Peptides 44 (2013) 139–148

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Truncated antimicrobial peptides from marine organisms retain anticancer activity and antibacterial activity against multidrug-resistant *Staphylococcus aureus*

Ming-Ching Lin[a], Cho-Fat Hui[b], Jyh-Yih Chen[c,*], Jen-Leih Wu[a,b,**]

[a] Department of Biochemical Science and Technology, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei 10617, Taiwan
[b] Institute of Cellular and Organismic Biology, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan
[c] Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, 23-10 Dahuen Road, Jiaushi, Ilan 262, Taiwan

ARTICLE INFO

Article history:
Received 4 March 2013
Received in revised form 8 April 2013
Accepted 8 April 2013
Available online 15 April 2013

Keywords:
Antimicrobial peptides
Epinecidin-1
Pardaxin-1
SALF
Antimicrobial
Anticancer

ABSTRACT

Antimicrobial peptides (AMPs) were recently determined to be potential candidates for treating drug-resistant bacterial infections. The aim of this study was to develop shorter AMP fragments that combine maximal bactericidal effect with minimal synthesis cost. We first synthesized a series of truncated forms of AMPs (anti-lipopolysaccharide factor from shrimp, epinecidin from grouper, and pardaxin from *Pardachirus marmoratus*). The minimum inhibitory concentrations (MICs) of modified AMPs against ten bacterial species were determined. We also examined the synergy between peptide and non-peptide antibiotics. In addition, we measured the inhibitory rate of cancer cells treated with AMPs by MTS assay. We found that two modified antibacterial peptides (epinecidin-8 and pardaxin-6) had a broad range of action against both gram-positive and gram-negative bacteria. Furthermore, epinecidin and pardaxin were demonstrated to have high antibacterial and anticancer activities, and both AMPs resulted in a significant synergistic improvement in the potencies of streptomycin and kanamycin against methicillin-resistant *Staphylococcus aureus*. Neither AMP induced significant hemolysis at their MICs. In addition, both AMPs inhibited human epithelial carcinoma (HeLa) and fibrosarcoma (HT-1080) cell growth. The functions of these truncated AMPs were similar to those of their full-length equivalents. In conclusion, we have successfully identified shorter, inexpensive fragments with maximal bactericidal activity. This study also provides an excellent basis for the investigation of potential synergies between peptide and non-peptide antibiotics, for a broad range of antimicrobial and anticancer activities.

1. Introduction

The widespread use of antibiotics in recent years has led to the rapid emergence of antibiotic-resistant bacteria [16]. Hence, it is very important to develop new classes of antimicrobial agents [38]. Naturally occurring, cationic antimicrobial peptides (AMPs) are suitable templates for the development of new therapeutic agents. AMPs have been isolated from a variety of organisms [2,8], and the likelihood of pathogens developing AMP resistance is very low [7]. In addition, AMPs have antimicrobial activity against a broad spectrum of pathogens, including gram-positive and gram-negative bacteria, fungi, and protozoa, and they exhibit antiviral and anticancer properties [6,26,38].

Infections with antibiotic-resistant bacteria require treatment with either new antibiotics or combination therapy with two or more drugs [31]. Synthetic combination therapy can reduce the drug dose required, and also prevent the development of resistance in bacteria [1,36]. In order to improve the activity and specificity of AMPs, several groups have altered their sequence, length, charge, and other properties [3,19,28,32]. High-throughput studies have generated synthetic or designer AMPs that are active against a broad range of pathogens [30,33]. These features have fostered renewed interest in studying synergy in antibiotic actions in several laboratories [4,5,20,27,29,37].

We have previously studied the biological activities of shrimp anti-lipopolysaccharide factor (SALF), epinecidin (Ep), and pardaxin [10,15,23]. Here, we investigated the possibility of producing low cost variants of these AMPs, and whether combination treatment with peptide and non-peptide antibiotics can (i) improve the antimicrobial activity of the peptides, (ii) increase the number of

* Corresponding author. Tel.: +886 920802111; fax: +886 39871035.
** Corresponding author at: Department of Biochemical Science and Technology, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei 10617, Taiwan. Tel.: +886 227899568; fax: +886 227824595.
E-mail addresses: zoocjy@gate.sinica.edu.tw (J.-Y. Chen), jlwu@gate.sinica.edu.tw (J.-L. Wu).

0196-9781/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2013.04.004

Table 1
Sequences and physicochemical properties of epinecidin variants used in this study.

Peptide	Sequence	Molecular weight	Isoelectric point	Charge
Epinecidin-1	GFIFHIIKGLFHAGKMIHGLV	2335.87	10.80	+5
Epinecidin-2	GFIFHIIKGLFHAGK	1685.03	10.80	+4
Epinecidin-3	GFIFHIIKG	1031.26	9.69	+2
Epinecidin-4	FIFHIIKGLFH	1371.68	9.69	+3
Epinecidin-5	FIFHIIKGLF	1234.54	9.69	+2
Epinecidin-6	FIFHIIKGLFHA	1442.75	9.69	+3
Epinecidin-7	FIFHIIKGLFHAG	1499.81	9.69	+3
Epinecidin-8	FIFHIIKGLFHAGKMI	1872.33	10.8	+4
Epinecidin-9	GFIFH	619.71	7.55	+1
Epinecidin-10	IKGLFHAGKMIHGLV	1621.01	10.8	+4
Epinecidin-11	KGLFHAGKMIH	1238.51	10.8	+4
Epinecidin-12	LFHAGKMIH	1053.29	9.68	+3
Epinecidin-13	MIHGLVTRR	1082.33	12.50	+3
Epinecidin-14	FHAGAM	632.73	7.55	+1
Epinecidin-15	AGKMIHGLV	925.15	9.69	+2
Epinecidin-16	HIIKGL	679.85	9.69	+2
Epinecidin-17	VTRRRHGV	980.13	12.80	+4
Epinecidin-18	IHGLV	537.65	7.55	+1

Table 2
Sequences and physicochemical properties of pardaxin variants used in this study.

Peptide	Sequence	Molecular weight	Isoelectric point	Charge
GE-1	GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE	3323.85	9.53	+1
GE-2	PKIISSPLFKTLLSAVGSALSSSGGQE	2675.05	9.53	+1
GE-3	PLFKTLLSAVGSALSSSGGQE	2049.29	6.34	0
GE-4	LSAVGSALSSSGGQE	1349.41	3.85	−1
GE-5	ALSSSGGQE	834.83	3.85	−1
GE-6	GFFALIPKIISSPLFKTLLSAVGSALS	2778.34	10.80	+2
GE-7	GFFALIPKIISSPLFKTLLSA	2263.77	10.80	+2
GE-8	GFFALIPKIISSPLF	1650.02	9.69	+1
GE-9	GFFALIPKI	1005.26	9.69	+1
GE-10	KTLLSAVGS	875.03	9.69	+1

candidates for antibacterial therapeutic drugs, and (iii) inhibit cancer cell growth.

2. Materials and methods

2.1. Antimicrobial agents

Peptides were synthesized by GL Biochemistry (Shanghai, China) using an Fmoc/tBu solid-phase procedure. We obtained crude peptides by extraction and lyophilization. The peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC). The molecular masses and purities of the purified peptides (with purity grades of >95%) were verified by mass spectroscopy (MS) and high-performance liquid chromatography (HPLC), respectively. The sequences of the different peptides of epinecidin-1, anti-lipopolysaccharide factor (ALF), and pardaxin are summarized in Tables 1–3.

2.2. Bacteria

The bacterial strains used were grouper *Vibrio alginolyticus* (from Dr. Kuo-Kau Lee, Department of Aquaculture, National Taiwan Ocean University, Taiwan), *Vibrio harveyi* (BCRC 13812), *Vibrio vulnificus* (204; from Dr. Chun-Yao Chen, Tzu Chi University, Hualien, Taiwan), *Micrococcus luteus* (BCRC 11034), *Staphylococcus aureus* (BCRC 10780), *Streptococcus pneumonia* (BCRC 10794), *Streptococcus agalactiae* (from Dr. Chun-Yao Chen), *Staphylococcus* sp. (BCRC 10451), *Pseudomonas aeruginosa*, and methicillin-resistant *Sta. aureus* (MRSA) (from Dr. Yih-Shyun E. Cheng). All strains were reconstituted according to suggested protocols.

2.3. Cell lines and culture

HeLa (human cervix adenocarcinoma), HT1080 (human fibrosarcoma), and MRC-5 (human lung fibroblast) cell lines were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). All cells were cultured using ATCC-suggested media.

2.4. Minimum inhibitory concentrations (MICs)

MICs against bacteria were determined using 96-well microtiter cell culture plates and a modified microdilution broth method for cationic AMPs, as previously described [24]. Briefly, bacterial cells

Table 3
Sequences and physicochemical properties of shrimp anti-lipopolysaccharide factor variants used in this study.

Peptide	Sequence	Molecular weight	Isoelectric point	Charge
SALF-1	ECKFTVKPYLKRFQVYYKGRMWCP	3069.68	10.07	+5
SALF-2	ECKFTVKPYLKRFQVYCP	2247.69	9.35	+3
SALF-3	ECKFTVKPYLCP	1425.72	8.22	+1
SALF-4	ECKFCP	723.86	6.23	0
SALF-5	ECYLKRFQVYYKGRMWCP	2368.81	9.28	+3
SALF-6	ECVYYKGRMWCP	1532.82	8.21	+1
SALF-7	ECMWCP	765.93	3.85	−1

grown overnight were diluted in medium broth to a cell density of 10^5 colony-forming units (CFU)/ml. In addition, peptides were dissolved in phosphate-buffered saline (PBS) to the desired concentration, and serial dilutions of the peptides were placed in 96-well polypropylene microtiter plates. Each well was seeded with 100 μl of test bacteria (10^5 CFU/well), and aliquots of an equal volume of the peptide were added and mixed. The plates were then incubated at 37 °C for 16 h in an incubator. Microbial sedimentation was confirmed by visual verification, and the absorbance readings at 600 nm (O.D. 600) were measured using a microtiter plate reader. The MIC was defined as the lowest concentration of a peptide that inhibited growth of the bacteria after overnight incubation. Each experiment was performed in triplicate and repeated at least three times.

2.5. Hemolytic-activity testing

Briefly, sheep blood cells (SBCs) in 10% citrate phosphate dextrose were harvested by centrifugation (1000× for 5 min at room temperature). SBCs were washed three times with PBS, and then diluted 25-fold with PBS to a blood cell concentration of approximately 4% (v/v). A portion of the SBC suspension (100 μl) was transferred to each well of a 96-well microtiter plate, and mixed with 100 μl of an AMP solution in PBS at the desired concentration. The microtiter plate was then incubated at 37 °C to allow hemolysis to occur. After 1 h of incubation, non-hemolyzed SBCs were separated by centrifugation (1000 × *g* for 5 min at room temperature). Aliquots (100 μl) of the supernatant were transferred to a new 96-well plate, and hemoglobin release was monitored by measuring the absorbance of the supernatant at 540 nm using a microtiter plate reader. An SBS solution treated with 0.1% TritonX-100 (to induce 100% lysis) was used as a positive control for this assay, and an untreated SBC suspension in PBS alone was used as a negative control. Each assay was performed in triplicate for three independent experiments, and data were expressed as the mean and standard deviation (SD) of triplicate analyses of three independent experiments. The percentage of hemolysis was calculated using the following formula: hemolysis (%) = [($O.D_{540\,nm}$ of the treated sample − $O.D_{540\,nm}$ of the negative control)/($O.D_{540\,nm}$ of the positive control − $O.D_{540\,nm}$ of the negative control)] × 100%.

2.6. Synergistic effect

Combinations of AMPs with antibiotics of different classes were tested for synergistic effects by the checkerboard titration method. The fractional inhibitory concentration (FIC) index (FICI) of each antimicrobial drug mixture (drugs A and B) was calculated according to the equation: FICI = FIC A + FIC B = (MIC A combination/MIC A alone) + (MIC B combination/MIC B alone), where MIC A combination and MIC B combination were MICs of drugs A and B tested in combination, MIC A alone and MIC B alone were the MICs of drugs A and B tested alone, and FIC A and FIC B were the FICs of drugs A and B, respectively. FICI values were interpreted as follows: an FICI of ≤0.5 indicated synergy; an FICI of >0.5 and ≤1 indicated additivity; an FICI of >1 to ≤4 indicated indifference (no interaction); and an FICI of >4 indicated antagonism.

2.7. Mammalian-cell cytotoxicity

AMP cytotoxicity in HeLa and HT1080 cells was determined individually using an MTS assay, as previously described [17]. Briefly, HeLa and HT1080 cells (5×10^3 cells/well) were cultured at 37 °C in 96-well plates overnight. After removal of the media, cells were incubated at 37 °C for 24 h with 0.1 ml of AMPs. At the end of the treatment period, 20 μl of a mixture of the tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and an electron-coupling reagent, phenazine methosulfate (PMS) (Promega, Mannheim, Germany), was added, and the cells were incubated for a further 2 h at 37 °C. A microtiter plate reader was then used to detect absorbance at 490 nm. All data were repeated in triplicate for three independent experiments. Results are expressed as a percentage of the inhibition rate of viable cells, and values of the PBS-treated group (negative control) were subtracted from the experimental results.

2.8. Statistical analysis

Student's *t*-test was used to graph and compare the data between the two groups. Multiple-group comparisons were evaluated by analysis of variance (ANOVA) using SPSS software (Chicago, IL, USA). Differences were defined as significant at $p < 0.05$.

3. Results

3.1. Characterization of peptide fragments

In order to reduce the costs associated with synthesizing AMPs, we attempted to identify truncated variants of AMPs that retained the biological activities of the full-length peptide. We randomly deleted peptide sequences from shrimp anti-lipopolysaccharide factor (SALF), epinecidin (Ep), and pardaxin (GE). The respective full-length sequences of Ep, GE, and SALF are Ac-GFIFHIIKGLFHAGKMIHGLV-NH2, Ac-GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE-NH2, and Ac-ECKFTVKPYLKRFQVYYKGRMWCP-NH2. We synthesized seventeen modified Ep peptides, nine modified GE peptides, and six modified SALF peptides; these modified AMPs exhibited differences in sequence, length, and the charge of several groups. The molecular weight, isoelectric point, and charge of the peptides were determined using GenScript's Peptide Property Calculator (https://www.genscript.com/ssl-bin/site2/peptide_calculation.cgi), and the results are shown in Tables 1–3.

3.2. MICs of the AMPs

The antimicrobial activities of the peptide fragments were tested using three gram-negative (grouper *V. alginolyticus*, *V. harveyi*, and *V. vulnificus*) and five gram-positive bacteria (*M. luteus*, *Sta. aureus*, *Str. pneumonia*, *Staphylococcus* sp., and *Str. agalactiae*). Among the Ep peptides, Ep-3 and Ep-9–18 had no activity against grouper *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, *M. luteus*, *Sta. aureus*, *Str. pneumonia*, *Staphylococcus* sp., or *Str. agalactiae* in three independent experiments. In contrast, Ep-1, -2, and -4–8 exhibited antibacterial activity against grouper *V. alginolyticus*, *V. harveyi*, *Staphylococcus* sp., and *Sta. aureus*. The MIC of Ep-4 against *Sta. aureus* was 6.25 mg/L, whereas the MICs of Ep-2 and -7 were 25 and 50 mg/L against *M. luteus*, and 50 and 25 mg/L against *Str. pneumonia*, respectively. Importantly, while the MIC of full-length Ep (Ep-1) against *Sta. aureus* and *Str. pneumonia* was 50 mg/L, the MIC of Ep-8 against *Sta. aureus* was 6.25 mg/L. That is to say, the MICs of Ep-1 were higher than those of Ep-8, and hence, Ep-8 is more effective at low concentrations against both the gram-negative *V. harveyi* and the gram-positive *Sta. aureus* and *Str. pneumoniae* (Table 4a).

Of the truncated GE peptides, GE-2–5 and –8 had no activity against the three gram-negative or five gram-positive bacteria in two independent experiments. However, the MICs of GE-7 were 100 mg/L against *M. luteus*, *Sta. aureus*, and *Str. pneumonia*, and 12.5 mg/L against *Staphylococcus* sp. The MICs of GE-1 (full-length pardaxin) were 50 mg/L against grouper *V. alginolyticus*, *V. harveyi*, and *Sta. aureus*, and 100 mg/L against *V. vulnificus*, *M. luteus*, *Str.*

Table 4
Antibacterial activities of 40 antimicrobial peptides against grouper *Vibrio alginolyticus*, *Vibrio harveyi*, *V. vulnificus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus pneumonia*, Str. agalactiae, and *Staphylococcus* sp.

Peptide	MIC (mg/L)							
	Gram negative			Gram positive				
	Grouper *Vibrio alginolyticus*	*Vibrio harveyi*	*Vibrio vulnificus*	*Micrococcus luteus*	*Staphylococcus aureus*	*Streptococcus pneumoniae*	*Streptococcus agalactiae*	*Staphylococcus* sp.
(a) Epinecidin								
Epinecidin-1	6.25	6.25	50	6.25	50	50	≧100	6.25
Epinecidin-2	25	12.5	100	25	25	50	50	6.25
Epinecidin-3	NA	NA	NA	NA	NA	NA	NA	NA
Epinecidin-4	6.25	6.25	NA	NA	6.25	NA	≧100	≧100
Epinecidin-5	6.25	6.25	NA	NA	6.25	NA	NA	6.25
Epinecidin-6	25	6.25	NA	NA	6.25	NA	NA	12.5
Epinecidin-7	6.25	6.25	NA	50	6.25	25	NA	6.25
Epinecidin-8	12.5	6.25	NA	NA	6.25	NA	NA	12.5
Epinecidin-9	NA	NA	NA	NA	≧100	≧100	NA	NA
Epinecidin-10	NA	NA	NA	NA	NA	NA	NA	NA
Epinecidin-11	NA	NA	NA	NA	≧100	≧100	NA	NA
Epinecidin-12	NA	NA	NA	NA	≧100	≧100	NA	NA
Epinecidin-13	NA	NA	NA	NA	≧100	≧100	NA	NA
Epinecidin-14	NA	NA	NA	NA	≧100	≧100	NA	NA
Epinecidin-15	NA	NA	NA	NA	NA	NA	NA	NA
Epinecidin-16	NA	NA	NA	NA	≧100	≧100	NA	NA
Epinecidin-17	NA	NA	NA	NA	≧100	≧100	NA	NA
Epinecidin-18	NA	NA	NA	NA	NA	≧100	NA	NA
(b) Pardaxin (GE)								
GE-1	50	50	100	100	50	100	100	6.25
GE-2	NA	NA	NA	NA	NA	NA	NA	100
GE-3	NA	NA	NA	NA	NA	NA	NA	NA
GE-4	NA	NA	NA	NA	NA	NA	NA	NA
GE-5	NA	NA	NA	NA	NA	NA	NA	NA
GE-6	12.5	12.5	50	25	50	50	50	6.25
GE-7	NA	NA	NA	100	100	100	NA	12.5
GE-8	NA	NA	NA	NA	NA	NA	NA	NA
GE-9	NA	NA	NA	NA	≧100	NA	NA	NA
GE-10	NA	NA	NA	NA	NA	NA	NA	NA
(c) SALF								
SALF-1	25	25	50	NA	NA	NA	NA	25
SALF-2	NA	NA	NA	NA	NA	NA	NA	NA
SALF-3	NA	NA	NA	NA	NA	NA	NA	NA
SALF-4	NA	NA	NA	NA	NA	NA	NA	NA
SALF-5	NA	100	NA	NA	NA	NA	NA	50
SALF-6	NA	NA	NA	NA	NA	NA	NA	NA
SALF-7	NA	NA	NA	NA	NA	NA	NA	NA

pneumonia, and *Str. agalactiae*. On the other hand, the MICs of GE-6 were 12.5 mg/L against grouper *V. vulnificus* and *V. harveyi*, 25 mg/L against *M. luteus*, and 50 mg/L against *V. vulnificus*, *Str. pneumonia*, and *Str. agalactiae*. Thus, at low concentrations, GE-6 was more effective than GE-1 against both gram-negative and gram-positive bacteria (Table 4b). Compared to SALF-1, no gain in activity of SALF-2–7 against bacteria was observed among the SALF fragments (Table 4c).

We proceeded to compare EP-1 to EP-8, and GE-1 to GE-6. We used the Schiffer-Edmundson helical wheel model to predict hydrophilic and hydrophobic regions in the four (EP-1, EP-8, GE-1, and GE-6) synthesized peptides (Fig. 1). EP-8 showed a hydrophobic region leaning to one side, and a positive region partially to the other side. Moreover, there were more hydrophilic regions in EP-1 than in EP-8. On the other hand, there were fewer hydrophobic regions in GE-1 than in GE-6. We proceeded to analyze the hemolytic, antimicrobial, and anticancer activities of EP-8 and GE-6, and compared them to those of EP-1 and GE-1, respectively.

3.3. Hemolytic analysis of peptides

In general, the safety of target applications and biomaterials need to be identified using various methods. Synthetic antimicrobial biomaterials should undergo hemolytic analysis, to determine their ability to lyse mammalian SBCs. When developing anti-infective agents, one must understand their hemolytic properties, to ensure that they do not cause adverse side effects at working concentrations. Hemolytic analysis of the four selected peptides (EP-1, EP-8, GE-1, and GE-6) was performed, to determine concentrations causing 50% blood cell lysis (HL50). EP-1 and EP-8 did not have a strong hemolytic effect at low doses (Fig. 2a and b). Moreover, the HL50 values of GE-1 and GE-6 (about 100 mg/L) were much higher than those of EP-1 and EP-8 (Fig. 2c and d). The HL50 value of EP-8 was about 400 mg/L, which was 64-times higher than the MIC for *Sta. aureus*. The HL50 of GE-6 was 100 mg/L, which was twice as high as the MIC for *Sta. aureus*. The hemolytic activities of these four peptides were ranked in the following order: GE-1 > GE-6 > EP-1 > EP-8.

3.4. Effect of synergy on peptide antimicrobial activity

Preliminary screening was performed to determine whether the antimicrobial peptides interacted with clinically-used antibiotics with different structures, and if so, whether these interactions were synergistic, additive, or antagonistic. As shown in Table 5, each AMP/antibiotic (streptomycin or kanamycin) combination was apportioned an FICI (see Section 2). FICI values of ≤0.5 were considered synergistic. In this study, no synergy was observed for

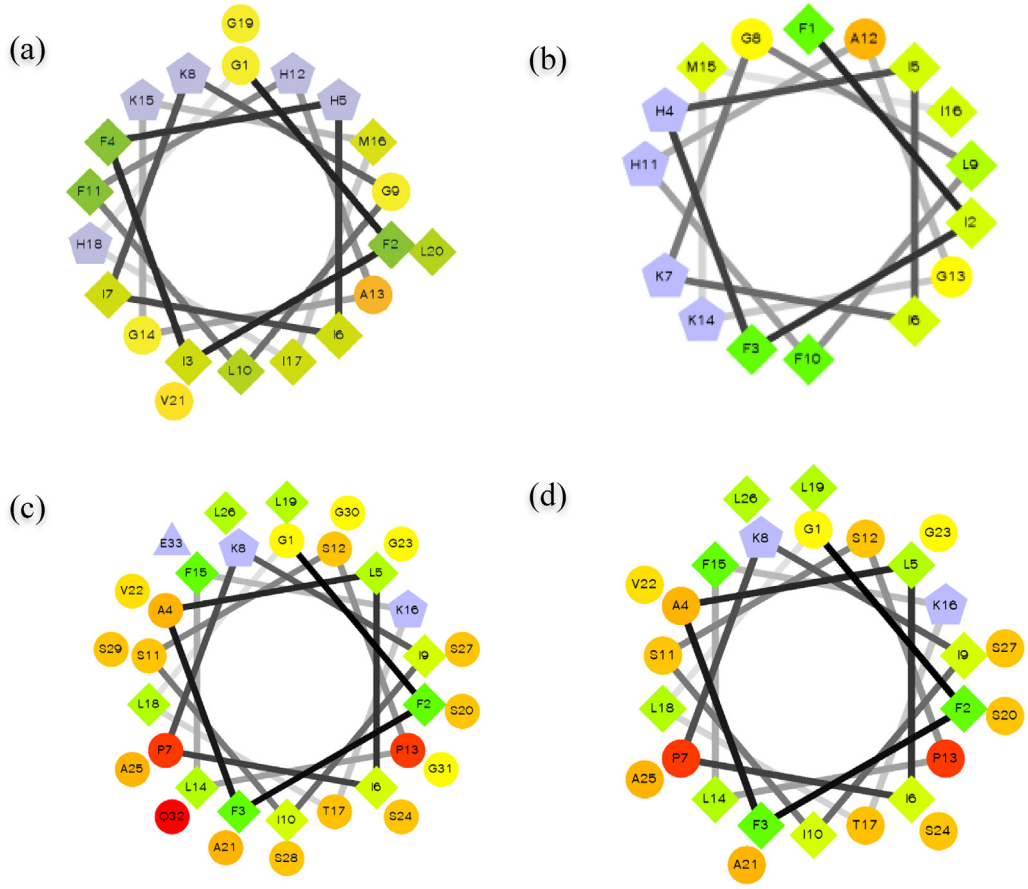

Fig. 1. Alpha-helix wheel projections of epinecidin-1 (a), epinecidin-8 (b), pardaxin-1 (c), and pardaxin-6 (d) peptides. Residues in circles indicate hydrophilic regions. Residues in diamonds indicate hydrophobic regions. Negatively charged residues are in triangles, and positively charged residues are in pentagons. Numbers are labeled from the N terminus to the C terminus. Green indicates the most hydrophobic residues. Red indicates the most hydrophilic residues. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

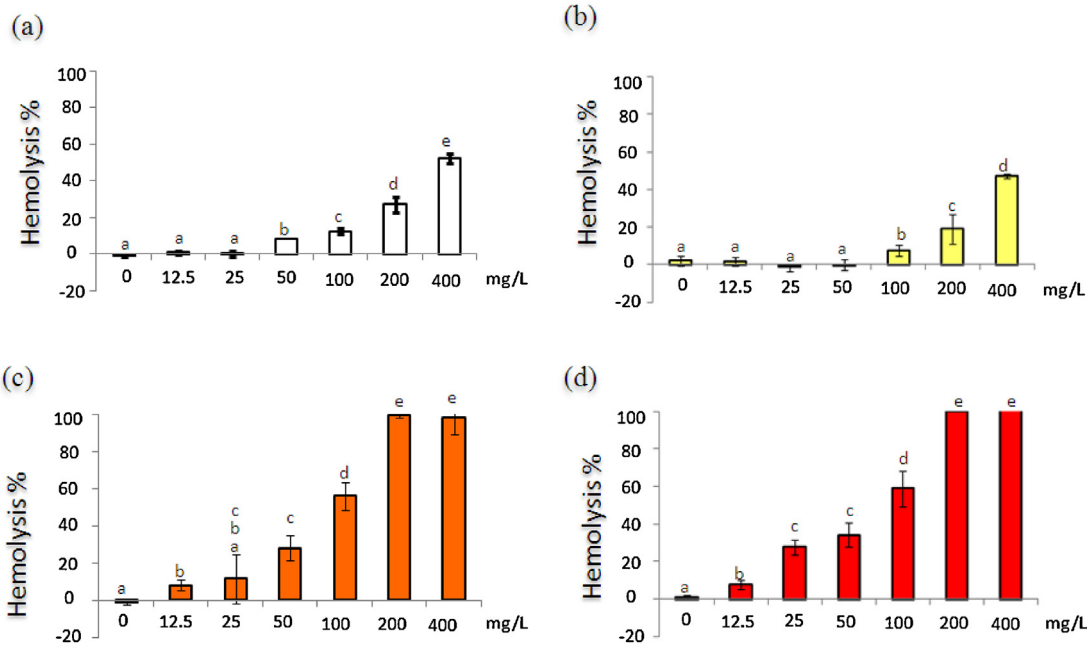

Fig. 2. Hemolytic activities of antimicrobial peptides incubated with sheep blood cells (SBCs) in PBS. Results for epinecidin-1 (a), epinecidin-8 (b), pardaxin-1 (c), and pardaxin-6 (d) peptides are expressed as percent hemolysis. SBCs incubated with Triton X-100 were considered to be 100% lysed. Different letters indicate a significant difference between two groups, while the same letter indicates no difference between two groups.

AMPs/antibiotic combinations against *Pse. aeruginosa* strains. Three AMPs (EP-1, GE-1, and GE-6) had FICI values of 1.3 when combined with streptomycin and kanamycin. Ep-8 was observed to be additive with antibiotics when used against *Pse. aeruginosa* strains, with an FICI of 0.8. Interestingly, the four antimicrobial peptides (EP-1, EP-8, GE-1, and GE-6) showed synergy with streptomycin and kanamycin in inhibiting MRSA growth. These results demonstrate that AMPs and non-peptide antibiotics improve antibacterial potency and thus the cost-effectiveness of the AMPs.

3.5. Toxic effects of peptides on inhibition of cancer cell growth

We next used MTS to examine the toxicities of EP-1, EP-8, GE-1, and GE-6 in HeLa and HT1080 cells. These cells were treated with 0, 3.125, 6.25, 12.5, 25, or 50 mg/L of one of the four peptides for 3, 6, 12, or 24 h. For both HeLa (Fig. 3a, b, e and f) and HT1080 cells (Fig. 3c, d, g and h), cell viability was not substantially affected by peptides at 3.125, 6.25, and 12.5 mg/L. However, over 50% cytotoxicity was observed upon treatment with peptides at 25 and 50 mg/L. Moreover, the AMPs affected cell morphology (Fig. 4). These results demonstrate that the truncated AMPs are able to inhibit cancer cell growth.

4. Discussion

In this work, we studied the synergy between AMPs and non-peptide antibiotics. Upon optimizing combination treatment conditions of a peptide with non-peptide antibiotics (kanamycin and streptomycin), we observed a dramatic increase in the antibacterial activity of the peptide. We also observed increased effectiveness of non-peptide antibiotics at low concentrations when they were used in combination with these peptides. Herein, we describe the *in vitro* activities of these peptides against bacteria. We also identified an optimized peptide against cervical carcinoma and a fibrosarcoma. Taken together, our results indicate that optimized peptides result in extreme synergistic enhancement of antibacterial activity, and also exhibit anticancer properties.

In order to identify optimal fragments with a low cost of synthesis and yet maximal bactericidal effect, we created many AMP fragments. We found that Ep-1 has antibacterial, antiviral, antiparasitic, and anticancer activities [18,22,25,34]. It is also involved in immune regulation [11,15,23]. Ep-8 retained the antibacterial activity of Ep-1, despite being shorter. In addition, GE-1 has antibacterial and anticancer activities, according to previous reports [10,12,21], and we found that its shorter variant, GE-6, also possessed antibacterial activity.

The goal of our *in vitro* study was to evaluate synergies among antibiotics and AMPs for treating MRSA infections. One of the greatest advantages of combining antibiotics and AMPs is the resulting decrease in therapeutic dosage, which decreases the possibility of adverse side effects, an important factor for clinical development. We report significant improvement in MIC values for the four peptides (Ep-1, Ep-8, GE-1, and GE-6) against many bacteria (Table 4). Thus, synergy resulted in dramatic decreases in MICs for Ep-1, GE-1, and GE-6 (from 12.5 to 4.3 mg/L) and Ep-8 (from 50 to 16.6 mg/L) against MRSA. In addition, antibiotics showed a 2–3-fold increase in potency in the presence of antibiotics (Table 5). Hence, synergistic combinations have the potential to make therapy more cost-effective, by decreasing the dosage of each agent used.

Streptomycin and kanamycin are different classes of antibiotics that affect translation processes [13,14], while AMPs induce membrane permeabilization *via* (1) carpet, (2) barrel stave, (3) toroidal pore, and (4) detergent-like models [9]. Schiffer-Edmundson helical wheel modeling revealed that Ep-8 has a hydrophobic region that leans to one side, and a positive region partially to the other side (Fig. 1). The region with a positive area may interact with the membrane and permeabilize it. The observation that both streptomycin and kanamycin had synergistic effects with AMPs suggests that different mechanisms may be involved.

To determine the effects of AMPs alone on mammalian cells, we studied their hemolytic potentials (Fig. 2) and their toxic effects on HeLa and HT1080 cell lines (Fig. 3). Ep-1 was previously shown to be cytotoxic to HeLa and HT1080 through inducing lysis [18]. Moreover, GE-1 demonstrated antitumor activity in human fibrosarcoma and epithelial carcinoma cells [10], which may be due to increased caspase-3/-7 activities, decreased MMP, and elevated reactive oxygen species (ROS) production [12]. As Ep-8 and GE-6 also inhibited HeLa and HT1080 cell lines (Fig. 3), these truncated peptides may be suitable therapeutic agents for future use against human fibrosarcoma and epithelial carcinoma cells.

Ep-8 contains fewer His, Gly, Leu, and Val residues than Ep-1. According to the AMP database, the ratios of His, Gly, Leu, and

Table 5
Synergistic activities of the tested antibiotics combined with antimicrobial peptides.

Peptide antibiotic	Nonpeptide antibiotic	MIC (μg/ml)				FIC index
		Peptide antibiotic		Nonpeptide antibiotic		
		Nonsynergy	Synergy	Nonsynergy	Synergy	
Pseudomonas aeruginosa						
Epinecidin-1	Streptomycin	12.5	8.3	12.5	8.3	1.3
	Kanamycin	12.5	8.3	12.5	8.3	1.3
Epinecidin-8	Streptomycin	100	16.6	12.5	8.3	0.8
	Kanamycin	100	16.6	12.5	8.3	0.8
Pardaxin-1	Streptomycin	12.5	8.3	12.5	8.3	1.3
	Kanamycin	12.5	8.3	12.5	8.3	1.3
Pardaxin-6	Streptomycin	12.5	8.3	12.5	8.3	1.3
	Kanamycin	12.5	8.3	12.5	8.3	1.3
Methicillin-resistant *Staphylococcus aureus* (MRSA)						
Epinecidin-1	Streptomycin	12.5	4.3	200	16.7	0.4
	Kanamycin	12.5	4.3	100	4.3	0.4
Epinecidin-8	Streptomycin	50	16.6	200	4.3	0.2
	Kanamycin	50	16.6	100	4.3	0.2
Pardaxin-1	Streptomycin	12.5	4.3	200	4.3	0.4
	Kanamycin	12.5	4.3	100	4.3	0.4
Pardaxin-6	Streptomycin	12.5	4.3	200	4.3	0.4
	Kanamycin	12.5	4.3	100	4.3	0.4

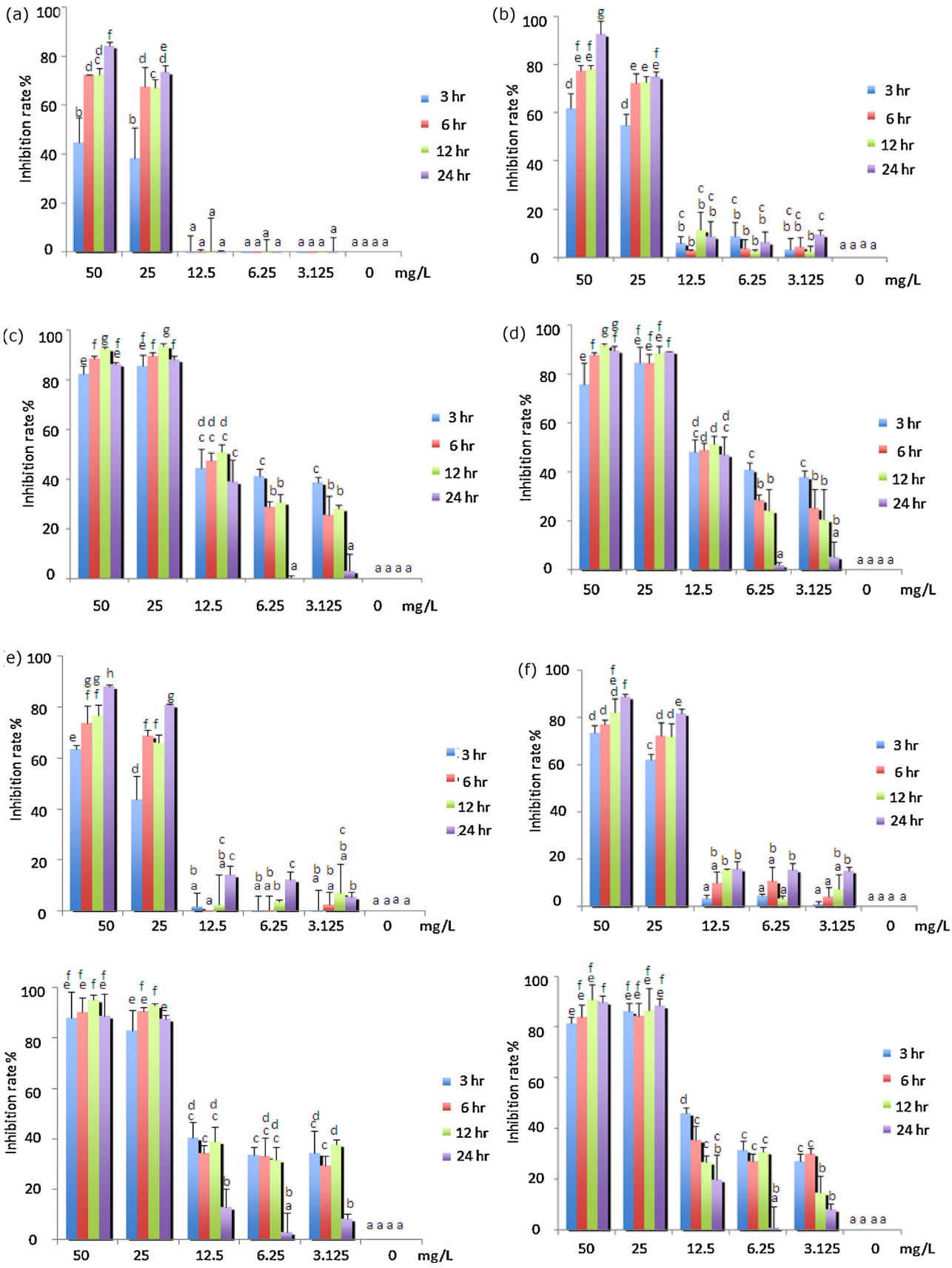

Fig. 3. Cytotoxicity (MTS assay) of full-length and truncated AMPs on mammalian cells. The inhibition rate of epinecidin-1 (a and c), epinecidin-8 (b and d), pardaxin-1 (e and g), and pardaxin-6 (f and h) against HT1080 (c, d, g, and h) and HeLa (a, b, e, and f) cells was determined at the indicated concentrations and times. Different letters indicate a significant difference between two groups, while the same letter indicates no difference between two groups.

Val in antibacterial peptides were 2.23%, 10.86%, 9.18%, and 6.38%, respectively. In anticancer peptides, the ratios for His, Gly, Leu, and Val were 3.90%, 8.40%, 9.60%, and 6.60%, respectively. GE-6 contains fewer Ser, Gly, Gln, and Glu residues than GE-1; interrogation of the AMP database revealed that the ratios of Ser, Gly, Gln, and Glu in antibacterial peptides were 4.98%, 10.86%, 2.51%, and 2.03%, respectively, and in anticancer peptides, 4.80%, 8.40%, 1.80%, and 1.20%, respectively. All of these residues are far less frequently included than hydrophobic residues, which are present at a ratio of 43.93% in antibacterial peptides, and 45.00% in anticancer pep-

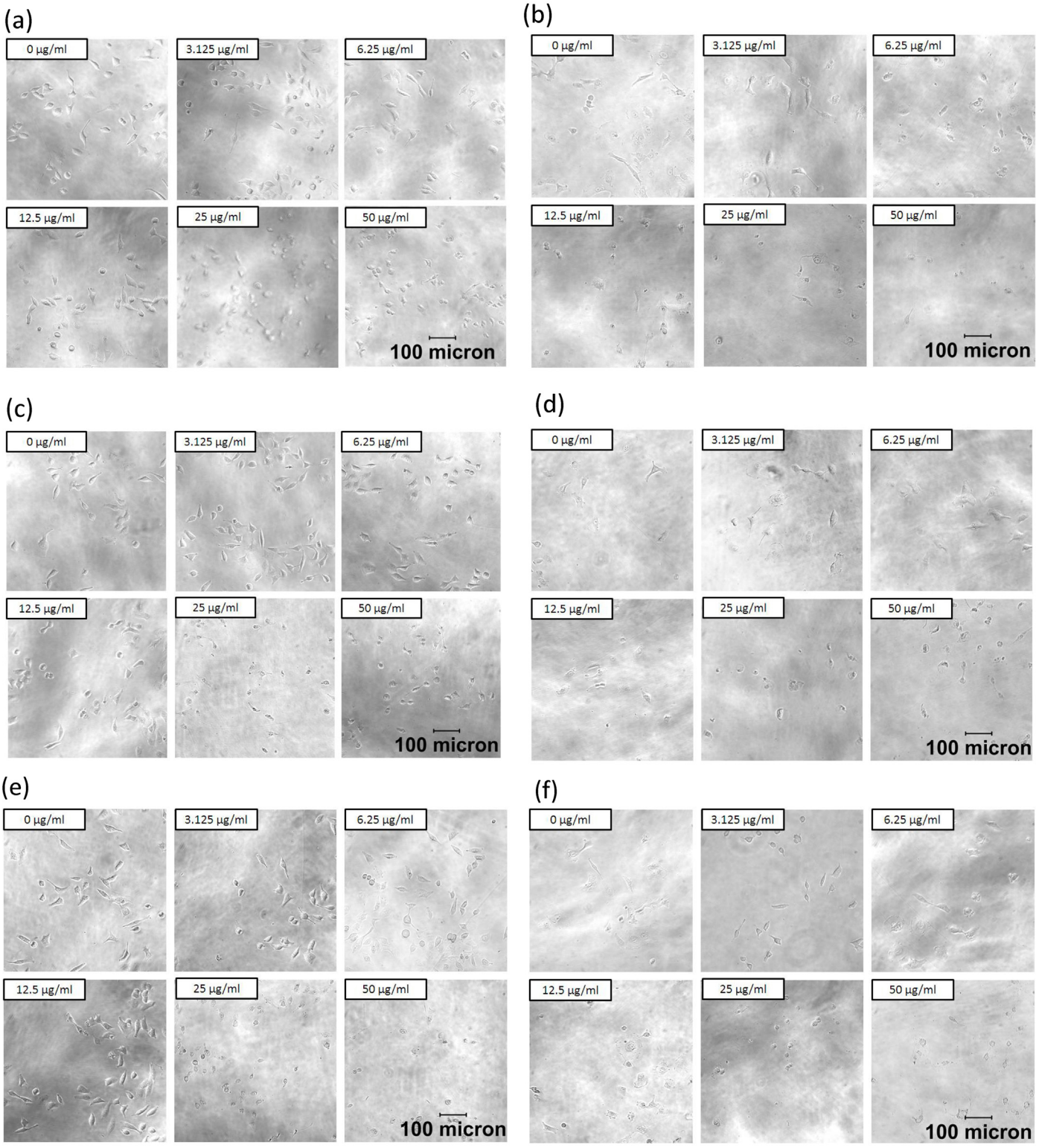

Fig. 4. The morphology of HT1080 (b, d, f, and h) and HeLa (a, c, e, and g) cells treated with the indicated concentrations of epinecidin-1 (a and b), epinecidin-8 (c and d), pardaxin-1 (e ane f), or pardaxin-6 (g and h) for 24 h.

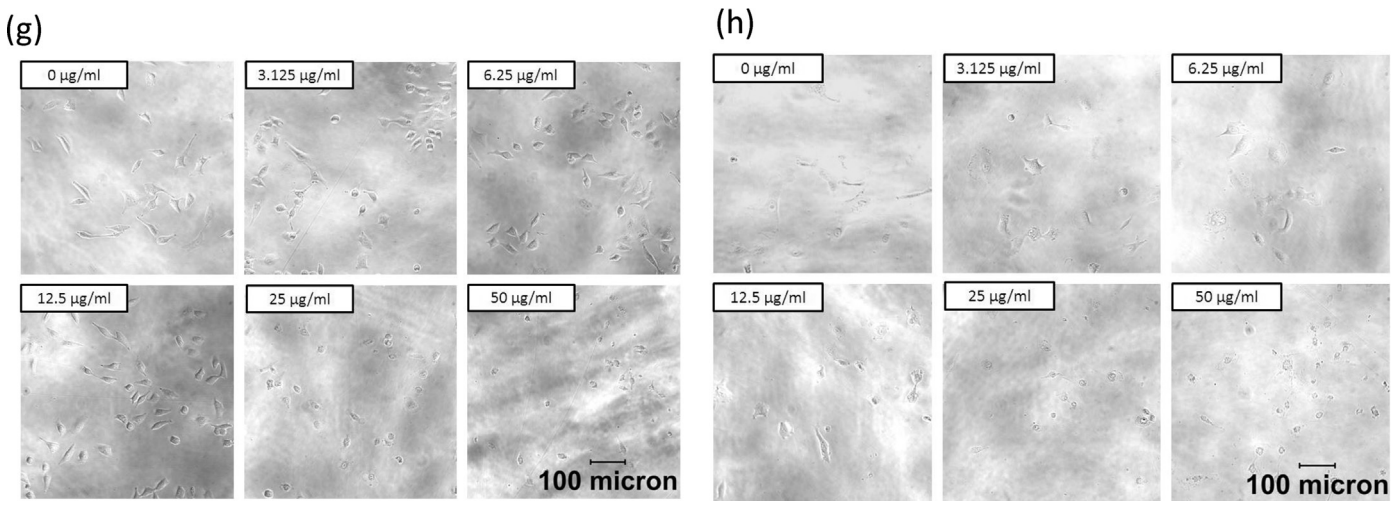

Fig. 4. (*Continued*)

tides [35]. It should also be noted that the isoelectric point of both Ep-1 and Ep-8 was 10.80, and Ep-1 and Ep-8 had charges of +5 and +4, respectively (Table 1). The isoelectric points of GE-1 and GE-6 were 9.53 and 10.80, and GE-1 and GE-6 had charges of +1 and +2, respectively (Table 2). Thus, the chemical properties of Ep-8 and GE-6 were similar to those of Ep-1 and GE-1, accounting for their similar therapeutic capabilities.

In conclusion, we generated truncated AMP fragments that retained maximal bactericidal effects, but could be synthesized at minimal costs. Our results indicate that Ep-8 and GE-6 exhibited similar activity against pathogens to Ep-1 and GE-1, suggesting that the former may aid in the development of antibacterial and anticancer drugs.

Acknowledgments

This study was supported by a grant from the Marine Research Station, ICOB, Academia Sinica, to Dr. Jyh-Yih Chen.

References

[1] Barriere SL. Bacterial resistance to beta-lactams and its prevention with combination antimicrobial therapy. Pharmacotherapy 1992;12:397–402.
[2] Bulet P, Stöcklin R, Menin L. Anti-microbial peptides: from invertebrates to vertebrates. Immunol Rev 2004;198:169–84.
[3] Chu-Kung AF, Bozzelli KN, Lockwood NA, Haseman JR, Mayo KH, Tirrell MV. Promotion of peptide antimicrobial activity by fatty acid conjugation. Bioconjug Chem 2004;15:530–5.
[4] Darveau RP, Cunningham MD, Seachord CL, Cassiano-Clough L, Cosand WL, Blake J, et al. Beta-lactam antibiotics potentiate magainin 2 antimicrobial activity in vitro and in vivo. Antimicrob Agents Chemother 1991;35:1153–9.
[5] Fassi Fehri L, Wróblewski H, Blanchard A. Activities of antimicrobial peptides and synergy with enrofloxacin against Mycoplasma pulmonis. Antimicrob Agent Chemother 2007;51:468–74.
[6] Gifford JL, Hunter HN, Vogel HJ. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. Cell Mol Life Sci 2005;62:2588–98.
[7] Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis 2001;1:156–64.
[8] Hancock RE, Chapple DS. Peptide antibiotics. Antimicrob Agents Chemother 1999;43:1317–23.
[9] Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta 2008;1778:357–75.
[10] Hsu JC, Lin LC, Tzen JT, Chen JY. Pardaxin-induced apoptosis enhances antitumor activity in HeLa cells. Peptides 2011;32:1110–6.
[11] Huang HN, Pan CY, Rajanbabu V, Chan YL, Wu CJ, Chen JY. Modulation of immune responses by the antimicrobial peptide, epinecidin (Epi)-1, and establishment of an Epi-1-based inactivated vaccine. Biomaterials 2011;32:3627–36.
[12] Huang TC, Lee JF, Chen JY. Pardaxin, an antimicrobial peptide, triggers caspase-dependent and ROS-mediated apoptosis in HT-1080 cells. Mar Drugs 2011;9:1995–2009.
[13] Jelenc PC, Kurland CG. Multiple effects of kanamycin on translational accuracy. Mol Gen Genet 1984;194:195–9.
[14] Lazar M, Gros F. Translation initiation defects in ribosomes from streptomycin dependent strains. Biochimie 1973;55:171–81.
[15] Lee SC, Pan CY, Chen JY. The antimicrobial peptide, epinecidin-1, mediates secretion of cytokines in the immune response to bacterial infection in mice. Peptides 2012;36:100–8.
[16] Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med 2004;10:S122–9.
[17] Lin MC, Hui CF, Chen JY, Wu JL. The antimicrobial peptide, shrimp anti-lipopolysaccharide factor (SALF), inhibits proinflammatory cytokine expressions through the MAPK and NF-B pathways in Trichomonas vaginalis adherent to HeLa cells. Peptides 2012;38:197–207.
[18] Lin WJ, Chien YL, Pan CY, Lin TL, Chen JY, Chiu SJ, et al. Epinecidin-1, an antimicrobial peptide from fish (Epinephelus coioides) which has an antitumor effect like lytic peptides in human fibrosarcoma cells. Peptides 2009;30:283–90.
[19] Makovitzki A, Avrahami D, Shai Y. Ultrashort antibacterial and antifungal lipopeptides. Proc Natl Acad Sci USA 2006;103:15997–6002.
[20] McCafferty DG, Cudic P, Yu MK, Behenna DC, Kruger R. Synergy and duality in peptide antibiotic mechanisms. Curr Opin Chem Biol 1999;3:672–80.
[21] Oren Z, Shai Y. A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from Moses sole fish Pardachirus marmoratus. Eur J Biochem 1996;237:303–10.
[22] Pan CY, Chen JY, Lin TL, Lin CH. In vitro activities of three synthetic peptides derived from epinecidin-1 and an anti-lipopolysaccharide factor against Propionibacterium acnes, Candida albicans, and Trichomonas vaginalis. Peptides 2009;30:1058–68.
[23] Pan CY, Chao TT, Chen JC, Chen JY, Liu WC, Lin CH, et al. Shrimp (Penaeus monodon) anti-lipopolysaccharide factor reduces the lethality of Pseudomonas aeruginosa sepsis in mice. Int Immunopharmacol 2007;5:687–700.
[24] Pan CY, Rajanbabu V, Chen JY, Her GM, Nan FH. Evaluation of the epinecidin-1 peptide as an active ingredient in cleaning solutions against pathogens. Peptides 2010;31:1449–58.
[25] Pan CY, Wu JL, Hui CF, Lin CH, Chen JY. Insights into the antibacterial and immunomodulatory functions of the antimicrobial peptide, epinecidin-1, against Vibrio vulnificus infection in zebrafish. Fish Shellfish Immunol 2011;31:1019–25.
[26] Papo N, Shai Y. Host defense peptides as new weapons in cancer treatment. Cell Mol Life Sci 2005;62:784–90.
[27] Park Y, Kim HJ, Hahm KS. Antibacterial synergism of novel antibiotic peptides with chloramphenicol. Biochem Biophys Res Commun 2004;321:109–15.
[28] Radzishevsky IS, Rotem S, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A. Improved antimicrobial peptides based on acyl-lysine oligomers. Nat Biotechnol 2007;25:657–9.
[29] Rand KH, Houck HJ. Daptomycin synergy with rifampicin and ampicillin against vancomycin-resistant enterococci. J Antimicrob Chemother 2004;53:530–2.
[30] Rathinakumar R, Wimley WC. Biomolecular engineering by combinatorial design and high-throughput screening: small, soluble peptides that permeabilize membranes. J Am Chem Soc 2008;130:9849–58.
[31] Rybak MJ, McGrath BJ. Combination antimicrobial therapy for bacterial infections. Guidelines for the clinician. Drugs 1996;52:390–405.
[32] Tencza SB, Creighton DJ, Yuan T, Vogel HJ, Montelaro RC, Mietzner TA. Lentivirus-derived antimicrobial peptides: increased potency by sequence engineering and dimerization. J Antimicrob Chemother 1999;44:33–41.

[33] Tew GN, Liu D, Chen B, Doerksen RJ, Kaplan J, Carroll PJ, et al. De novo design of biomimetic antimicrobial polymers. Proc Natl Acad Sci USA 2002;99:5110–4.
[34] Wang YD, Kung CW, Chen JY. Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1-5 against nervous necrosis virus in medaka. Peptides 2010;31:1026–33.
[35] Wang Z, Wang G. APD: the antimicrobial peptide database. Nucleic Acids Res 2004;32:590–2.
[36] Wu YL, Scott EM, Po AL, Tariq VN. Ability of azlocillin and tobramycin in combination to delay or prevent resistance development in Pseudomonas aeruginosa. J Antimicrob Chemother 1999;44:389–92.
[37] Zasloff M. Antimicrobial peptides of multicellular organisms. Nature 2002;415:389–95.
[38] Yan H, Hancock RE. Synergistic interactions between mammalian antimicrobial defense peptides. Antimicrob Agents Chemother 2001;45:1558–60.