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Anticancer Activity of Undecapeptide Analogues Derived from Antimicrobial Peptide, Brevinin-1EMa

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In spite of great advances in cancer therapy, cancer remains the major cause of death throughout the world. The increasing resistance of cancer cells towards current anticancer drugs requires development of anticancer agents with a new mode of action. Some antimicrobial peptides have become therapeutic candidates as new anticancer agents. As part of an effort to develop new antimicrobial and/or anticancer agents from natural peptides with low molecular weights, we have investigated the shortest bioactive analogues, which were derived from a 24-residue antimicrobial peptide, Brevinin-1EMa. Recently, we found four bioactive undecapeptides derived from a cationic, amphipathic α-helical, 11-residue peptide (named herein GA-W2: FLGWLFKWASK-NH_2) (Won et al., 2011). In order to identify the potential of these peptides as anticancer agents, we investigated the anticancer activity of four undecapeptides against seven tumor cell lines such as A498 (kidney), A549 (lung), HCT116 (colon), MKN45 (stomach), PC-3 (prostate), SK-MEL-2 (skin) and SK-OV-3 (ovary). GA-K4 (FLKWLFKWAKK-NH_2), which had the most potent antimicrobial activity of the four undecapeptides, also exhibited the most potent anticancer activity and synergistic effect in combination with doxorubicin. Therefore, GA-K4 peptide may be a potentially useful candidate as an anticancer peptide agent.

Key words: Anticancer peptides, Antimicrobial peptides, Synergistic effects, Combination index, Dose reduction index

INTRODUCTION

Although a growing number of studies for the treatment of cancer have been advanced recently, these could not catch up the increasing resistance to anticancer drugs in cancer cells. Instead, the general population with cancers including the skin, prostate, breast, and kidney continues to increase (Hoskin and Ramamoorthy, 2008). Multidrug resistance (MDR) is known to be responsible for chemotherapy failure in numerous cancers (Kim et al., 2003). Therefore, it is important to develop new anticancer agents that can overcome MDR mechanism for existing anticancer drugs. Many antimicrobial peptides damage the cellular membrane as part of their killing mechanism (Kim et al., 2003; Won et al., 2006). Furthermore, it has been reported that some antimicrobial peptides show anticancer activity against MDR cancer cells with little toxicity against non-tumor cells (Hoskin and Ramamoorthy, 2008). For example, the combination of antimicrobial peptide cecropin A with the conventional chemotherapeutic agents, 5-flurouracil and cytarabine, at certain doses, shows a synergistic cytotoxic effect on CCRF-SB human lymphoblastic leukemia cells (Hui et al., 2002). Like this, antimicrobial peptides have become a therapeutic candidate as new anticancer agents.

Most organisms produce membrane-active peptides that exhibit antibiotic, fungicidal, hemolytic, virucidal and even tumoricidal activities (Gabay, 1994; Boman, 1995; Nicolas and Mor, 1995; Bechinger, 1997; Hancock and Scott, 2000; Zasloff, 2002; Yeaman and Yount, 2003; Dennison et al., 2005; Yount and Yeaman, 2005). Many membrane-active antimicrobial peptides have little toxicity against animal cells, whereas they

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exhibit broad spectrum of antimicrobial activity against diverse microorganisms (Ohsaki et al., 1992; Mor and Nicolas, 1994; Goraya et al., 1999; Hancock and Scott, 2000; Rinaldi, 2002, Won et al., 2004a, 2004b). Most antimicrobial peptides are believed to kill pathogens by disrupting their membranes. These molecules commonly carry a net positive charge and most of them have the propensity to form an amphipathic α-helix. Recently, the number of antimicrobial peptides as potential therapeutic agents have increased, and several antimicrobial peptides have been successfully used in pharmaceutical and commercial development (Cruciani et al., 1991). In addition, some antimicrobial peptides were found to be cytotoxic against MDR cancer cells (Cruciani et al., 1991; Rozek et al., 2000; Kim et al., 2003; Papo and Shai, 2005). The anuran skin has served as an exceedingly rich source of antimicrobial peptides, and a number of cationic α-helical antimicrobial peptides has been isolated from the granular glands of the amphibian skin (Barra and Simmaco, 1995; Simmaco et al., 1998; Rinaldi, 2002; Zasloff, 2002). Thus, anuran-skin antimicrobial peptides have been considered as useful target molecules for developing new antibiotic and/or anticancer drugs (Nicolas and Mor, 1995; Andreu and Rivas, 1998; Won et al., 2002; Kim et al., 2003).

In our previous study, we have studied the shortest bioactive analogues of brevinin-1EMa, formerly known as gaegurin 5 (Park et al., 1994). Brevinin-1EMa is a 24-reside antimicrobial peptide isolated from the skins of *Glandirana emeljanovi* (Conlon, 2008), formerly classified as *Rana rugosa* inhabiting the Korean peninsula, and its antimicrobial and hemolytic activity was investigated (Won et al., 2004a, 2006). As a result, we identified the antimicrobial activities of some undecapeptides derived from an inactive N-terminal fragment (residue 1-11) of brevinin-1EMa (Conlon, 2008) and the anticancer activities of some of them additionally (Won et al., 2006). Furthermore, their sequences were modified in order to develop better antimicrobial activities with lower hemolytic activities. Finally, four bioactive undecapeptides were derived from GA-W2 (sequence: FLGWLFKWASK-NH_2) which was the most potent antimicrobial peptide in our previous study (Won et al., 2011). In order to develop new anticancer agents originating from natural antimicrobial peptides, the anticancer activities of the four bioactive undecapeptides were examined against various cancer cell lines: A498 (kidney), A549 (lung), HCT116 (colon), MKN45 (stomach), PC-3 (prostate), SK-MEL-2 (skin), and SK-OV-3 (ovary). Among them, GA-K4 (sequence: FLKWLFKWAKK-NH_2) exhibited the most potent anticancer activity. Furthermore, to evaluate its potential as a therapeutic agent, the combination effect of the four bioactive undecapeptides and doxorubicin was tested. Doxorubicin is a DNA alkylating agent used extensively in combination chemotherapy. The combination index (CI) and the dose reduction index (DRI) were calculated to evaluate the potential synergy (Dasmahapatra et al., 2004). Consequently, GA-K4 exhibited the most potent anticancer activity and synergistic effect with doxorubicin and thus could be a candidate for the development of a new peptide anticancer agent.

MATERIALS AND METHODS

Preparation of peptide

Peptides were synthesized automatically on a peptide synthesizer (Model 90 manufactured by Advanced Chemtech, Inc.) by solid-phase methods using standard Fmoc chemistry (Wellings and Atherton, 1997; Amblard et al., 2006). Fmoc (9-fluorenylmethyloxycarbonyl)-protected amino acids and Rink Resins were obtained from Advanced Chemtech, Inc. and HPLC solvents were obtained from Fisher Scientific. All other chemicals were either analytical or biotechnological grade and purchased from various manufacturers. To obtain the peptide amide, 4-(2',4'-dimethoxyphenyl-Fmoc-amino-methyl) phenoxy resin was used. Side-chain protecting groups included Fmoc-O-t-butyl-L-Serine and N-Fmoc-N-Boc-L-Lysine. Double coupling procedures were used with diisopropylcarbodiimide/1-hydroxybenzotriazole activation. Fmoc group removal from the peptide chain was performed with 25% piperidine in dimethylformamide. Cleavage of the resin and the protecting group were done with 10% trifluoroacetic acid in dichloromethane. The end of the peptide synthesis was verified by Ninhydrin reaction (Meyer, 1957). Purification and analysis of the products were done by analytical reversed-phase HPLC on C-18 column from Merck-Hitachi. Acetonitrile/water mixed with 0.1% trifluoroacetic acid was used as eluent and a gradient of 20-80% acetonitrile was applied at a flow rate of 1 mL/min. The correct mass of the product peptides was identified by mass spectrometry. The same peptides were also purchased from the peptide manufacturer company ANYZEN (URL, http://www.anygen.com), to check reproducibility of the activity test. Both the synthesized and purchased peptides were applied to the anticancer activity test.

Anticancer assay

The anticancer activities of the present peptides were assessed by their cytotoxicity against seven tumor cell lines from different tissues: A498 (kidney), A549

(lung), HCT116 (colon), MKN45 (stomach), PC-3 (prostate), SK-MEL-2 (skin), and SK-OV-3 (ovary). A normal breast cell line (MCF10a) was used as a negative control cell line, and a conventional drug (paclitaxel) was used as a positive control agent. The cell lines were obtained from the Korean Cell Line Bank (URL, http://cellbank.snu.ac.kr). All cancer cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine and antibiotics. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. The cytotoxicity assay was done using the typical microculture MTS method. Briefly, the cells were seeded at 0.25-0.5 × 10^4 cells/mL/well in 96-well microtiter plates, and then incubated at 37°C. After 72 h incubation with the drug, 40 μM of MTS reagent (in PBS at pH 7.0) was added to each well and incubated for 2 h. The plates were then centrifuged at 100 × g for 5 min. After removing the supernatant by aspiration, 150 μL of dimethyl sulfoxide was added to each well, followed by gentle shaking to dissolve the formazan crystals that remained in the wells. The absorbance was immediately recorded using a microplate reader at 490 nm. Wells without drugs were used as a control for cell viability, and wells without cells were used to blank the spectrophotometer. Inhibition of cell growth was calculated using the formula % inhibition = (1 (absorbency of treated cells/absorbency of untreated cells)) × 100. Concentration for 50% inhibition of cell growth (IC_{50}) value for each cell line was evaluated at treatment dose causing a 50% reduction in absorbance compared to the untreated control cells. Each experiment was repeated three times. Dose-response curves were plotted using the Prism (version 4.0) software packages (Graph Pad, Inc.).

Determination of combination effect

Doxorubicin was purchased from Sigma Chemical Company. Doxorubicin initially was dissolved according to the manufacturer's instructions and further diluted in RPMI 1640 medium. The peptides in this work were dissolved in water and diluted in RPMI 1640 medium. Cells were seeded into 96-well plates, and after adherence, drugs were added (alone or in combination) for 72 h. Ten-fold serial dilutions were done for both single drugs and the combinations. The concentrations were all based on the IC_{50} values of each individual drug and the combined drug regimens were evaluated at the equivalent to the ratio of their IC_{50} values.

To examine the synergistic effect of GA-K4 peptide and doxorubicin, the median-effect principle by T-C Chou was used. Median-effect computer software (Calcusyn for Windows, Biosoft) was used to generate the CI and DRI. The CI was calculated by the Chou-Talalay equation, which takes into account both the potency (D_m or IC_{50}) and the shape of the dose-effect curve. The general equation for the classic isobologram (CI = 1) is given by:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad \text{(A)}$$

where $(D_x)_1$ and $(D_x)_2$ in the denominators are the doses (or concentrations) for $(D)_1$ and $(D)_2$ alone that give 50% inhibition, whereas $(D)_1$ and $(D)_2$ in the numerators are the doses of agents in combination that also give 50% inhibition. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. The $(D_x)_1$ and $(D_x)_2$ can be readily calculated from the median-effect equation of Chou and Talalay:

$$D_x = D_m\left[\frac{f_a}{(1-f_a)}\right]^{1/m} \quad \text{(B)}$$

where D_m is the median-effect dose that is obtained from the anti-log of the X-intercept of the median-effect plot, X = log (D) *vs* Y = log[$f_a/(1-f_a)$] and m is the slope of the median-effect plot. The computer software of Chou and Talalay allows automated calculation of m, D_m, D_x, and CI values. From $(D_x)_1$, $(D_x)_2$, and $D_1 + D_2$, it becomes easy to construct isobolograms automatically based on Eq. (A).

To measure how many fold the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone, DRI values were also calculated as follow.

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2} \quad \text{(C)}$$

RESULTS

Design of peptides

Fig. 1 shows amino acid sequence of peptides used in this work. These peptides was designed based on the rule in our previous work, that is, two tryptophan residues were conserved at the critical amphipathic interface shown as the helical wheel in Fig. 2.

Anticancer activities

Anticancer activities of the peptides were examined against various tumor cell lines from different tissues: A498 (kidney), A549 (lung), HCT116 (colon), MKN45 (stomach), PC-3 (prostate), SK-MEL-2 (skin) and SK-OV-3 (ovary). The IC_{50} values of GA-W3, GA-W4, GA-K3, and GA-K4 against seven tumor cell lines are

Amino acid sequence												name
1		3		5		7		9		11		
F	L	G	W	L	F	K	W	A	S	K	– NH_2	GA–W2
F	L	G	W	L	F	K	W	A	W	K	– NH_2	GA–W3
F	L	W	W	L	F	K	W	A	W	K	– NH_2	GA–W4
F	L	G	W	L	F	K	W	A	K	K	– NH_2	GA–K3
F	L	K	W	L	F	K	W	A	K	K	– NH_2	GA–K4

Fig. 1. Amino acid sequences of undecapeptides used in this study. Each peptide sequence is labeled with the peptide name on the right. The regions conserved in all peptides are boxed, and amino acid variations from the starting peptide (GA-W2) are indicated in bold letters.

presented in Table I and Fig. 3. The ranges of the IC_{50} values varied from 12.55 to 47.69 μM. Comparing two-point substitutions (GA-W4 and GA-K4) with one-point substitutions (GA-W3 and GA-K3) at position 3 and 10, the IC_{50} values of the double substitutions were smaller than those of the one substitutions overall. GA-W3 and GA-K4 had almost similar anticancer activity.

Combination effect of GA-K4 and doxorubicin

In order to evaluate the potential synergy, GA-K4 and doxorubicin were used in combination with each other. The IC_{50} values of doxorubicin were reduced when combination treatments were used (Fig. 4). Combination effect of doxorubicin and GA-K4 was

Table I. IC_{50} values of GA-W3, GA-W4, GA-K3, and GA-K4 against 7 anticancer cell lines

Tumor cell lines	IC_{50} (uM)			
	GA-W3	GA-W4	GA-K3	GA-K4
A498 (kidney)	22.88	22.59	41.21	21.48
A549 (lung)	20.36	15.88	25.20	14.53
HCT116 (colon)	24.63	14.80	27.00	14.80
MKN45 (stomach)	24.31	13.77	26.70	22.51
PC-3 (prostate)	46.51	24.32	47.69	29.10
SK-MEL-2 (skin)	13.97	13.16	27.39	22.25
SK-OV-3 (ovary)	22.06	14.58	24.76	12.55

IC_{50}: 50% inhibition concentration

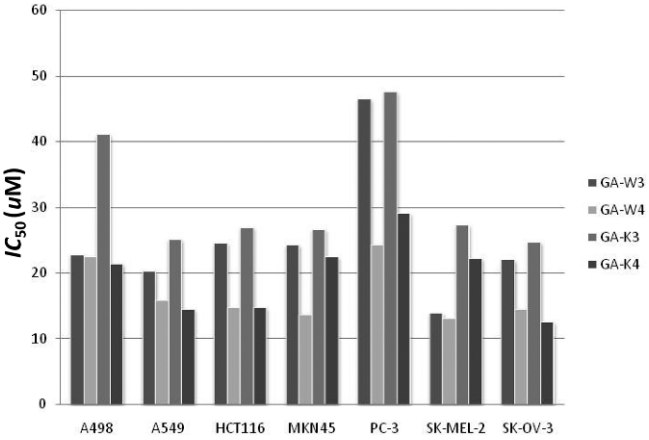

Fig. 3. Anticancer activities of GA-W3 (■), GA-W4 (■), GA-K3 (■) and GA-K4 (■) against 7 cancer cell lines.

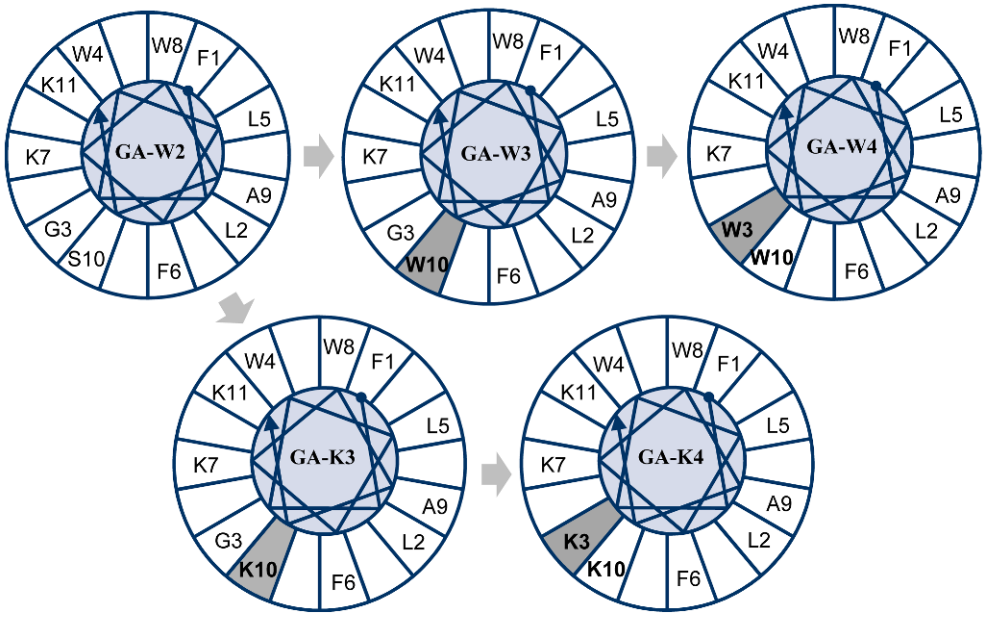

Fig. 2. Helical wheel diagrams for the undecapeptides used in this study. The peptide name was presented in the middle of each diagram. The order of design is depicted by the flow of the gray arrow. The amino acid variations from the preceding peptides are indicated in bold letters with gray background.

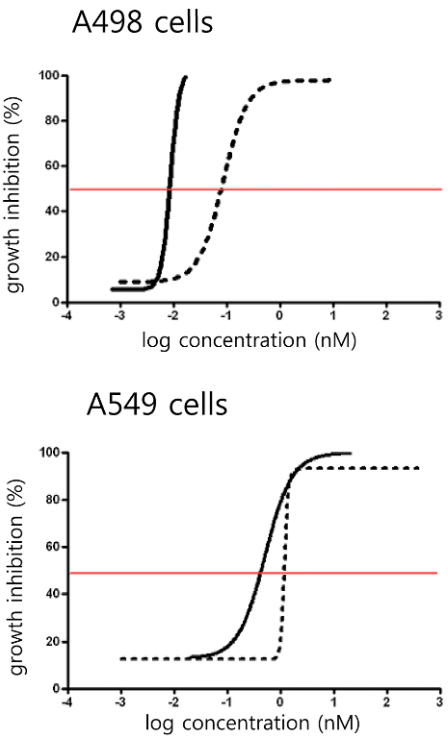

Fig. 4. Relative growth inhibition curves of GA-K4. Relative growth inhibition curves when exposed to doxorubicin alone (dotted line) and in combination with GA-K4 (solid line) in A498 (top, a fixed ratio 1:400 of doxorubicin and GA-K4) and A549 cells (bottom, a fixed ratio 1:15 of doxorubicin and GA-K4).

Table II. Dose-effect relationship of doxorubicin alone and in combination with GA-K4 on the growth of A498 and A549 cells

Cells	IC_{50} (μM)	
	Doxorubicin + GA-K4	Doxorubicin alone
A498	0.008	0.071
A549	0.229	1.256

IC_{50}: 50% inhibition concentration

estimated as nine times more effective against A498 cells and five times more effective against A549 cells (Table II). By the computer-simulated CI values, the CI values in A498 cells were shown to be <1 at 20-50% inhibition levels and >1 at other inhibition levels, and the CI values in A549 cells were shown to be <1 at 45-99% inhibition levels and >1 at other inhibition levels (Fig. 5 and Table III). The DRI values for the same inhibition levels with a value <1 exhibited a favorable dose reduction with a value >1 (Table IV).

DISCUSSION

Previously, as a part of an effort to develop new thera-

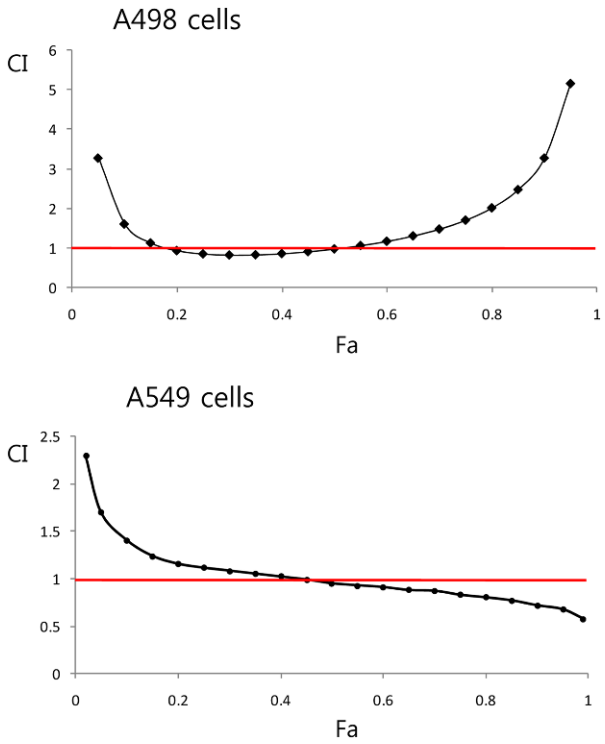

Fig. 5. Computer-generated plots of the combination index of doxorubicin and GA-K4 in A498 (top) and A549 (bottom) cells. Combination index <1 indicates synergism, whereas combination index >1 indicates antagonism, and combination index = 1 indicates simple additivity.

Table III. Computer-simulated combination index (CI) for doxorubicin and GA-K4 in A498 and A549 cells

A498		A549	
Inhibition (%)	Combination Index	Inhibition (%)	Combination Index
20	0.933	45	0.987
25	0.850	55	0.926
30	0.822	65	0.876
35	0.828	75	0.825
40	0.858	85	0.766
45	0.908	95	0.672
50	0.976	99	0.572

Table IV. Computer-simulated dose-reduction index (DRI) for doxorubicin and GA-K4 in A498 and A549 cells

A498		A549	
Inhibition (%)	DRI	Inhibition (%)	DRI
20	1.757	45	2.104
25	2.407	55	2.585
30	3.168	65	3.003
35	4.067	75	3.455
40	5.138	85	4.058
45	6.428	95	5.234
50	8.006	99	9.125

peutic agents from natural peptides, we reported the antimicrobial activities of novel undecapeptides derived from an inactive N-terminal fragment (residue 1-11) of brevinin-1EMa, formerly known as gaegurin 5 (GGN5), a 24-residue antimicrobial peptide isolated from the skin of an Asian frog, *Glandirana emeljanovi,* formerly classified as *Rana rugosa*. In addition, we have recently documented the anticancer activities of these analogues, as well. In order to suppress hemolytic activity while maintaining or enhancing antimicrobial activity, we attempted further sequence modification from GA-W2 (sequence: FLGWLFKWASK-NH_2), which was the most potent antimicrobial peptide in the previous study. As a result, we generated four analogues, GA-W3, GA-W4, GA-K3, and GA-K4, and the GA-K4 peptide showed superior bioactivity compared to the starting molecule, GA-W2. In continuing the studies, we examined the anticancer activities of the four improved analogues based on GA-W2. Amino acid sequences of the peptide analogues tested in this study are summarized in Fig. 1. To design each peptide sequence, we used helical wheel projections shown in Fig. 2. Based on our previous study, two tryptophan residues were fundamentally conserved at the critical amphipathic interface between the end of the hydrophobic side and the start of the hydrophilic side, as seen in the helical wheel projection. In the other amphipathic interface between the end of the hydrophilic side and the start of the hydrophobic side, the non-positive, hydrophilic S10 and the neutral G3 were substituted to W and/or K.

Cancer has become a major health problem in the world. Chemotherapy is still the preferred anticancer treatment. Despite excellent clinical success of anticancer drugs, the emergence of drug resistance diminishes their efficacy leading to treatment failure. Furthermore, side effects are dose-dependent and may be aggravated by increasing the dose and some combination of anticancer drugs. Thus, natural peptides with little toxicity are considered as useful agents for combination therapy. Thus, we tested the possibility of antimicrobial peptides which we have studied to develop anticancer agents.

In order to determine the potential anticancer activity of the designed peptide analogues on cell growth, we used MTS assay against seven anticancer cells (A498, A549, HCT116, MKN45, PC-3, SK-MEL-2 and SK-OV-3) (Lopez et al., 1985; Martello et al., 2000). Table I summarizes the IC_{50} values of GA-W3, GA-W4, GA-K3, and GA-K4 against the seven cancer cell lines. Experimental validation was done by confirming that the IC_{50} values for the positive control agent, paclitaxel, agreed well with values from the literatures. As a result, four brevinin-1EMa analogue peptides exhibited moderate anticancer activities with IC_{50} values ranging from 12.55 to 47.69 uM against various tumor cell lines. Judging from Table I, one-point substitutions at position 3 and 10, such as GA-W3 and GA-K3, exhibited higher IC_{50} values than the double substitutions at position 3 and 10, such as GA-W4 and GA-K4. It seems that the tightly organized amphipathicity by the double substitutions stabilizes membrane interactions, while it is likely that the presence of the flexible glycine is favorable for discriminating against different membrane surface charges. These double-substituted peptide analogues, GA-W4 and GA-K4, had almost similar anticancer activity. However, from our previous study, we found that the GA-K4 peptide exhibited stronger antimicrobial activity. In particular, the IC_{50} values of the GA-K4 peptide against tumor cell lines were the smallest of the four analogue peptides except for MKN45, PC-3 and SK-MEL-2. Because the GA-K4 peptide, which showed the most superior antimicrobial activity in the previous works, also exhibited the most potent anticancer activity, this peptide is noteworthy as a potential compound for the therapeutic development of an anticancer agent.

The combined effect of GA-K4 and doxorubicin, which is a DNA alkylating agent and is used extensively in combination chemotherapy, was examined. To evaluate the potential synergy, we treated combination of GA-K4 and doxorubicin at constant ratios with respect to their respective IC_{50} values for each cell line (Chou, 1998; Drewinko et al., 1979). Fig. 4 shows the representative relative growth inhibition curves when exposed to doxorubicin alone and in combination with GA-K4. A498 cells and A549 cells were exposed to a fixed 1:400 ratio and 1:15 ratio (doxorubicin:GA-K4), respectively. The dose-effect curves of the combination of GA-K4 and doxorubicin both in A498 and A549 cells were shaped with a shift to the lower concentration range, and the inhibition of cell growth was greater than that observed with doxorubicin alone. This means that the combination of GA-K4 with doxorubicin reduced the required dose for the same effect. The IC_{50} values of doxorubicin were also reduced when combination treatments was used (Table II). The IC_{50} values of doxorubicin were 0.071 μM and 1.256 μM for A498 and A549 cells, respectively. These values were reduced to 0.008 μM for A498 cells and to 0.229 μM for A549 cells. The dose-effect curves of the combination with GA-K4 in A498 and A549 cells were shaped with a shift to the lower concentration range and the IC_{50} values of doxorubicin were reduced. These results indicate that the addition of the GA-K4 enhances the cytotoxicity of doxorubicin. It has been widely re-

ported that some antimicrobial peptides show a synergic effect with conventional cancer-therapeutic agents. In many cases, antimicrobial peptides penetrate cell membrane by attaching and disrupting it (Papo and Shai, 2005). It is because cell wall of cancer cell consists of negative charge whereas antimicrobial peptides are usually cationic. This permeability of antimicrobial peptides may help that anticancer used combinationaly agents can go easily into the cell and work rapidly. We can expect that the GA-K4 peptide may have this mechanism for synergism with doxorubicin.

Transformation of the data as described by Chou and Talalay (Chou and Talalay, 1984; Chou et al., 1994; Chou, 1998) can be used to calculate the CI, a parameter that indicates whether the doses of the agents required to produce a particular effect in combination are larger than, equal to, or smaller than the doses required to produce that same degree of cytotoxicity. Combination index <1 indicates synergism, whereas combination index >1 indicates antagonism, and combination index = 1 indicates simple additivity. The computer-simulated CI values of doxorubicin are given in Table III and the plots of CI versus Fa (Fractions affected) are given in Fig. 5. The CI values varied over a ranges of Fa studied; at a Fa range from 0.2 to 0.5, the CI values was less than 1 for the A498 cells and at a Fa range from 0.45 to 0.99, the CI values was less than 1 for the A549 cells. That is, the degree of synergism of GA-K4 and doxorubicin varied at different Fa. According to the CI values, the combined effect of analogue GA-K4 and doxorubicin was moderate synergism at 20-50% inhibition levels in A498 cells and was moderate synergism at 45-99% inhibition levels in A549 cells. In addition, the synergistic effect in A549 cells was shown at all of the inhibition levels in A549 cells. The combined effect showed antagonism at 51-99% inhibition levels in A498 cells. However, this antagonism does not necessarily mean that the action of GA-K4 reduces the action of doxorubicin. Depending on the effects of these combinations on normal tissue versus tumor tissues, some of the 'antagonistic' combinations may be useful clinically.

In order to determine whether the dose of doxorubicin in combination at a given degree of inhibition is reduced when compared with the dose required for doxorubicin alone, DRI values were estimated (Chou, 2008). For therapeutic development, the synergistic interaction must result in a dose reduction of the anticancer drugs. The DRI, therefore, provides a quantitative measure of how many fold a dose of a drug can be reduced. DRI >1 indicates favorable dose reduction, whereas DRI <1 indicates unfavorable dose reduction. The greater DRI value indicates a greater dose reduction for a given therapeutic effect. As shown in Table IV, the DRI values of doxorubicin in A498 cells ranged from 1.757 to 8.006 at 20-50% inhibition levels in A498 cells and ranged from 2.104 to 9.125 at 45-99% inhibition levels in A549 cells. The DRI values for doxorubicin in A498 and A549 cells suggest that the presence of GA-K4 can reduce the dose requirement of doxorubicin. A reduction of the dose without changing the degree of effect may lead to reduced toxicity and improved therapeutic efficacy. Dose-dependent side effects are shown in chemotherapy, in such cases, any reduction in dose, even as low as twofold, may enhance the therapeutic effect of the anticancer drugs and prove to be beneficial. Accordingly, these results of CI values and DRI values demonstrate that the combined effect of GA-K4 and doxorubicin results in more greatly enhanced anticancer action than that of doxorubicin acting alone, and can be useful clinically.

In summary, four undecapeptides, GA-W3, GA-W4, GA-K3, and GA-K4, derived from the parent molecule, GA-W2, showed anticancer activities as well as antimicrobial activities. Especially, the GA-K4 peptide exhibited the most potent antimicrobial activity and the least hemolytic activity in our previous study. In this study, this peptide additionally showed the most potent anticancer activity and exhibited moderate synergism in combination with doxorubicin judging from the combination index and the dose reduction index. We could identify the possibility as an anticancer agent of GA-K4. Therefore, we can conclude that our sequence-optimized GA-K4 peptide in this study can be a potentially useful candidate for new anticancer agents originating from antimicrobial peptides. It may provide a new method for overcoming MDR which is a major problem in cancer therapy.

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