

# In vivo antimicrobial activity of the hybrid peptide H4: a follow-up study

Ammar Almaaytah<sup>1</sup>  
Qosay Albalas<sup>2</sup>  
Karem H Alzoubi<sup>3</sup>

<sup>1</sup>Department of Pharmaceutical Technology, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan; <sup>2</sup>Department of Medicinal Chemistry and Pharmacognosy, Jordan University of Science and Technology, Irbid, Jordan; <sup>3</sup>Department of Clinical Pharmacy, Jordan University of Science and Technology, Irbid, Jordan

**Background:** The consistent upsurge in antimicrobial resistance globally is threatening the world population with the prospect of facing the post-antibiotic era. Dry pipelines and a drastic decrease of antimicrobial drug development accompany this rise in antimicrobial resistance. Governments and health authorities are calling for the development of novel classes of antimicrobial agents that would tackle this problem. Antimicrobial peptides represent a promising group of molecules for antimicrobial drug development due to their potency and rapid mode of killing. However, several obstacles, such as high mammalian cell toxicity and lack of target selectivity, have challenged the development of such agents.

**Methods:** We have recently designed a novel hybrid peptide named H4 that exhibits potent antimicrobial activity and low toxicity in vitro. In order to confirm the potential therapeutic efficacy and safety of the peptide, we evaluated the in vivo activity and toxicity of H4 against *Staphylococcus aureus* peritonitis mice model.

**Results:** Our results indicate that H4 is highly potent in eradicating bacterial infections in vivo with an effective dose<sub>50</sub> value of 4.55±0.89 mg/kg. Additionally, the acute systemic toxicity results indicate that the peptide exhibits a high therapeutic index with no significant negative effects on the function of major body organs.

**Conclusion:** H4 is a novel hybrid peptide with great potential for antimicrobial drug development.

**Keywords:** antimicrobial peptides, hybrid peptides, in vivo activity, hybridization, antimicrobial resistance, drug development

## Introduction

The issue of bacterial resistance continues to haunt all world health authorities. The human population is currently facing microbial resistance on an unprecedented scale, with some bacterial strains becoming pan-resistant and combating all types of antibiotics available in the clinic.<sup>1</sup> With over 3.5 billion years of continuous evolution, bacteria have managed to develop highly efficient tools for evading the antimicrobial activity of conventional antibiotics.<sup>2</sup> This remarkable inherent evolutionary trait of bacteria, accompanied by the overuse and misuse of antibiotics by humans in recent decades, has led to the emergence of this scale of microbial resistance worldwide.<sup>3</sup> Conventional antibiotics, which were considered as “wonder drugs” in the previous century, are not as effective anymore and the world is currently set to face the prospect of a post-antibiotic era with catastrophic health and financial outcomes.<sup>4,5</sup> Additionally, this increasing trend of antimicrobial resistance is accompanied by a sharp decrease in the number of antibiotics developed by the pharmaceutical industry and approved

Correspondence: Ammar Almaaytah  
Department of Pharmaceutical  
Technology, Faculty of Pharmacy, Jordan  
University of Science and Technology, PO  
Box 3030, Irbid 22110, Jordan  
Tel +962 7 7765 8820  
Fax +962 2 720 1075  
Email amalmaaytah@just.edu.jo

by health regulatory bodies.<sup>6</sup> Therefore, the development of new classes of antibiotics is urgently needed.

Antimicrobial peptides (AMPs) represent an attractive class of molecules for antimicrobial drug development. These molecules are usually composed of short sequences of peptides, ranging from 10 to 50 amino acids, and exhibiting an amphipathic nature. AMPs have been proven to be very effective in killing most types of bacterial strains rapidly and efficiently, including the multi-drug resistant forms, *in vitro*.<sup>7</sup> Despite the attractive characteristics of AMPs for antimicrobial drug development, they have faced several obstacles that are mainly attributed to their significant cytotoxicity toward mammalian cells, hampering their clinical development.<sup>8</sup> We have recently reported the design of a novel hybrid peptide AMP named H4 by merging two individual  $\alpha$ -helical fragments from two predefined native peptides.<sup>9</sup> This hybridization strategy managed to reduce hemolytic and mammalian cell toxicity of the resultant synthetic peptide (H4) significantly, while achieving potent antimicrobial activity against standard and multi-drug resistant bacterial strains. H4 is capable of eliminating the growth of multi-drug resistant bacteria with minimum inhibitory concentration values as low as 5  $\mu$ M. In this study, we have evaluated the activity of H4 *in vivo* in mouse models of peritonitis. Additionally, in order to evaluate the peptide's toxicity and confirm the previously reported *in vitro* results, *in vivo* studies of H4 and its potential acute systemic toxicity effects were evaluated accordingly.

## Methods

### Animals

The *in vivo* experiments conducted in this study employed Balb/C mice (male, 18–22 g) and in order to establish the infection model, the mice were immunosuppressed by intraperitoneal administration of cyclophosphamide (200 mg/kg) 4 days before the establishment of the infection. Ketamine (100 mg/kg) and xylazine (10 mg/kg) were administered to the mice to ensure complete anesthesia before conducting any invasive operation. All animal experiments were conducted in accordance with institutional guidelines and approved by the Animal Studies Committee at the Jordan University of Sciences and Technology. The mice were randomly marked to permit individual identification.

### Establishment of the peritonitis infection model and determination of effective dose<sub>50</sub> (ED<sub>50</sub>)

The establishment of the peritonitis mouse model was performed by administering the immunocompromised

mice intraperitoneally with 0.5 mL of overnight cultured and harvested *Staphylococcus aureus* bacterial suspension. Several different doses of the bacterial suspension ( $1.0 \times 10^7$  CFU/mL,  $1.0 \times 10^8$  CFU/mL,  $1.0 \times 10^9$  CFU/mL, and 0.5 mL, six mice per group) were administered to the mice in order to identify the lethal bacterial inoculum concentration which was capable of causing a 100% mortality after 48 hours of infection. Additionally, and in order to identify the ED<sub>50</sub> of the hybrid peptide H4, the lethal dose ((LD<sub>50</sub>) was administered to each mice followed by the administration of H4 in different doses (0.5, 1, 2, 4, 6, and 12 mg/kg, six mice per group), 1 and 6 hours post-infection. Mice survival was monitored for 7 days post-infection for determination of the ED<sub>50</sub>.

### In vivo toxicity studies

The median LD<sub>50</sub> of H4 was employed for the determination of the peptide's acute systemic toxicity in mice. The mice employed in the study were randomly distributed into six groups, each with six mice. PBS dissolved H4 peptide was administered intraperitoneally at different concentrations (12.5, 14.9, 17.7, 21.0, and 25.0 mg/kg and 0.2 mL/20 g). Mice survival was determined after 7 days of peptide treatment and LD<sub>50</sub> determined accordingly. Additionally, the potential toxicity of H4 in the major organs of the mice, including the kidney and the liver, was determined by randomly distributing the mice into a PBS control group and H4 treated group, with ten mice incorporated into each group. The mice in the treated group received the ED<sub>50</sub> intraperitoneally twice daily for 3 days. Blood samples were collected from all the mice included in the treatment and control groups 72 hours after initial administration for blood analysis, including alanine transaminase (ALT), aspartate transaminase (AST), creatinine, urea nitrogen and sodium ion levels.

## Results

### In vivo efficacy of H4 against *S. aureus* peritonitis mouse model

The effectiveness of the *in vivo* antimicrobial activity of H4 was based on the *S. aureus* mouse model. Mice were subjected to cyclophosphamide in order to compromise their immunity and induce severe immunosuppression. The LD<sub>50</sub> causing 100% mortality was determined by administering different microbial concentrations and measuring the survival rate 48 hours post-infection. Accordingly, the lethal bacterial dose was determined to be  $1 \times 10^8$  CFU/mL. This lethal bacterial dose was employed for all subsequent efficacy experiments by introducing this inoculum size while administering the H4 peptide. H4 managed to display

significant anti-infective activity with an ED<sub>50</sub> value of 4.55±0.89 mg/kg (Table 1).

### In vivo evaluation of acute toxicity

The in vivo acute toxicity of H4 was evaluated by determining the median LD<sub>50</sub>. Accordingly, and as detailed in the methodology section, the LD<sub>50</sub> value of peptide H4 was 192.4 mg/kg, which corresponds to the dose at which half of the mice are killed after peptide treatment (Table 2). Additionally, and in order to evaluate the potential toxicity of H4 on the major organs of the mice, including the liver and kidney in addition to the balance of electrolytes, several levels of major enzymes such as ALT and AST were compared between the H4 treated group vs the control group. Other physiological indicators such as the levels of creatinine, urea nitrogen and sodium were also monitored between the two groups. The treatment regimens consisted of administering the ED<sub>50</sub> of H4, administered intraperitoneally at the ED<sub>50</sub> twice a day for 3 days; the results are summarized in Table 2. No significant changes in the levels of the major liver enzymes or other functional parameters in the blood were detected between the H4 treated group and the control.

### Discussion

In the present study, we have evaluated the in vivo efficacy and toxicity of a recent in house designed antimicrobial peptide named H4. The hybrid peptide was designed based on hybridization of two α-helical fragments that were identified from the primary sequence of two peptides named BMAP-27 and OP-145 respectively. The rationale behind designing such a peptide was to capture the α-helical parts of the parent peptides to generate a novel peptide with uninterrupted helical activity and increased selectivity towards microbial cells. Our results proved that the peptide was highly effective in killing several strains of Gram-positive and Gram-negative

bacteria and in exhibiting a significant high therapeutic index in vitro. To confirm the previously reported in vitro results, we have evaluated the efficacy of H4 against *S. aureus* in a peritonitis infection mouse model. The ED<sub>50</sub> value reported for was 4.55±0.89 while the LD<sub>50</sub> value was reported to be 192.4 mg/kg. These results clearly show that H4 is highly efficient in eradicating the bacterial infection in parallel with a high therapeutic index. Other cationic antimicrobial peptides, such as polymixin B, exhibit LD<sub>50</sub> values in the range of 8–10 mg/kg which clearly shows the relatively safe and effective antimicrobial behavior of H4.<sup>10</sup> Additionally, the acute systemic toxicity of H4 and its effects on major body organs such as the liver and kidney as well as the electrolyte balance was evaluated and compared with the control samples. As the results in Table 2 show, mice that were treated twice daily for 3 days with the ED<sub>50</sub> concentration of H4 did not display any significant differences with the control group and retained the normal functions of the liver and kidney in addition to other physiological parameters. These results imply that H4 neither adversely affects the liver and kidney functions nor disturbs the balance of electrolytes in the blood under the tested regimen. In addition, all the mice treated with the peptides were found to be in good condition, without observable weight loss. Based on the results of our previous study on the in vitro activity of H4 against clinical isolates of multidrug resistant bacteria, we believe that the hybridization of H4 as a design strategy improved its selectivity towards microbial cells and consequently, increased the number of peptides targeting microbial cells and inducing bacterial membrane destruction and cell lysis while relieving the mammalian cells from the destructive pressure and keeping their membranes intact.

These results confirm the results of the in vitro studies and indicate that H4 exhibits a strong potential for being developed as a successful antimicrobial therapeutic agent.

**Table 1** The efficacy of H4 hybrid peptide against *Staphylococcus aureus* in the peritonitis infection mouse model

Bacterial strain	Minimum LD <sub>50</sub> (CFU/mouse)	H4 peptide sequence	In vitro MIC (μM)	LD <sub>50</sub> (mg/kg)	ED <sub>50</sub> (mg/kg)
<i>S. aureus</i>	1×10 <sup>8</sup>	KFKKLFKKLSPVIGKEFKRIVERIKRFLR	10	192.4 mg/kg	4.55±0.89

**Abbreviations:** MIC, minimum inhibitory concentration; LD<sub>50</sub>, lethal dose; ED<sub>50</sub>, effective dose.

**Table 2** Liver and kidney functions as well as sodium ion concentration in the blood of mice in the control and treated groups

Treatment group	ALT (IU/L)	AST (IU/L)	Creatinine (μM/L)	Urea nitrogen (mmol/L)	Sodium (mmol/L)
Control	22.33±4.25	92.56±10.54	18.54±6.54	5.33±3.25	147.78±10.25
Peptide H4	25.54±6.22	79.99±15.55	16.54±7.55	7.55±3.48	155.48±15.78

**Note:** Data shown as mean ± standard deviation.

**Abbreviations:** ALT, alanine transaminase; AST, aspartate transaminase.

Several additional studies are needed to confirm the overall efficacy and toxicity of the peptide but our preliminary results can be considered very promising.

## Acknowledgments

This study was generously supported by the Jordanian Scientific Research Fund (grant no. MPH/1/15/2014). The authors would also like to thank the Deanship of Research at Jordan University of Science and Technology for their logistical and administrative support of this project.

## Disclosure

The authors report no conflicts of interest in this work.

## References

1. Chen L, Todd R, Kiehlbauch J, Walters M, Kallen A. Notes from the Field: Pan-Resistant New Delhi Metallo-Beta-Lactamase-Producing *Klebsiella pneumoniae* - Washoe County, Nevada, 2016. *MMWR Morb Mortal Wkly Rep*. 2017;66(1):33–33
2. Sykes R. The 2009 Garrod Lecture: The evolution of antimicrobial resistance: a Darwinian perspective. *J Antimicrob Chemother*. 2010;65(9):1842–1852.
3. Padiyara P, Inoue H, Sprenger M. Global Governance Mechanisms to Address Antimicrobial Resistance. *Infect Dis*. 2018;11:1178633718767887.
4. Shankar PR, Balasubramaniam R. Antimicrobial resistance: global report on surveillance 2014. *Australasian Medical Journal*. 2014;7(5):237.
5. World Bank Group. Drug resistant infections: a threat to our economic future. Final Report. Available from: <http://documents.worldbank.org/curated/en/323311493396993758/pdf/114679-REVISED-v2-Drug-Resistant-Infections-Final-Report.pdf>. Accessed March 2017.
6. Luepke KH, Mohr JF. The antibiotic pipeline: reviving research and development and speeding drugs to market. *Expert Rev Anti Infect Ther*. 2017;15(5):425–433.
7. Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol*. 2006;24(12):1551–1557.
8. Kang SJ, Park SJ, Mishig-Ochir T, Lee BJ. Antimicrobial peptides: therapeutic potentials. *Expert Rev Anti Infect Ther*. 2014;12(12):1477–1486.
9. Almaaytah A, Qaoud MT, Abualhajjaa A, Al-Balas Q, Alzoubi KH. Hybridization and antibiotic synergism as a tool for reducing the cytotoxicity of antimicrobial peptides. *Infect Drug Resist*. 2018;11:835–847.
10. Barnett M, Bushby SRM, Wilkinson S. Sodium sulphomethyl derivatives of polymyxins. *Br J Pharmacol Chemother*. 1964;23(3):552–574.

### Infection and Drug Resistance

#### Publish your work in this journal

Infection and Drug Resistance is an international, peer-reviewed open-access journal that focuses on the optimal treatment of infection (bacterial, fungal and viral) and the development and institution of preventive strategies to minimize the development and spread of resistance. The journal is specifically concerned with the epidemiology of antibiotic

resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/infection-and-drug-resistance-journal>

Dovepress