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Bactericidal oncocin derivatives with superior serum stabilities

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ABSTRACT

The proline-rich antimicrobial peptide oncocin is remarkably active in vitro against a number of important Gram-negative bacteria of concern to humans owing to their increasing resistance to antibiotics, i.e. Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*) and non-fermenting species (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*). Degradation of oncocin in mouse serum was investigated in this study. Several approaches to stabilise the main cleavage sites (C-terminal to Arg-15 and N-terminal to Arg-19) by substituting either or both arginine (Arg) residues with non-proteinogenic amino acids, i.e. α -amino-3-guanidino-propionic acid, homoarginine, nitro-arginine, *N*-methyl-arginine, β -homoarginine, *D*-arginine (*D*-Arg) or ornithine (Orn), were tested. These modifications were found to increase the half-life of oncocin in full mouse serum. For oncocin with two Orn residues in positions 15 and 19, the half-life in full serum increased from 25 min to 3 h. An increase of >8 h was observed for oncocin with two *D*-Arg residues at these same positions. The antibacterial activities of these modified sequences were slightly better than the original oncocin sequence. Moreover, the three most stable analogues were found to be bactericidal against *E. coli* and were not toxic to HeLa cells or haemolytic to human erythrocytes.

1. Introduction

Infectious diseases are a major cause of death worldwide. Despite the considerable progress made in hygiene and antibiotic treatment strategies during the last century, bacterial infections still contribute substantially to high rates of mortality and morbidity. What is perhaps even more worrying is that the incidence of serious bacterial infections caused by pathogens resistant to the current antibiotics is rapidly increasing. A recent report from the European Centre for Disease Prevention and Control indicated that an estimated three million hospitalised patients acquire nosocomial infections every year, accounting for ca. 50 000 deaths [1]. Similar numbers were estimated by the US Centers for Disease Control and Prevention (CDC) for the USA, accounting for more than US\$3.5 billion in additional healthcare costs. According to the CDC, ca. 70% of these nosocomial infections are caused by bacteria resistant to at least one of the current antibiotics. Gram-negative bacteria account for ca. 40% of all nosocomial infections, nearly twice as many as those caused by Gram-positive bacteria [2]. Currently, three species of Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*) and two non-fermenting species (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) are causing great concern due to the rapid spread of multiresistant or extremely resistant strains. A very recent and even more frightening development has been the incidence of pan-resistant *A. baumannii*, *E. coli* and *K. pneumoniae* strains reported in outbreaks in India, Pakistan, the UK and the USA that were uniformly susceptible only to polymyxin treatment [3,4]. Although prudent use of current antibiotics may slow down further resistance development, innovative antimicrobial drug classes with novel modes of action are urgently required. Inducible gene-encoded antimicrobial peptides (AMPs) may represent such a promising class of antibiotics. Whereas most

AMPs act via disruptive lytic or pore-forming mechanisms directly on the bacterial membrane, only a few inhibit specific intracellular targets. The latter group appears especially promising as agents with which to treat systemic infections, as they will most likely not cross-react with the corresponding human proteins owing to low sequence homologies and structural differences [5].

Mammalian AMPs kill bacteria *in vitro* but also probably stimulate the innate immune system and might even link it to the adaptive immune system [6], which could cause severe problems if AMPs are administered at higher therapeutic doses. Thus, an ideal antimicrobial drug lead should be neither toxic to human cells nor modulate the human immune system. Insects are so evolutionarily removed from humans that their AMPs will most likely not modulate the human immune system. Thus, insect-derived, proline-rich AMPs, which are neither toxic to human cell lines nor haemolytic, appear to meet all the requirements of an ideal antibiotic.

In light of these facts, several laboratories have recently designed and optimised novel proline-rich AMP analogues with interesting antibacterial activities and with no toxic effects on mammalian cell lines or erythrocytes [7–9]. The high proline content also provides fairly good stability against proteolytic degradation in mammalian serum, which can be even further reduced by stabilising the cleavage sites. Recently, the efficiency of these optimised peptides was also proven for mouse models with systemic infections of *E. coli*, *A. baumannii* and *Salmonella enterica* serotype Typhimurium [10,11]. A3-APO was even more effective than conventional antibiotics administered as positive controls [12].

This study investigated the efficiency of oncocin, which has recently been developed by our laboratory to treat systemic *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* infections [13]. The minimum inhibitory concentrations (MICs) of this 19mer sequence ranged from 0.25 µg/L to 4 µg/mL for a panel of 32 bacterial strains. For the purpose of this investigation, the stability of oncocin against serum proteases using mouse serum, which in our experience degrades peptides equally as fast as human serum, was optimised. The half-life of oncocin in full mouse serum was increased from only 25 min to >8 h. The new peptide derivatives did not show any cytotoxic effects on human cell lines and were not haemolytic. Importantly, they were equally as active, or even more active, than oncocin.

2. Material and methods

2.1. Peptide synthesis

Peptides were synthesised on solid phase and were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) as described previously [13]. α -Amino-3-guanidino-propionic acid (Agp) (Iris Biotech, Marktredwitz, Germany), nitro-arginine (Nir), *N*-methyl-arginine (Nmr), homoarginine (Har) (all from Bachem, Weil am Rhein, Germany) and β -homoarginine (β Hr) (Fluka, Buchs, Switzerland) were coupled manually with diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) activation. Side chains of trifunctional amino acids were protected with the following: 2,2,4,5,7-pentamethyldihydrobenzofuran-5-sulfonyl for arginine (Arg) and D-arginine (D-Arg); 2,2,5,7,8-pentamethylchroman-6-sulfonyl for Har and β Hr; 4-methoxy-2,3,6-trimethylphenyl-sulfonyl for Nmr; *tert*-butyl for aspartic acid (Asp) and tyrosine (Tyr); trityl for asparagine (Asn) and histidine (His); and *tert*-butyloxycarbonyl for Agp,

lysine (Lys) and ornithine (Orn). For a peptidyl propylamide, Arg was coupled to 4-sulfamylbutyryl AM resin (Novabiochem[®]; Merck KGaA, Darmstadt, Germany) with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the presence of *N,N*-diisopropylethylamine (DIPEA) at $-20\text{ }^{\circ}\text{C}$. The peptide–acylsulphonamide resins were activated first with iodoacetonitrile (67 eq) and DIPEA (13 eq) in *N,N*-dimethylformamide (DMF) and were then cleaved with propylamine (50 eq) in DMF to yield the peptide propylamide [14].

2.2. Antibacterial activity

The non-pathogenic bacterial strains *E. coli* BL21AI and *Micrococcus luteus* ATCC 10240 were cultured in nutrient broth (Carl Roth GmbH, Karlsruhe, Germany), and pathogenic *E. coli* DSM 1103 and *K. pneumoniae* DSM 681 were grown overnight on nutrient agar (Carl Roth GmbH) at $37\text{ }^{\circ}\text{C}$. MIC values were determined in triplicate by a liquid broth microdilution assay in sterile 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) using a total volume of $100\text{ }\mu\text{L}$ /well. Aqueous peptide solutions (1 mg/mL) were serially two-fold diluted in 1% tryptic soy broth (TSB) starting at a concentration of $128\text{ }\mu\text{g/mL}$ and typically reducing to $0.5\text{ }\mu\text{g/mL}$ in eight steps. Overnight bacterial cultures were diluted with 1% TSB to 1.5×10^7 cells/mL (*E. coli* BL21AI), 4×10^7 cells/mL (*M. luteus* ATCC 10240) or 5×10^5 cells/mL (*E. coli* DSM 1103 and *K. pneumoniae* DSM 681). Then, $50\text{ }\mu\text{L}$ of these solutions was added to each well. The plates were incubated at $37\text{ }^{\circ}\text{C}$ and the absorbance of each well was measured at 595 nm after $20 \pm 2\text{ h}$. The MIC was defined as the lowest peptide concentration where the absorbance value did not exceed that of the medium only.

2.3. Time–kill assay

Time–kill assays were performed for *E. coli* BL21AI in sterile polypropylene tubes containing a final volume of 2 mL of 1% TSB. The peptide concentrations were four-fold the corresponding MIC. The positive control did not contain any antibiotic. The inocula to be tested were prepared by adjusting the turbidity of an actively growing broth culture in 1% TSB to 1×10^7 colony-forming units (CFU)/mL. Tubes were continuously shaken on an orbital incubator at 37 °C and aliquots of 1 μ L were taken in triplicate after 0, 1, 2, 4, 6 and 24 h. These aliquots were then spread with an inoculating loop directly, or after appropriate dilution, in triplicate onto an agarose plate containing 1% TSB. Colonies were counted after an incubation period of 24 h at 37 °C.

2.4. Serum stability

Peptides (7.5 μ g) were dissolved in 25% (v/v) aqueous or undiluted pooled mouse serum (PAA Laboratories GmbH, Pasching, Austria) (100 μ L) and were incubated at 37 °C [7]. Aliquots taken in duplicate or triplicate after 0, 30, 60, 120 and 240 min (and also 480 min for peptide O24) were precipitated by addition of trichloroacetic acid to a final concentration of 3% (v/v). After 10 min on ice, the samples were centrifuged and the supernatant was neutralised with sodium hydroxide solution (1 mol/L) and stored at –20 °C. Samples were analysed on an analytical Jupiter C₁₈ column using a linear aqueous acetonitrile or methanol gradient containing 0.1% (v/v) trifluoroacetic acid. Metabolites were identified by matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI-TOF-MS).

2.5. Haemolytic activity

Peptides were serially diluted from 600 $\mu\text{g}/\text{mL}$ to 5 $\mu\text{g}/\text{mL}$ in phosphate-buffered saline (PBS) (GIBCO, Invitrogen, Darmstadt, Germany) in a V-bottomed, 96-well polypropylene plate (Greiner Bio-One GmbH) to a final volume of 50 μL .

Concentrated human erythrocytes were washed and suspended in PBS to a final concentration of 2%. Aliquots of this suspension (50 μL) were added to the peptide solution in each well and were incubated at 37 °C for 1 h. After centrifugation (1000 \times g), the absorbance of the supernatants was determined in a fresh, flat-bottomed, 384-well plate (Greiner Bio-One GmbH) at 405 nm in a Paradigm™ microplate reader (Beckman Coulter, Wals, Austria).

2.6. Cytotoxicity

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 medium with 10% (v/v) fetal bovine serum containing 1% (w/v) streptomycin, penicillin or neomycin. Then, $1-1.5 \times 10^4$ cells were seeded in the same medium into 96-well plates and were incubated overnight (37 °C, 5% CO₂). Subsequently, the peptide solutions (600 $\mu\text{g}/\text{mL}$) were added (100 $\mu\text{L}/\text{well}$) and were incubated again in identical conditions for 20 h. Cell viability was determined with the methyl thiazolyl diphenyltetrazolium bromide (MTT) Cell Proliferation Assay I (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

3. Results and discussion

Oncocin is a chemically optimised analogue of the 2 kDa *Oncopeltus* antibacterial peptide 4, which was originally isolated from *Oncopeltus fasciatus* [15]. Its high

antibacterial activity against several Gram-negative human pathogens makes it a promising lead compound for systemic administration. As described recently, native oncocin possesses two major degradation sites in mouse serum [13]. The cleavage after Arg-14 probably results from trypsin-like proteases producing oncocin(1–14), whereas cleavage after Asn-18 might be explained by a carboxypeptidase activity removing the C-terminal Arg residue. Either degradation abolishes the antibacterial activity. With a half-life of 2 h in 25% mouse serum, oncocin(1–18) (peptide O2 in Table 1) is significantly more stable than oncocin (peptide O1 in Table 1, half-life of 68 min). As oncocin targets intracellular bacterial objects, most likely DnaK, it required ca. 30 min to kill the bacteria or at least to cause enough damage to ensure subsequent death. For systemic treatment, it would therefore be advantageous to increase the half-life of oncocin in serum to several hours. Thus, we studied conservative substitutions at both sites in order to reduce enzymatic degradation in this region without compromising antibacterial activity.

First, the C-terminal position was studied. Substitution of Arg-19 by alanine (Ala) (O3) reduced the activity against *E. coli* at least eight-fold, whereas Asn (O4) and His (O5) reduced it four-fold (Table 1). The MIC values for *M. luteus* were increased by a factor of two to eight. Substitutions with Orn, Agp, Nir, Nmr, Har and D-Arg affected the activities against *E. coli* and *M. luteus* only slightly (O6–O11; Table 1). The half-lives for these six analogues in 25% aqueous mouse serum increased from 68 min (oncocin) to ≥ 80 min (Nir and Nmr), or even 160 min (D-Arg) under the best circumstances. A similar effect was seen for the C-terminal propylamide (O12), which had a half-life of >2 h in diluted mouse serum (Table 1) and which also did not affect the MIC values against *E. coli* and *M. luteus*. This may indicate that the C-terminus of oncocin is not critical for its antibacterial activity, although the free acid is less active

[13]. Although the serum stabilities were only slightly improved, it is important to note that all monosubstituted oncocin derivatives were only degraded between Arg-14 and Arg-15, yielding oncocin(1–14) as the only stable metabolite. Thus, stabilisation of this position was considered as crucial to reduce further the metabolism rates.

Although enzymatic degradation occurred after Arg-14, this residue was not substituted, but the following arginine in position 15 was. It is well known that for trypsin the cleavage of an Arg–Lys/Arg motif is reduced between, and after, both residues. Replacing the first position by an amino acid that is not targeted by the protease would protect this position against proteases. However, at the same time, it would probably also form a new cleavage site between Arg-15 and isoleucine (Ile)-16. For this reason, substitutions at Arg-15 were investigated first. Ala in this position (O13) abolished the antibacterial activity, whereas both Orn (O14) and β Hr analogues (O15) were almost as active as oncocin. The half-lives increased to 90 min (O14) and 150 min (O15), with the only stable metabolite resulting from C-terminal cleavage of Arg-19. The D-Arg-15 analogue (O16) was equally as stable in serum and was equally as active against *E. coli* as oncocin, but was slightly more active against *M. luteus* (Table 1). This unexpected activity indicates that Arg-15 is probably not involved in any stereospecific binding towards the bacterial target (i.e. DnaK) or is a likely protein transporter in bringing oncocin from the periplasmic space into the intracellular region. As substitution of Arg-15 completely protected the digestion after Arg-14, substitutions in position 14 were not tested further.

Having identified several substitutions both for Arg-15 and Arg-19 that completely prevented enzymatic cleavage in serum of the peptide chain next to the modified site, several combinations with Agp, Har, Orn and D-Arg at both positions were

subsequently tested (Table 1). Within the typical error range of peptide quantification and antibacterial tests, the MIC values of all double-substituted oncocin derivatives were identical to the original oncocin sequence, i.e. 4 $\mu\text{g/mL}$ against *E. coli* and 8 $\mu\text{g/mL}$ against *M. luteus*. Owing to the expected higher serum stabilities of the double-substituted oncocin analogues, all further studies were conducted in full mouse serum. Under these conditions, the half-life of oncocin was 25 min. The double Har analogue (O17) had a half-life of 55 min, which was gradually further increased by substituting one or both positions with Agp, finally reaching 170 min for peptide O20 (Table 1). Replacing Har or Agp in position 15 with Orn yielded very similar serum stabilities for the corresponding double-substituted analogues, i.e. peptides O21 and O22 versus peptides O19 and O20 (Table 1). Based on the finding that D-Arg in position 15 and 19 did not reduce the antibacterial activity, the double D-Arg analogue was also tested. Surprisingly, the MIC value against *E. coli* BL21AI was identical to oncocin, and the resulting half-life of >8 h was better than any other oncocin derivative yet tested. In contrast, the C-terminal propylamide of oncocin(Arg15Orn) was only moderately stable and therefore this modification was not investigated further.

The three most stable lead compounds O20, O23 and O24 were further tested in a time–kill assay to evaluate their bacteriostatic or bactericidal activity. Viable cell counts of all three inocula decreased within the first 30 min (Fig. 1). A 1000-fold reduction in the number of CFU was obtained after 8, 4 and 1 h for peptides O23, O20 and O24, respectively. After an incubation time of 24 h, the bacterial counts decreased by four or five orders of magnitude. Interestingly, the time–kill assay followed the serum stability for these three oncocin analogues, with the most stable peptide (O24) yielding the lowest bacterial count of only 100 CFU/mL. It is tempting

to speculate that the increased serum stability also reduced the peptide degradation within bacterial cells. If true, the increased stability against bacterial proteases might also prevent possible resistance mechanisms based on increased expression of proteases.

Next, the same three peptides were tested against a human HeLa cell line for any possible cytotoxic effects. The MTT test did not indicate any toxicity at peptide concentrations of 600 $\mu\text{g/mL}$ (Fig. 2a). Furthermore, there was no indication of any haemolytic activity up to the highest peptide concentration tested, i.e. 600 $\mu\text{g/mL}$ (Fig. 2b).

Although it is difficult to compare and judge in vitro data gathered in different laboratories for different peptide families, it is probably fair to say that the new oncocin derivatives presented here currently belong to the most promising lead compounds based on gene-encoded AMPs. Both the MIC values and the serum stabilities are comparable with, and sometimes even better than, peptide derivatives reported in recent years. Future studies will have to show whether this promising in vitro profile will correctly predict their in vivo efficacy.

4. Conclusion

Oncocin is relatively stable against serum proteases despite its short sequence and unordered structure owing to its relatively high proline content. Only two degradation sites (C-terminal to Arg-14 and Asn-18) have been identified in mouse serum. Both were successfully stabilised by substituting Arg-15 and Arg-19 with Agp, Har, Orn or D-Arg, resulting in serum half-lives of >8 h for oncocin(Arg15D-Arg, Arg19D-Arg) and

ca. 3 h for oncocin(Arg15Agp, Arg19Agp) and oncocin(Arg15Orn, Arg19Orn). Considering that the viable cell counts for *E. coli* BL21AI had already decreased within the first 30 min, the obtained serum stabilities appear to be sufficient for effective treatment of systemic infections. Within the half-life of peptide O24 (>8 h), the CFU dropped by more than five orders of magnitude. Moreover, peptides O20, O23 and O24 were slightly more active against the tested *E. coli*, *K. pneumoniae* and *M. luteus* strains than oncocin and exhibited bactericidal activity against the tested *E. coli* strain. As these three oncocin analogues were also non-toxic to HeLa cells and were not haemolytic against human erythrocytes, they represent valid lead compounds for future in vivo studies.

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Competing interests

RH is co-founder of AMP-Therapeutics GmbH & Co. KG, which currently holds the patents for oncocin. All other authors declare no competing interests.

Ethical approval

Not required.

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Fig. 1. Time–kill curves for *Escherichia coli* BL21AI using a peptide concentration corresponding to the four times the minimum inhibitory concentration (MIC) of O20 (●, 32 µg/mL), O23 (○, 32 µg/mL) and O24 (◇, 16 µg/mL). No antibiotic was added to the positive control (×). Bactericidal activity (dotted line) was defined as a >1000-fold decrease in the number of viable colony-forming units (CFU) after 24 h. CFU counts were determined in triplicate on agarose plates.

Fig. 2. (a) Cytotoxic effects and (b) haemolytic activity of oncocin analogues. (a) Cytotoxicity was studied for HeLa cells using the MTT cell proliferation assay. The HeLa cell line was incubated with the peptides at a concentration of 600 µg/mL for 20 h at 37 °C. Positive controls consisted of dimethyl sulphoxide (DMSO) (12%) and melittin (120 µg/mL). Values were normalised to phosphate-buffered saline (PBS) (12%) as the negative control. (b) Human erythrocytes were incubated with a two-fold serial dilution of peptides O1 (◆), O17 (□), O20 (●), O23 (○), O24 (◇) and O25 (Δ) from 600 µg/mL to 5 µg/mL at 37 °C for 60 min. Triton X-100 (0.1%; ×) and melittin (150–1.25 µg/mL; ■) were the positive controls, and PBS was the negative control (▲). Error bars indicate the standard deviations from three independent experiments. OD, optical density.

Table 1

Sequences, half-lives in serum ($t_{1/2}$) and minimum inhibitory concentrations (MICs) of oncocin and oncocin analogues derived by substitutions in positions 15 and 19

| No. | Amino acid sequence | $t_{1/2}$ (min) ^a | | MIC ($\mu\text{g/mL}$) ^b | | | |
|-----|--|------------------------------|------------|---------------------------------------|------------------|----------------|----------------------|
| | | 25% aqueous serum | Full serum | <i>E. coli</i> | <i>M. luteus</i> | <i>E. coli</i> | <i>K. pneumoniae</i> |
| | | | | BL21AI | ATCC 10240 | DSM 1103 | DSM 681 |
| O1 | VDKPPYLPRPRPPR-Arg-IYN-Arg-NH ₂ | 68 ± 15 | 25 ± 4 | 4 | 8 | 8 | 4 |
| O2 | VDKPPYLPRPRPPR-Arg-IYN-OH | 120 | | 64 | 128 | N/T | N/T |
| O3 | VDKPPYLPRPRPPR-Arg-IYN-Ala-NH ₂ | N/T | | 32 | 16 | N/T | N/T |
| O4 | VDKPPYLPRPRPPR-Arg-IYN-Asn-NH ₂ | 120 | | 16 | 64 | N/T | N/T |

| | | | | | | |
|-----|--|----------|----|-----|-----|-----|
| O5 | VDKPPYLPRPRPPR-Arg-IYN- His-NH ₂ | 90 ± 1 | 16 | 16 | N/T | N/T |
| O6 | VDKPPYLPRPRPPR-Arg-IYN- Orn-NH ₂ | 210 ± 60 | 8 | 16 | N/T | N/T |
| O7 | VDKPPYLPRPRPPR-Arg-IYN- Agp-NH ₂ | 98 ± 6 | 8 | 8 | N/T | N/T |
| O8 | VDKPPYLPRPRPPR-Arg-IYN- Nir-NH ₂ | 78 ± 2 | 8 | 16 | N/T | N/T |
| O9 | VDKPPYLPRPRPPR-Arg-IYN- Nmr-NH ₂ | 79 ± 3 | 8 | 4 | N/T | N/T |
| O10 | VDKPPYLPRPRPPR-Arg-IYN- Har-NH ₂ | 94 ± 1 | 8 | 8 | N/T | N/T |
| O11 | VDKPPYLPRPRPPR-Arg-IYN- arg-NH ₂ | 160 ± 5 | 4 | N/T | N/T | N/T |

| | | | | | | |
|-----|---|--------|----|----|-----|-----|
| O12 | VDKPPYLPRPRPPR-Arg-IYN- Arg-NH _{pr} | >120 | 4 | 8 | N/T | N/T |
| O13 | VDKPPYLPRPRPPR-Ala-IYN- Arg-NH ₂ | N/T | 32 | 16 | N/T | N/T |
| O14 | VDKPPYLPRPRPPR-Orn-IYN- Arg-NH ₂ | 90 | 8 | 16 | N/T | N/T |
| O15 | VDKPPYLPRPRPPR-βHr-IYN- Arg-NH ₂ | 150 | 4 | 16 | N/T | N/T |
| O16 | VDKPPYLPRPRPPR-arg-IYN- Arg-NH ₂ | 58 ± 2 | 4 | 4 | N/T | N/T |
| O17 | VDKPPYLPRPRPPR-Har-IYN- Har-NH ₂ | 55 ± 0 | 4 | 4 | 8 | 2 |
| O18 | VDKPPYLPRPRPPR-Har-IYN- Agp-NH ₂ | 62 ± 4 | 4 | 4 | N/T | N/T |

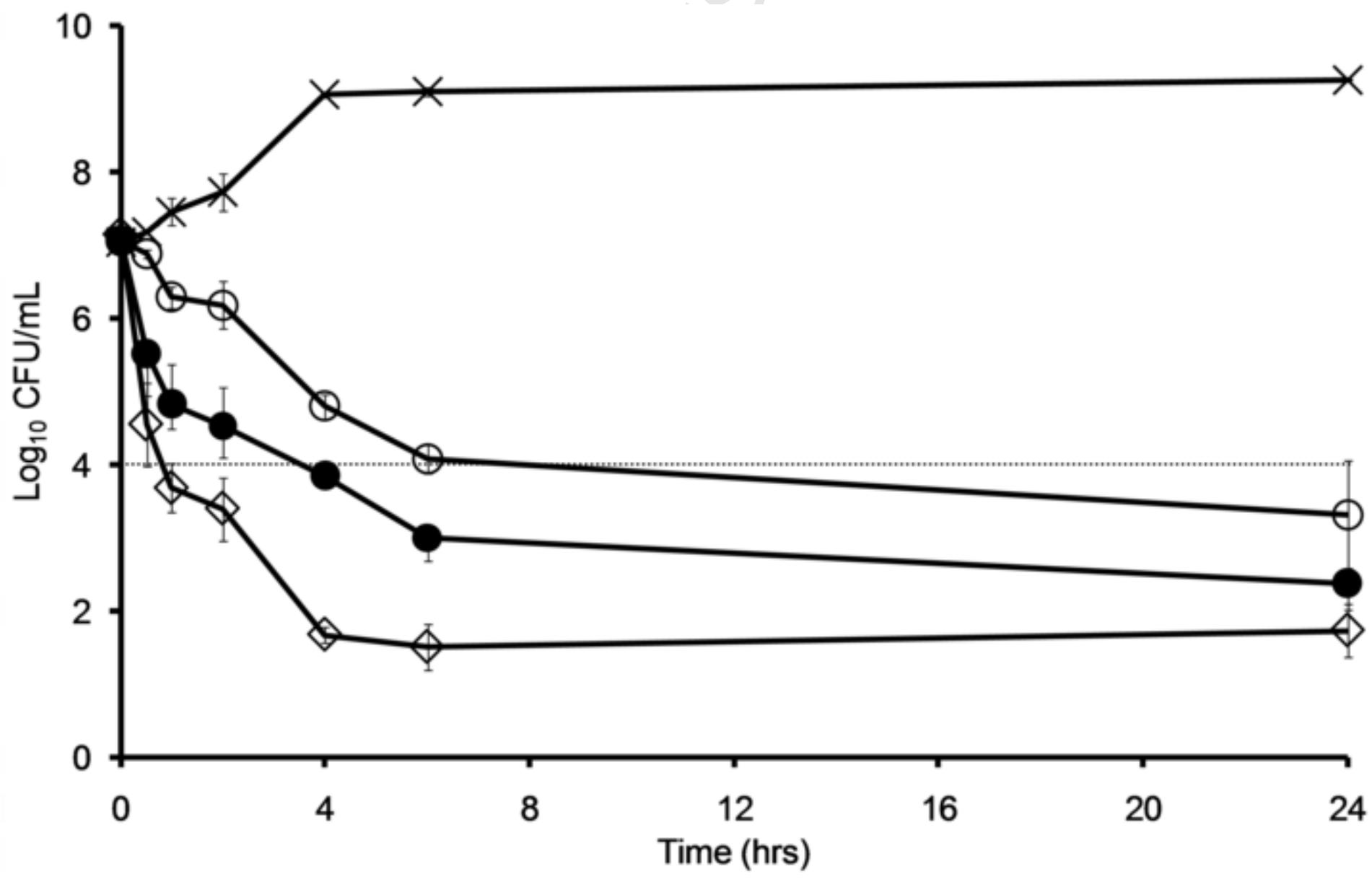
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|-----|--|-----------------------|---|-----|-----|-----|
| O19 | VDKPPYLPRPRPPR- Agp -IYN- Har-NH ₂ | 111 ± 6 | 4 | 2 | N/T | N/T |
| O20 | VDKPPYLPRPRPPR- Agp -IYN- Agp-NH ₂ | 171 ± 20 | 8 | 4 | 4 | 4 |
| O21 | VDKPPYLPRPRPPR- Orn -IYN- Har-NH ₂ | 75 ± 1 | 4 | 4 | N/T | N/T |
| O22 | VDKPPYLPRPRPPR- Orn -IYN- Agp-NH ₂ | 103 ± 5 | 4 | 4 | N/T | N/T |
| O23 | VDKPPYLPRPRPPR- Orn -IYN- Orn-NH ₂ | 176 ± 8 | 8 | 8 | 4 | 2 |
| O24 | VDKPPYLPRPRPPR- arg -IYN- arg-NH ₂ | >480 (79.6 ± 0.2%) | 4 | N/T | 2 | 2 |
| O25 | VDKPPYLPRPRPPR- Orn -IYN- Arg-NH _{pr} | 102 ± 2 | 4 | 8 | 8 | 4 |

N/T, not tested; Arg, arginine; Ala, alanine; Asn, asparagine; His, histidine; Orn, ornithine; Agp, α -amino-3-guanidino-propionic acid;

Nir, nitro-arginine; Nmr, *N*-methyl-arginine; Har, homoarginine; arg, D-arginine; pr, propyl; β Hr, β -homoarginine.

^a Serum stabilities were determined in 25% aqueous mouse serum (O1–O16) and full mouse serum (O1, O17–O25) in duplicate. Half-life was determined in a single experiment.

^b Antibacterial activity was determined against *Escherichia coli*, *Micrococcus luteus* and *Klebsiella pneumoniae* strains using a serial dilution of each peptide in 1% tryptic soy broth in at least two independent experiments (in triplicate) with a standard abbreviation of one dilution step.



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