATP released upon treatment with RN7-IN7 and RN7-IN6. The experiment was done in triplicate.

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

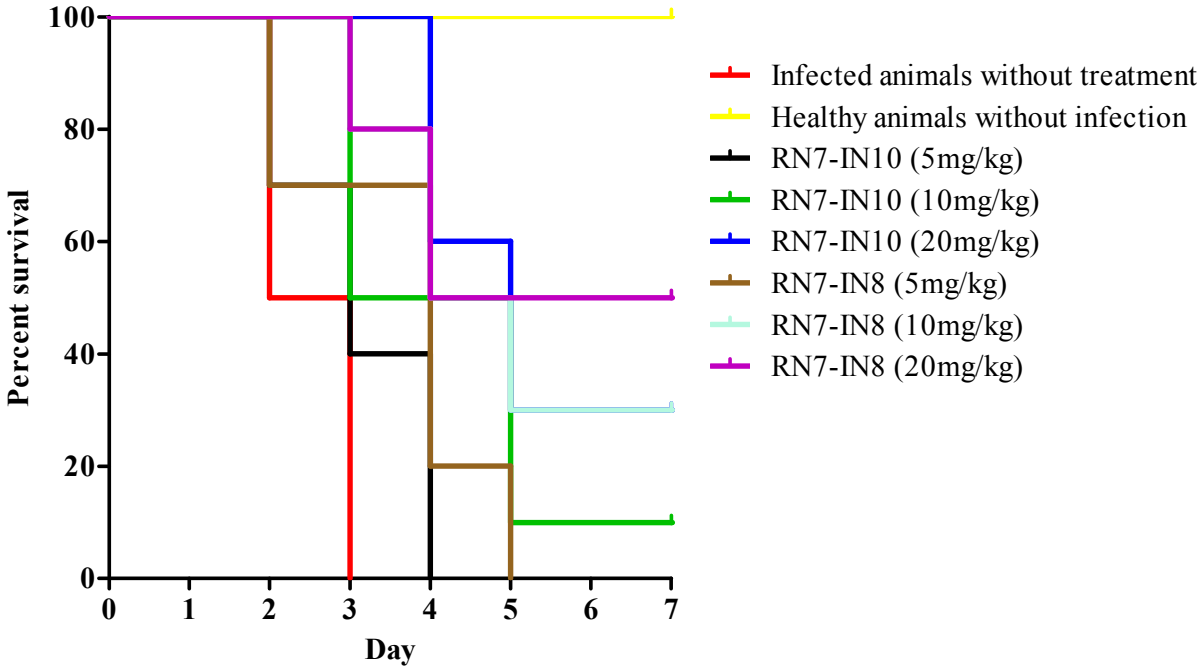

Fig 5. Survival curve of infected mice treated with RN7-IN10 and RN7-IN8. Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical for all treated groups and the untreated control using. Treatment with RN7-IN8 at 20mg/kg displayed the highest survival rate of 50% up to 7 days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

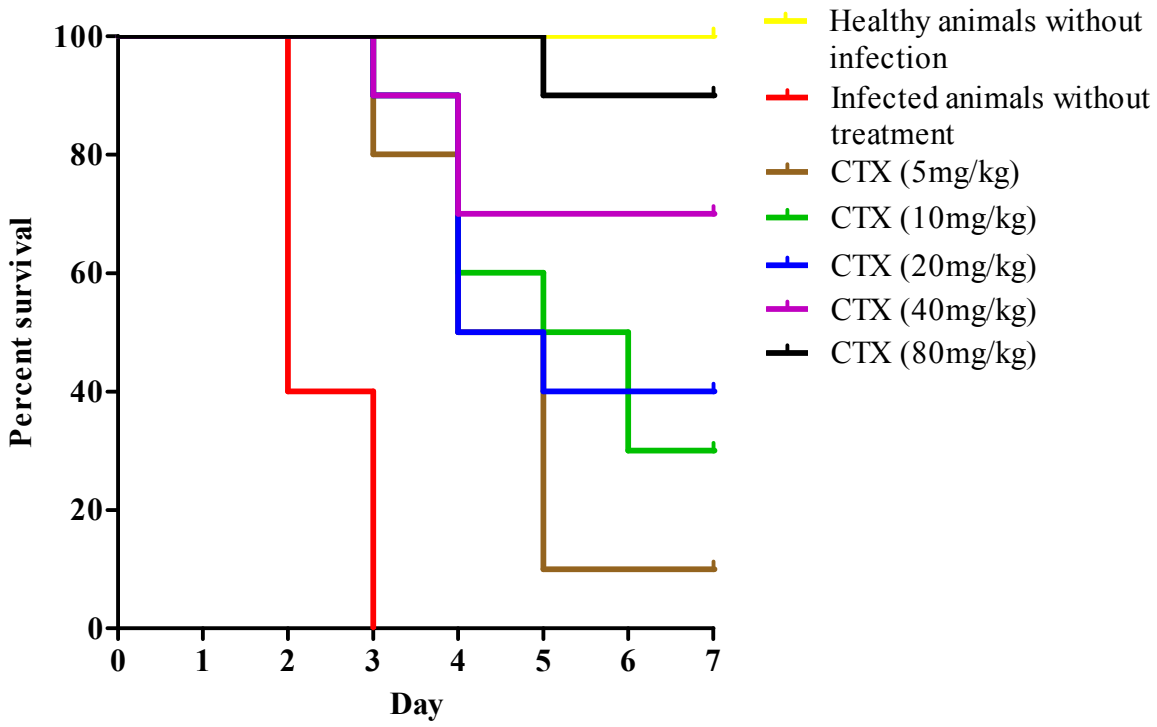

Fig 6. Survival curve of infected mice treated with ceftriaxone (CTX). Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group and the untreated control.

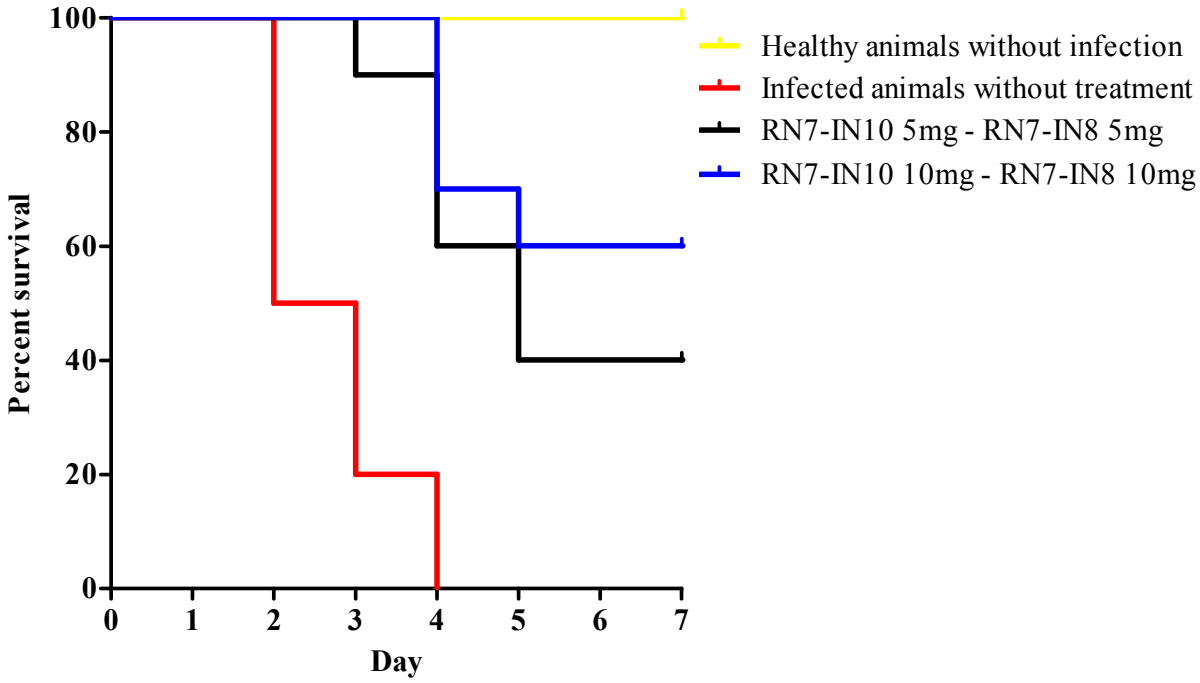

Fig 7. Survival curve of infected mice treated with combinations of RN7-IN10 and RN7-IN8. Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group versus the untreated control.

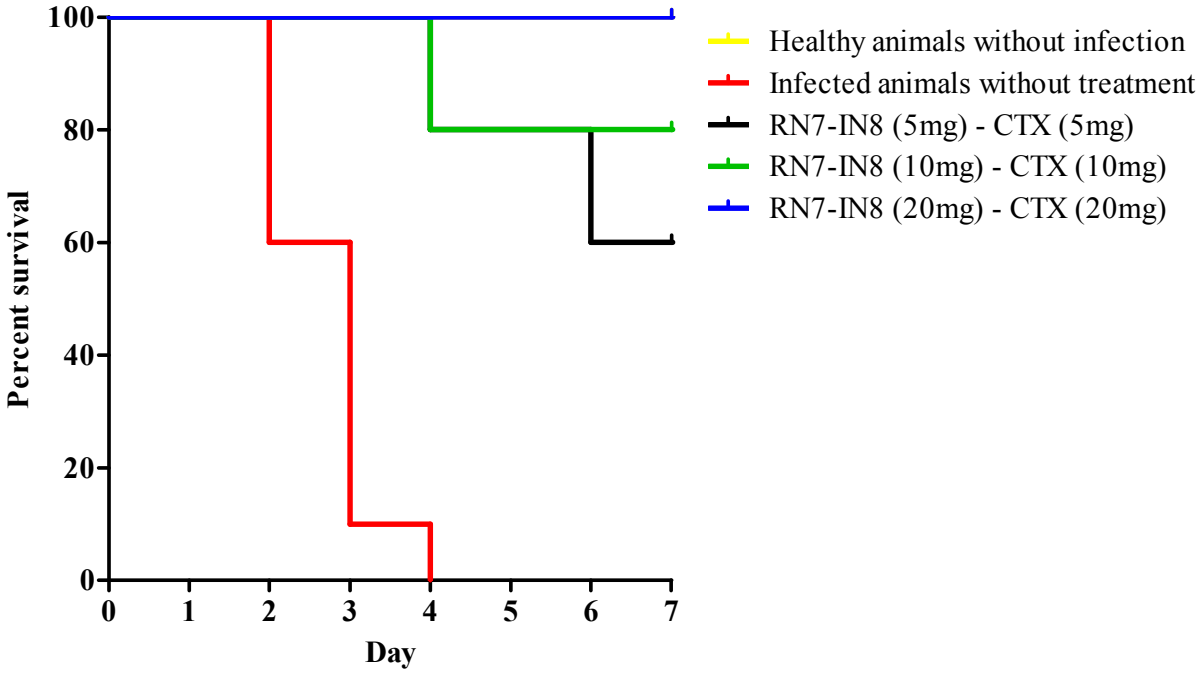

Fig 8. Survival curve of infected mice treated with combinations of RN7-IN8 and ceftriaxone (CTX). Kaplan-Meier with log-rank test (Mantel-Cox) was used to perform the statistical analysis for all treated group and the untreated control. Combination of RN7-IN and ceftriaxone at (20mg/kg – 20mg/kg) showed 100% survival ($P < 0.0001$).

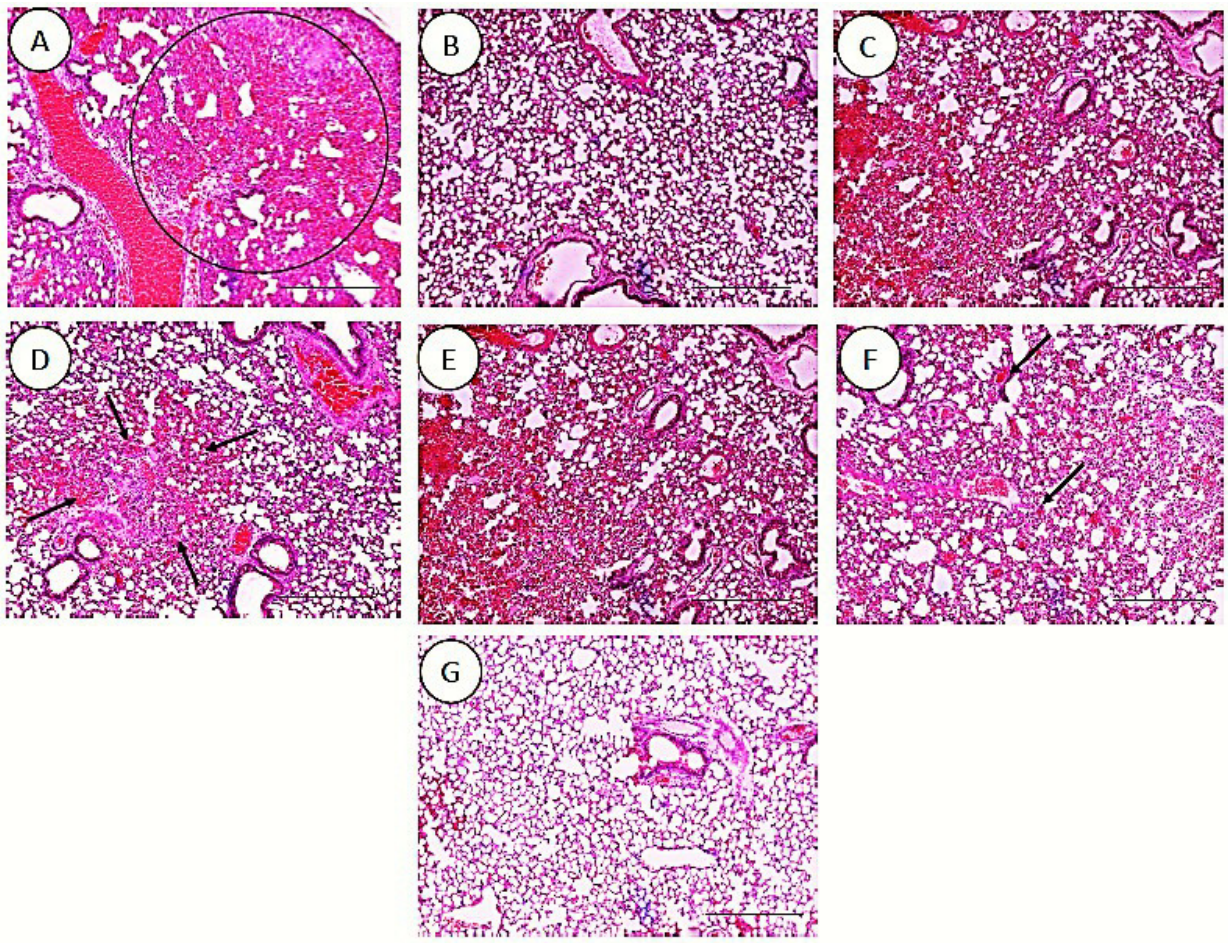

Fig 9. Histology of lungs harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated with combination 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 μM.

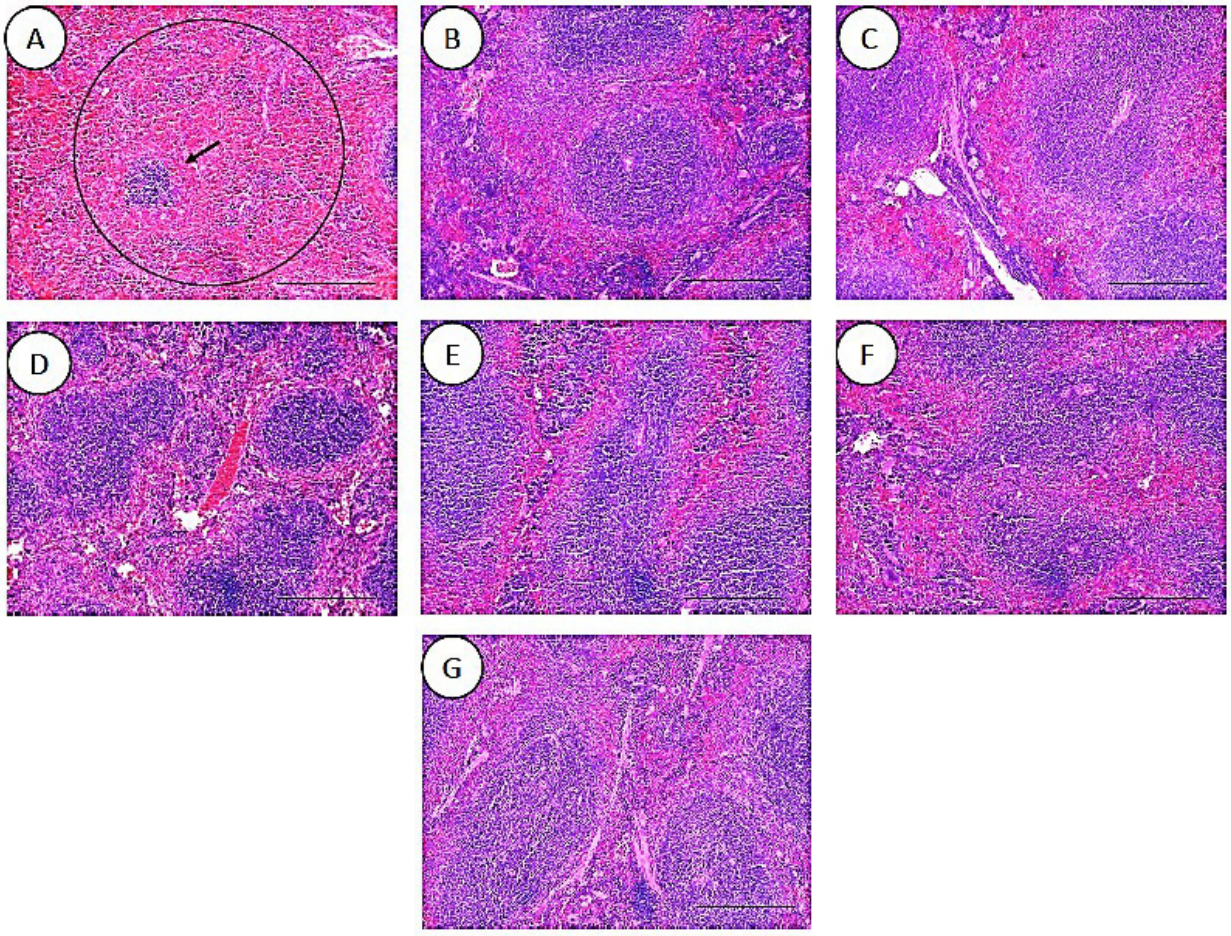

Fig 10. Histology of spleens harvested from mice infected with *S. pneumoniae* receiving treatments. (A) infected mice, (B) uninfected mice (control), (C) mice treated with RN7-IN8 (20mg/kg), (D) mice treated with combination of RN7-IN10 and RN7-IN8 (10mg/kg + 10mg/kg), (E) mice treated with combination 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 μM.

Peptide	Sequence	MIC[a]	aa[b]	MW[c]	Q[d]	Pho%[e]
Indolicidin	ILP**WKWPWWPWRR**-NH2	15.62-31.25	13	1907.30	+4	53%
Ranalexin	**FLGGLIK**IVPAMICAVTKKC-OH	62.5	20	2105.70	+3	65%
RN7-IN10	FLGGLIKWKWPWWPWRR-NH2	7.81-15.62	17	2300.791	+5	52 %
RN7-IN9	FLGGLIKKWPWWPWRR-NH2	7.81-15.62	16	2114.578	+5	50 %
RN7-IN8	FLGGLIKWPWWPWRR-NH2	7.81-15.62	15	1986.408	+4	53 %
RN7-IN7	FLGGLIKPWWPWRR-NH2	62.5	14	1800.195	+4	50 %
RN7-IN6	FLGGLIKWWPWRR-NH2	7.81-15.62	13	1709.078	+4	53 %

Table 1: Sequences and physicochemical properties of the template and hybrid AMPs.

841 [a] Minimum inhibitory concentration (μg/ml).
842 [b] Number of amino acids.
843 [c] Molecular weight.
844 [d] Net charge. Lys (K), Arg (R), and C-terminal amidation (NH_2) was assigned with +1 charge.
845 [e] hydrophobic residues%.
846

847 **Table 2:** FIC index of various combinations of hybrid peptides with each other and with standard
848 antibiotics against susceptible and resistant *S. pneumoniae*.

Combination			**Susceptible *S. pneumoniae***			**Resistant *S. pneumoniae***	
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**

[a]FIC index ≤ 0.5 represents synergy; > 0.5 – ≤4.0 represents indifference; > 4.0 represents antagonism.

Highlighted in bold: peptide-antibiotic combination with synergistic effect.