Novel Antimicrobial Peptides Designed Using Recurrent Neural Network Reduce Mortality in Experimental Sepsis

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Research Article

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Abstract

The amino acid sequences of 198 novel peptides were obtained using a generative long short-term memory recurrent neural network (LTSM RNN). To assess their antimicrobial effect, I synthesized 5 out of 198 generated peptides. The PEP-38 and PEP-137 peptides were active *in vitro* against carbapenem-resistant isolates of *Klebsiella aerogenes* (n=12) and *K. pneumoniae* (n=18). PEP-137 was also active against *Pseudomonas aeruginosa* (n=17). The remaining three peptides (PEP-36, PEP-136 and PEP-174) showed no antibacterial effect. Then I investigated the effect of PEP-38 and PEP-137 (a single intraperitoneal administration of a 100 μg dose 30 min after infection) on animal survival in an experimental murine model of *K. pneumoniae*-induced sepsis. As a control, I used two groups of mice: one received sterile saline, and the other received inactive *in vitro* PEP-36 (a single 100 μg dose). The PEP-36 peptide was shown to provide the highest survival rate (66.7%). PEP-137 showed a survival rate of 50%. PEP-38 was found to be ineffective. The data obtained can be used to develop new antibacterial peptide drugs to combat antibiotic resistance.

1. Introduction

In recent decades, the humankind is facing the global problem of antimicrobial resistance (AMR), the main reason for which is natural evolutionary selection due to the excessive use of antimicrobials in medicine and agriculture [1]. Widespread use of antibiotics during COVID-19 pandemic is likely to further accelerate the AMR spread [2]. According to a recent systematic review, even though no more than 7% of COVID-19 patients had bacterial co-infection, 72% of patients received antibiotics [3]. It is obvious that the most effective solution to the AMR problem is the development of new antimicrobial drugs. But in recent decades, the number of new approved antibiotics was very limited [4]. Pharmaceutical companies are reluctant to invest in the development of new antibacterial drugs because drug development is very expensive, and the potential profits may not cover the costs due to the rapid development of resistance [5]. From this point of view, it is necessary to look for such compounds to which bacteria do not form resistance or form no sooner than after 20-30 years. Perhaps then the development of new antibiotics will become attractive for investments.

In recent years, of great interest are the so-called antimicrobial peptides (AMPs) which are produced by all living species. AMPs are known to have pronounced antibacterial, antiviral, antifungal, antiparasitic and antitumor effects [6]. However, unfortunately, AMPs are much more expensive to synthesize than small molecules; moreover, some peptides are rapidly degraded when used *in vivo*, which can negatively affect their pharmacokinetic properties [7].

On the other hand, it is important to note that the market for peptide drugs is now actively growing and [8]. Moreover, the likelihood of developing resistance to AMPs is much lower than to conventional antibiotics [9]. The mechanism of action of AMPs is based on direct action on the bacterial cell wall via membrane permeabilization [10]. This makes AMPs equally effective against susceptible and multidrug-resistant bacteria [4]. Previously, I examined the strategy of using AMPs in combination with conventional antibiotics and showed that the antibiotic resistance phenotype of bacteria does not affect the effectiveness of AMPs [11]. In addition, in this work I showed that natural human AMPs in combination with rifampicin and aminoglycosides demonstrate synergistic action against methicillin-resistant staphylococci and carbapenem-resistant strains of

E. coli, respectively. An alternative approach to the use of AMPs can be the design of completely new peptides with antimicrobial activity. Such *de novo* design of AMPs could be a potentially effective strategy for combating antibiotic resistance; moreover, it can be commercially attractive. Earlier, Alex T Müller et al. suggested using generative long short-term memory (LSTM) recurrent neural network (RNN) for combinatorial *de novo* peptide design [12].

In this short communication, I demonstrated the feasibility of designing completely new peptides using LSTM RNN and for the first time investigated their antibacterial activity against multidrug-resistant strains *in vitro* and *in vivo*.

2. Materials And Methods

2.1. Development of novel peptides with potential antimicrobial effect

For combinatorial *de novo* antimicrobial peptide design, I used generative long short-term memory (LSTM) recurrent neural network (RNN), the principle of which was suggested by Alex T Müller et al. in 2018 [12]. RNN models identify patterns in consecutive data and generate new data from the analyzed context. Amino acid sequences of peptides are good consecutive input data for such machine learning models. Thus, training of generative models on the sequences of antimicrobial peptides can make it possible to design new peptides with unique amino acid sequences [12].

To train the RNN model, I used the amino acid sequences of AMPs from the APD3 database (https://aps.unmc.edu/) [13]. Unlike Alex T Müller et al., I used all AMPs from the APD3 database, not only helical peptides. Peptides shorter than 7 amino acid residues were excluded from the training set. The final training set comprised 3,100 sequences with mean sequence length of 33.6 ± 22.2 and median length of 29 amino acid residues (file “Training dataset.csv” in the supplementary information). When starting the training, I used the following parameters: 2 layers, 256 neurons and 167 epochs.

Upon completion of training, 198 novel peptide sequences were generated (Figure 1, file “Sampled peptides.csv” in the supplementary information).

The generated 198 sequences were assessed using the CAMP AMP prediction tool (http://camp.bicnirrh.res.in/predict/) [14] (all four available verification algorithms were used: SVM, Random Forest, Artificial Neural Network, Discriminant Analysis). In addition, I ran 198 novel designed peptide sequences through the prediction algorithms proposed by B. Vishnepolsky and M. Pirtskhalava (http://www.biomedicine.org.ge/dbaasp/) [15]: "Prediction of general antibacterial activity" and "Prediction of activity against specific microbial species". As a result, for the subsequent synthesis I selected 5 out of 198 sequences (Table 1).

Peptides were synthesized on a commercial basis at AtaGenix Laboratories (China) by solid-phase peptide synthesis, with purity>95%. Amino acid sequences and some physicochemical properties of the synthesized peptides are presented in Table 1.

2.2. Bacteria

The strains of bacteria were isolated in 2021 from patients in the intensive care unit of the Stavropol State Regional Clinical Hospital. Identification and determination of antibiotic resistance of bacterial isolates was carried out using the disk diffusion method as part of a routine microbiological study in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocols in the Department of Clinical Microbiology of the Center of Clinical Pharmacology and Pharmacotherapy [16].

In preliminary experiments, the synthesized peptides did not show activity against gram-positive bacteria; therefore, in this work for the *in vitro* experiments I used carbapenem-resistant isolates of *Klebsiella aerogenes* (n=12), *K. pneumoniae* (n=18), and *Pseudomonas aeruginosa* (n=17). According to EUCAST breakpoints, all isolates of *Klebsiella spp*. were sensitive only to tigecycline, whereas the *P. aeruginosa* strains were sensitive only to ceftazidime/avibactam. Colistin sensitivity was not tested (this antibiotic is not included in routine microbiological testing in Russia).

2.3. *In vitro* study of antibacterial activity

To study the antimicrobial action of the synthesized peptides, I used the standard broth dilution method [17] according to the EUCAST guidelines [18].

Briefly, pure bacterial cultures were cultured on a solid nutrient media (mannitol salt agar, BioMedia, Russia). From a fresh morning culture, I prepared a suspension in sterile saline corresponding to a McFarland turbidity standard of 0.5 (which is equivalent to 1-2 × 10^8 CFU/mL). The resulting suspension was dissolved in the BBL™ Mueller-Hinton broth (Becton, Dickinson and Company, USA) to obtain an inoculum with an approximate concentration of 5 × 10^5 CFU/mL. The inoculum (100 µl per well) was added to the wells of a sterile 96-well microtiter plate with a U-shaped bottom (Medpolymer, Russia). After that, serial two-fold dilutions of tested peptides (100 µl per well) were added to the wells. There were also sterility control wells (Mueller-Hinton broth only, without bacteria) and growth control wells (bacterial inoculum without peptides). Then the plates were incubated in a thermostat at 37°C. After 18-20 h, the minimum inhibitory concentration (MIC) values were determined. The MIC was taken as the minimum peptide concentration at which there was no visual growth in the corresponding well [17].

The range of concentrations of the studied peptides was from 0 to 8 µg/ml (the upper value was chosen empirically). The peptides were dissolved in the Mueller-Hinton broth. The experiments with each peptide and bacterial isolate were performed in triplicate (in different plates). MIC values were calculated as median values of three independent replicates (calculations are presented in Supplementary Tables S1, S2 and S3).

2.4. A murine experimental model of sepsis

To assess the effectiveness of the studied peptides, a murine model of lethal generalized infection was used. The model of sepsis makes it possible to perform a simple and rapid screening assessment of the effectiveness of new antimicrobial agents *in vivo* [19,20].

Animal studies were approved by the local ethics committee of the Stavropol State Medical University (Protocol No. 95 dated 18.02.2021) and were performed in accordance with the Code of Ethics of the World

Medical Association (Declaration of Helsinki, EU Directive 2010/63/EU for animal experiments). Animal studies are reported in compliance with the ARRIVE guidelines [21]. In order to generate animal groups of equal size, a randomization of animals between groups was carried out. Blinding in this study was not possible as it was performed by one investigator.

The ICR (CD-1) laboratory mice (females with an average weight of 30 g) were maintained in the vivarium of the Stavropol State Medical University. The mice (4 animals per cage) were housed in temperature-controlled rooms at 24°C and 50–60% humidity with a 12h/12h light/dark cycle and water and food availability *ad libitum.*

The animals were injected with bacterial suspensions of one of the carbapenem-resistant isolates of *K. pneumoniae* (isolate #7, Supplementary Table S2) prepared from a fresh morning culture in accordance with the McFarland turbidity standard of 15 (which corresponds to an approximate concentration of 4.5×10^9 CFU/ml). To determine the turbidity of the suspension, a DEN-1 densitometer (Biosan, Latvia) was used. The bacterial suspension and peptides were dissolved in sterile saline and injected intraperitoneally (the total volume of injected fluid did not exceed 250 µl per mice).

In the experiment there were 4 groups of 12 mice in each: the first group was a control one (it received saline); the second group was an additional control (it received PEP-36 which was not active *in vitro*); the third group received PEP-38; the fourth group received PEP-137. At t = 0 min, all animals were infected with *K. pneumoniae*: 150 µl of a 4.5×10^9 CFU/ml suspension (approx. 6.75×10^8 CFU per mice). 30 min after infection, the mice received a single injection of peptides at a dose of 100 µg. Mortality was assessed every 24 h for 5 days. Moribund animals were killed humanely to avoid unnecessary distress.

2.5. Modeling the structure of novel peptides

To model highly accurate structures of synthesized peptides, I used the recently published algorithm AlphaFold [22] (a slightly simplified version of AlphaFold v2.1.0 available online). To generate the images, the PyMOL Molecular Graphics System Version 2.5.2 (Schrödinger, USA) was used. The .pdb files of PEP-36, PEP-38 and PEP-137 peptides are available in supplementary information).

2.6. Statistical analysis

Calculations of MICs (median, first and third quartile) were performed in the Microsoft Excel (Supplementary Tables S1, S2 and S3).

Survival analysis by the Kaplan-Meier method and Log-rank (Mantel-Cox) test was performed using the GraphPad Prism version 9.2.0 for macOS (GraphPad Software, USA, www.graphpad.com).

3. Results

3.1. PEP-38 and PEP-137 are active against carbapenem-resistant gram-negative bacteria *in vitro*

I investigated the antibacterial activity of 5 synthesized peptides (Table 1) which were selected from 198 amino acid sequences generated using LSTM RNN.

Table 1
Amino acid sequences and characteristics of the studied peptides.

Peptide ID	Amino acid sequence	Length (amino-acid residues)	Molecular weight (g/mol)	Charge	Hydrophobic residues (%)
PEP-36	GIFSKLAGKKIKNLLISGLKNIGKEVGM	28	2958	+5	43
PEP-38	GLKDWVKKALGSLWKLANSQKAIISGKKS	29	3156	+6	41
PEP-136	KWKLFKKIWSSVKLKS	16	2007	+6	44
PEP-137	KWKSFIKKLAKFGFKVIKKFAKKHGSKIAKNQ	32	3764	+12.25	41
PEP-174	GILSSFKGVLKGAGKNLLGSLKDKLKN	27	2786	+5	37

The PEP-36, PEP-136, PEP-174 peptides were not active against the studied isolates. The PEP-38 and PEP-137 peptides had a high level of antibacterial activity against the studied multidrug-resistant bacteria (Supplementary Tables S1, S2 and S3).

MIC of PEP-38 against *K. aerogenes* was 6 (4-8) μg/ml (hereinafter MIC values are presented as median, in brackets – first and third quartile); against *K. pneumoniae*, it was 8 (4-8) μg/ml. PEP-38 was not active against *P. aeruginosa*.

PEP-137 demonstrated a higher antimicrobial activity against the studied isolates. MIC against *K. aerogenes* was 2 (1-2.5) μg/ml; against *K. pneumoniae* – 2 (1-4) μg/ml; against *P. aeruginosa* – 2 (2-4) μg/ml.

3.2. PEP-36 and PEP-137 peptides reduce mortality in experimental sepsis

In an experimental murine model of *K. pneumoniae*-induced sepsis, I investigated the effect of a single injection of PEP-38 and PEP-137 on the survival rate in comparison with the control (sterile saline solution); as an additional control, I used the PEP-36 peptide (which did not demonstrate antimicrobial effect *in vitro*).

In the control group, the probability of survival was 0% on the third day after infection (Figure 2, Supplementary Table S4, Figures S1 - S3).

The most active in the *in vitro* experiments PEP-137 had significant differences from the control group ($p=0.02694$): the survival proportion by the end of the observation period was 50% (Figure 2, Supplementary Table S4, Figure S3).

PEP-38 which was also effective *in vitro* did not reduce mortality in an experimental model of sepsis *in vivo* and did not significantly differ from the control group (Figure 2, Supplementary Table S4, Figure S2).

Unexpected results were obtained on survival rate in the analysis of the effect of the PEP-36 peptide which was completely inactive *in vitro*. After a single injection of this peptide, 8 out of 12 mice survived by the end of the observation period – a probability of survival of 66.7% (p=0.00051 compared to the control) (Figure 2, Supplementary Table S4, Figure S1).

3.3. PEP-36, PEP-38 and PEP-137 peptides have similarity in their spatial structure

For fast modeling of the spatial structure of the synthesized peptides, I used the recently developed AlphaFold v2.1.0 algorithm. Since I did not need to analyze the physicochemical properties of the obtained spatial structures in depth, as a simple example for comparison, I used the structure of human cathelicidin LL-37 from the Protein Data Bank (PDB ID: 2K6O).[23] Figure 3 shows obvious similarities in the spatial structures of all studied molecules and LL-37.

4. Discussion

In this short communication, I for the first time experimentally (*in vitro* and *in vivo*) tested the method suggested by Alex T Müller et al. [12] to generate novel peptides with presumable antimicrobial activity. I slightly modified the input data for the neural network to use all 3,100 antimicrobial peptides (with a length of more than 7 amino acid residues) from the APD3 database (https://aps.unmc.edu/) [13]. The neural network generated 198 sequences that I ran through various *in silico* screening systems, after which, based on my own subjective opinion, I selected 5 peptides for synthesis (PEP-36, PEP-38, PEP-136, PEP-137 and PEP-174).

The first stage of the experimental screening was the study of the minimum inhibitory concentrations of these peptides by the method of serial dilutions. PEP-38 was found to be active against *K. aerogenes* and *K. pneumoniae*. PEP-137 was more effective against *Klebsiella spp.*, as well as against *P. aeruginosa*. The PEP-36, PEP-136 and PEP-174 peptides were not active against these bacteria. It should be noted, however, that one of the limitations of this work was that I did not study the effectiveness of concentrations above 8 μg/ml.

The data obtained prompted me to test the effectiveness of the PEP-38 and PEP-137 peptides in the simplest experimental model of sepsis in mice. Moreover, as an additional control, I randomly selected PEP-36 which is not active *in vitro*. It should be noted that I used a simple and easily reproducible sepsis model with a single injection of the studied peptides at a dose of 100 μg/mouse.

Surprisingly, the PEP-36 peptide was found to be the most effective – the animal survival rate was 66.7%. PEP-137 showed a survival rate of 50%. PEP-38 was proven to be ineffective.

In their work on LL-37, Guangshun Wang et al. using nuclear magnetic resonance structural analysis identified a short three-turn amphipathic helix rich in positively charged side chains, which helps to effectively compete for anionic phosphatidylglycerols in bacterial membranes. [23]

The modeled spatial structures of the novel peptides are very similar to the LL-37 molecule. It seems likely that the helix-rich structure of the PEP-36, PEP-38 and PEP-137 peptides may be an important contributor to the demonstrated antimicrobial effect. It remains unclear why PEP-38 was active *in vitro*, but did not affect

mortality in experimental sepsis; it may be necessary to investigate larger doses of this peptide and other routes of administration.

It is also unclear, on the other hand, why PEP-36 did not have antimicrobial activity *in vitro*, but was most effective *in vivo*. There can be two explanations: after entering a living organism, PEP-36 becomes somehow modified and acquires antimicrobial activity; or this peptide has some kind of immunomodulatory effect. To clarify the nature of the data obtained, more research is required. Earlier, in the *P. aeruginosa*-induced sepsis model, epinecidin-1 (Epi-1; by *Epinephelus coioides*) due to its antimicrobial and pronounced immunomodulatory effect has been shown to reduce mortality in mice [19]. Epi-1 enhances the production of IgG antibodies by activating the Th2-cell response [24], reduces the level of tumor necrosis factor alpha by reducing the level of endotoxins [19].

The data obtained in this work require a wide range of additional experiments: the study of pharmacodynamics and pharmacokinetics, toxicity, as well as the study of the combined action of the obtained peptides with conventional antibiotics.

Thus, I conducted a simple and easily reproducible study with an experimental assessment of the feasibility of using the generative long-term memory recurrent neural network to generate novel peptides that demonstrate antimicrobial activity *in vitro* and reduce mortality in experimental sepsis *in vivo*. The peptides I obtained can be used to develop new antibacterial drugs for the treatment of infections caused by carbapenem-resistant gram-negative bacteria.

5. Conclusion

In this study, I for the first time evaluated the effectiveness of novel peptides generated using a recurrent neural network in *in vitro* experiments and in a murine model of sepsis. The obtained data open up new possibilities for the design of new antibacterial drugs to combat carbapenem-resistant gram-negative bacteria.

Declarations

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Competing Interests

The author declares that he has no competing interests.

Author's Contributions

Albert Bolatchiev conceived, designed and performed the experiments, analyzed the data, prepared figures and tables, and wrote the manuscript.

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Figures

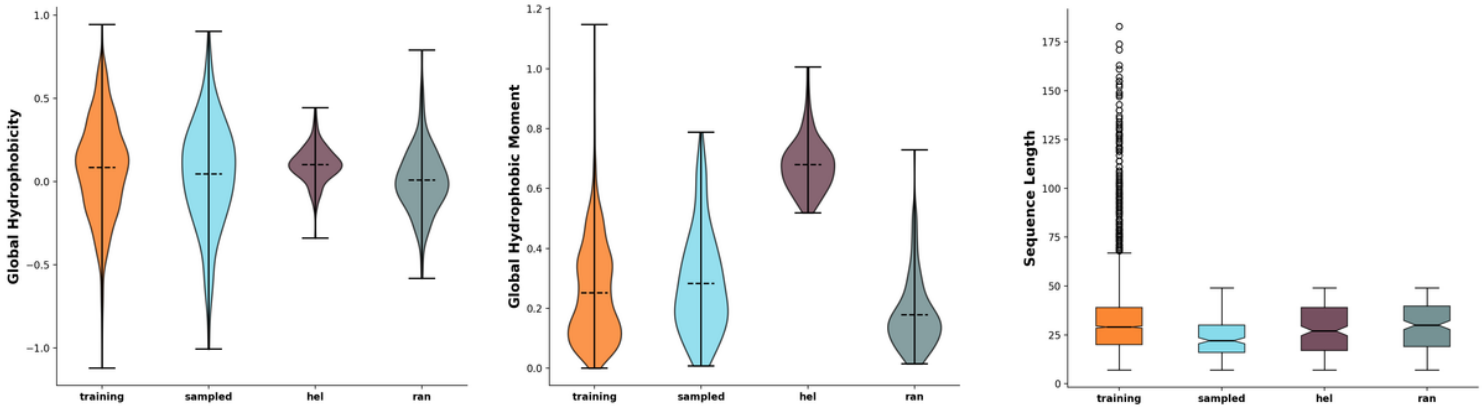

Figure 1

Comparison of peptide characteristics between the training data (Training, orange), the generated sequences (Sampled, blue), the pseudo-random sequences with the same amino acid distribution as in the training set (Ran, purple), and the manually created hypothetical amphipathic helices (Hel, green). The horizontal dashed lines represent the mean (violin plots) and median (box plots) values; the whiskers extend to the outermost non-outlier data points. Graphs from left to right: Eisenberg hydrophobicity, Eisenberg hydrophobic moment, and sequence length. The figure was generated using the modlAMP's GlobalAnalysis.plot_summary method in Python 12.

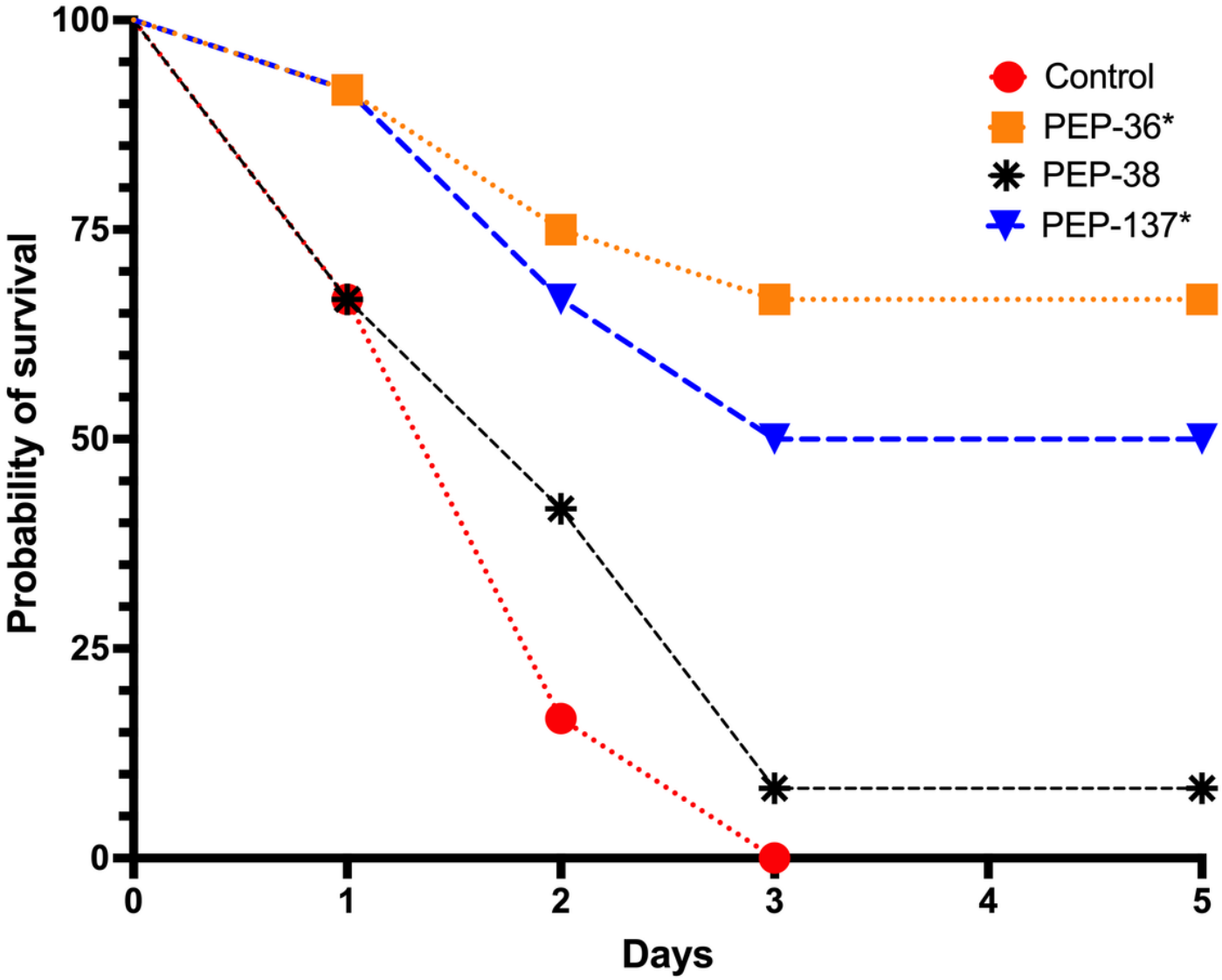

Figure 2

Comparison of probability of survival in each group: control (sterile saline), PEP-36, PEP-38 and PEP-137. The peptides were injected once in a dose of 100 µg 30 min after infection of mice with 6.75 × 108 CFU suspension of a carbapenem-resistant isolate of K. pneumoniae. * – significant differences from the control group using the Kaplan-Meier method and Log-rank (Mantel-Cox) test.

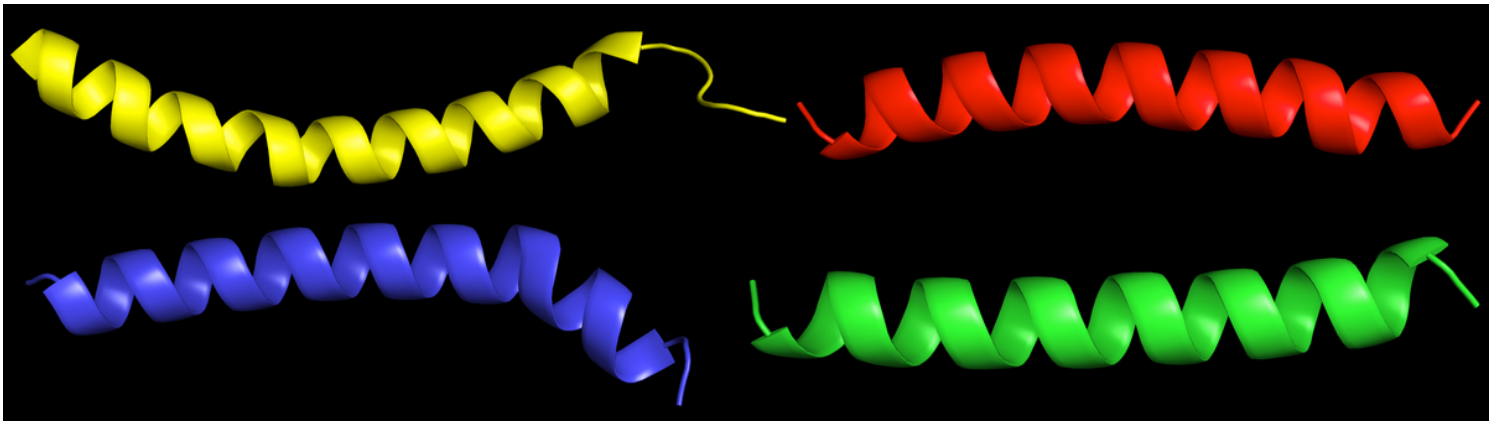

Figure 3

Visual comparison of the structures of LL-37 (yellow; PDB ID: 2K6O) and synthesized peptides: PEP-137 – blue, PEP-38 – red, PEP-36 – green (by AlphaFold).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- PEP36.pdb
- PEP38.pdb
- PEP137.pdb
- Sampledpeptides.csv
- Supplementaryinformation.docx
- Trainingdataset.csv