Bioorganic & Medicinal Chemistry Letters 23 (2013) 5744–5747

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Anticancer activities of an antimicrobial peptide derivative of Ixosin-B amide

Yu-Cheng Hsiao [a], Kai-Shiuan Wang [a], Shu-Huai Tsai [b], Wei-Ting Chao [b,c], Feng-Di T. Lung [a,c,*]

[a] Department of Chemistry, Tunghai University, Taichung, Taiwan, ROC
[b] Department of Life Science, Tunghai University, Taichung, Taiwan, ROC
[c] Life Science Research Center, Tunghai University, Taichung, Taiwan, ROC

ARTICLE INFO

Article history:
Received 9 May 2013
Revised 10 July 2013
Accepted 20 July 2013
Available online 9 August 2013

Keywords:
Antimicrobial peptides
Ixosin-B
Antimicrobial activity
Anticancer activity

ABSTRACT

In nature, antimicrobial peptides (AMPs) represent the first line of defense against infection by pathogens; thus, they are generally good candidates for the development of antimicrobial agents. Recently, we reported two potent antimicrobial peptides, KWLRRVWRWWR-amide (MAP-04-03) and KRLRRVWRRWR-amide (MAP-04-04), which were derived from a fragment of Ixosin-B-amide (KSDVRRWRSRY). Since some cationic AMPs exhibited cytotoxic activity against cancer cells, in the current study, we further investigated the anticancer activity of these potent antimicrobial peptides by antiproliferative assays and wound-healing assays, and the effect of peptide on the cytoskeleton alteration and cell morphology were analyzed by confocal microscopy. Results indicated that MAP-04-03 not only exhibited inhibitory effects on the proliferation (IC_{50} = 61.5 μM) and on the cell migration of MCF-7 breast cancer cells (at a concentration of 5 μM), but also affected the cytoskeleton at the concentration of 25 μM. These results demonstrated that MAP-04-03 can serve as a lead peptide analog for developing potent anticancer agents.

Bacterial resistance to conventional antibiotics is a widespread public health concern. Thus, the need for alternative antimicrobial agents is urgent. Antimicrobial peptides (AMPs) are considered as a potential alternative class of antimicrobial agents due to their selectivity for prokaryotic cells and minimized bacterial resistance.[1–5]

Ixosin-B, QLKVDLWGTRSGIQPEQHSSGKSDVRRWRSRY, exhibited antimicrobial activities against the Gram-negative bacterium *Escherichia coli*, Gram-positive bacterium *Staphylococcus aureus*, and fungus *Candida albicans*.[6] We assayed a series of peptide analogs derived from Ixosin-B amide and developed two antimicrobial peptide amides, MAP-04-03 (KWLRRVWRWWR) and MAP-04-04 (KRLRRVWRRWR).[7]

As some of the cationic AMPs that are toxic to bacteria but not to normal mammalian cells were found to exhibit cytotoxic activity against cancer cells,[8,9] in the current study, anticancer activities of MAP-04-03 and MAP-04-04 were investigated by the antiproliferative assay and the wound-healing assay. Effects of peptides on the alteration of cytoskeleton filaments and cell morphology were analyzed by confocal microscopy because microtubules and actin filaments are cytoskeletal protein polymers critical for cell growth and division, motility, signaling, and the development of cell shape.[10,11]

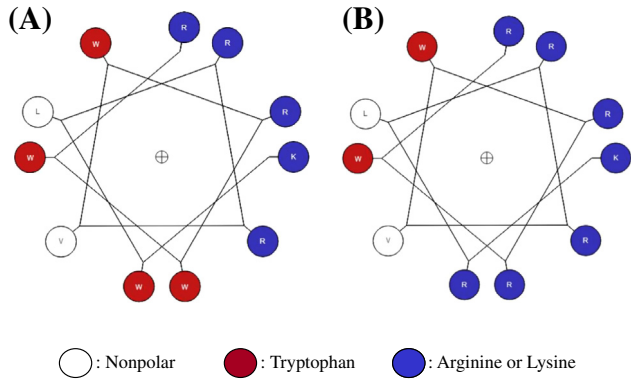
MAP-04-03 and MAP-04-04 were synthesized manually by solid phase method[12] using Fmoc/t-Bu chemistry[13] as previously reported.[14] Briefly, the Fmoc-protected Rink amide AM resin was swelled in DMF for 10 min at room temperature and then treated with 20% piperidine in DMF for 15 min to remove the Fmoc protecting group. The N-Fmoc, side-chain-protected amino acid was preactivated with the coupling reagent mixture (HOBt/HBTU/DIEA, 1:1:2) for 5 min and then mixed with resin at room temperature for coupling reaction (1.5 h). Cycles of removing Fmoc and coupling with the subsequent amino acids were repeated to produce the desired peptide-bound resin. The crude peptide was cleaved from resin by TFA cleavage mixture and lyophilization, and then purified by RP-HPLC followed by lyophilization and characterized by MALDI-TOF MS and RP-HPLC (Fig. 1).

As shown in Figure 2, characterized peptides were examined for their inhibitory effect on proliferation of MCF-7 breast cancer cells by the antiprolifeartive assay.

While MAP-04-03 showed antiproliferation effect in MCF-7 breast cancer cells (IC_{50} = 61.5 μM), MAP-04-04 did not exhibit antiproliferative activity in cancer cells at the concentration of 100 μM, demonstrating that the hydrophobicity of tryptophan (W) in MAP-04-03 ($K^1W^2L^3R^4R^5V^6W^7R^8W^9W^{10}R^{11}$-amide) is crucial for the antiproliferative activity, while the cationic charge of arginine (R) in MAP-04-04 ($K^1R^2L^3R^4R^5V^6W^7R^8R^9W^{10}R^{11}$-amide) is crucial for the antibacterial activity, particularly against *S. aureus*. The α-helical contents of MAP-04-03 and MAP-04-04 in SDS solution were determined as 87.6% and 44.6%, respectively, by

* Corresponding author. Department of Chemistry, Tunghai University, Taichung, Taiwan, ROC, Tel.: +886 4 23590121x32244; fax: +886 23590426.
E-mail address: fdlung@thu.edu.tw (F.-D.T. Lung).

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.07.063



	MAP-04-03	MAP-04-04
Mass (theoretical)	1727.1 Da	1667.0 Da
Mass (detected)	1727.9 Da	1668.4 Da
T_R (retention time)	10.88 min	8.98 min

Figure 1. Helical wheel projections and physicochemical characterization of synthesized peptides, (A) MAP-04-03 and (B) MAP-04-04.

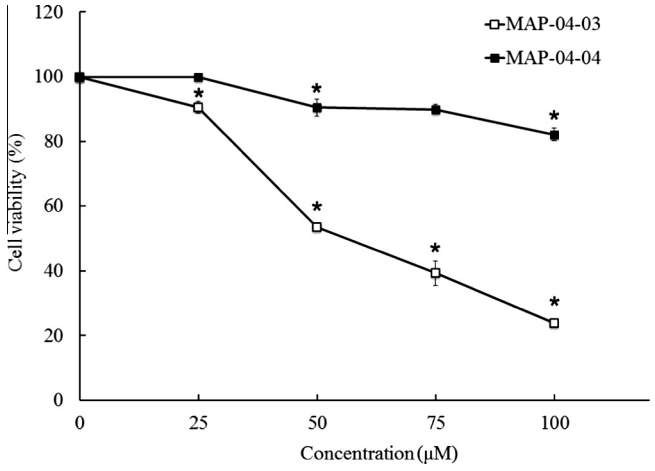


Figure 2. Human breast cancer cells (MCF-7) treated with 25, 50 75, and 100 μM of MAP-04-03 and MAP-04-04 for 48 h. The cell viability was determined by MTT assay. Data were reported as the mean value ± SD (standard deviation) of three independent experiments. $^*p < 0.05$ compared with the control.

circular dichroism (CD) spectroscopy,[7] suggesting that the helical conformation and the amphipathic property may contribute to the antimicrobial and/or anticancer activities of these peptides.

Because cell migration is a critical process in the development and maintenance of living organism, and errors during this process can cause serious events, such as vascular disease[15,16] and cancer,[17,18] these prompted us to further investigate the inhibitory effect of MAP-04-03 on the migration of cancer cells by the wound-healing assay.[19] Breast cancer cells MCF-7 were cultured in six-well plate for 24 h. The density of cells in a monolayer was more than 95% confluent. The cell monolayer was scraped with a 200 μL pipette tip to generate the wound of 6–7 mm. The removed culture medium and MCF-7 cells were treated with 1, 5, and 25 μM of MAP-04-03 or without peptide, and then re-cultured in fresh medium for 24 h and 48 h. The change of wound width caused by cell migration was observed under a phase contrast microscope following treatment with MAP-04-03 for 24 h and 48 h. Photographs were taken at 24 and 48 h on the same position of the wound (Fig. 3A).

There is significant difference on the wound width between the 5 μM peptide-48 h treated cells and the untreated control (~40%) in Figure 3B, while there is no visible difference on the wound width between the 1 μM peptide-24 h treated cells and the control, suggesting MAP-04-03 exhibited inhibitory effect on migration of the treated cells. However, treatment of MCF-7 cancer cells with 25 μM of MAP-04-03 caused the cells to detach from the bottom of dish, suggesting treatment with 25 μM of MAP-04-03 may cause the death of MCF-7 cell and making accurate measurement of wound width at this concentration is very difficult.

Although the IC_{50} of MAP-04-03 is 61.5 μM in the cell viability assay, yet it is effective at inhibiting the cell migration at 5 μM which is ten times more potent than the IC_{50}. In order to investigate if the cell migration inhibition by the peptide was caused by

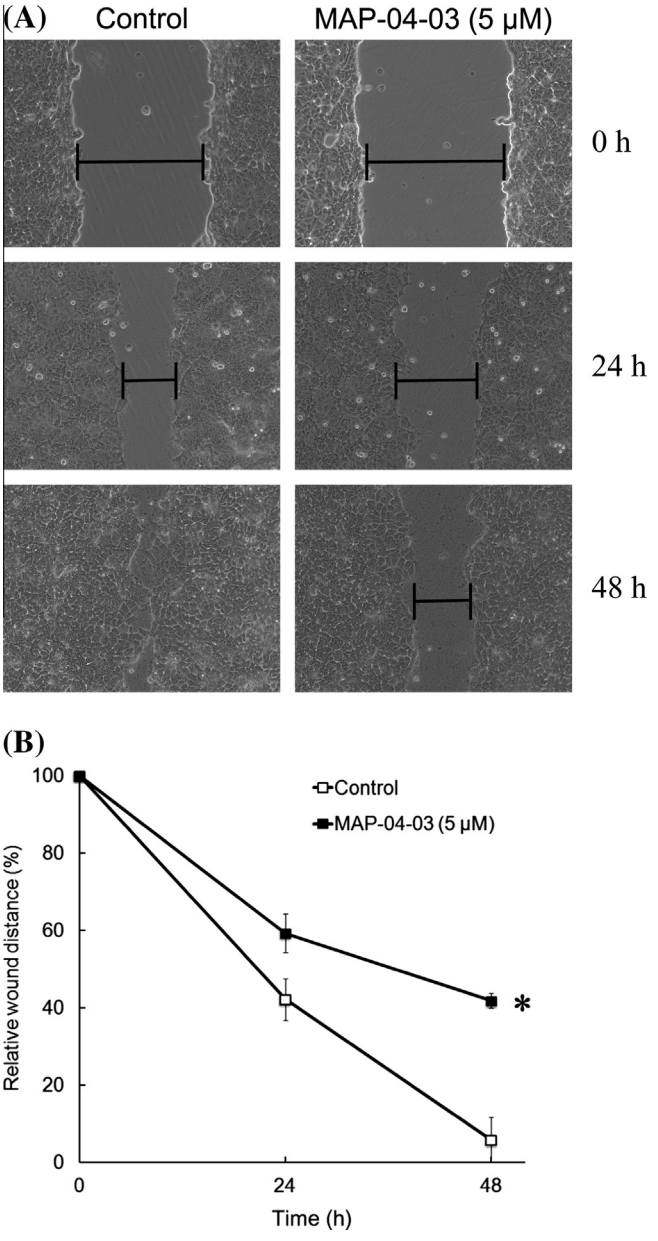


Figure 3. Effect of MAP-04-03 on cell migration by wound-healing assay in breast cells. (A) The MCF-7 cells scraped by a pipette tip to generate wounds of 6–7 mm. Then the cells were treated with 5 μM MAP-04-03 and then cultured for 24 and 48 h. (B) Results were obtained from 3 separate experiments. The bar represents the mean ± SE $^*p < 0.05$ indicates a significant difference between the control and MAP-04-03 treated samples.

the perturbing effects of the amphipathic peptides on cell membranes, we used MAP-04-04 as the control in the cell migration assay for comparison (data not shown). After 48 h, there is no significant difference on the wound width between the 5 μM MAP-04-04 treated cells and the untreated control, suggesting the amino acid residues W^2 and W^9 in MAP-04-03 play important role in affecting the cell migration, while R^2 and R^9 in MAP-04-04 did not show significant effect on the cell migration. It was reported that W has a distinct preference for the interfacial region of lipid bilayers, while R residues endow the peptides with cationic charges and hydrogen bonding properties necessary for interaction with the abundant anionic components of bacterial membranes.[20] These results suggested that 5 μM of MAP-04-03 may affect the migration of peptide-treated cells by perturbing cell membranes.

The wound width (the distances of scratch) of cancer cells treated with or without peptides was summarized in Figure 3B. It was demonstrated that ~40% of cell migration was inhibited by treatment with 5 μM of MAP-04-03 for 48 h.

E-cadherin is a transmembrane protein acting in the cell-to-cell junction. Previous studies have shown in the process of embryonic development that this protein can influence epithelial-mesenchymal transition.[21,22] Previous findings have supported a model that the loss of E-cadherin–based cell adhesion is considered to be an important factor in tumor invasiveness.[23,24] In cancer cells, the lack of E-cadherin expression will result in a loss of function in the cell–cell junction, resulting in cancer metastasis.[25] Although it was indicated that exogenous expression of N-cadherin would induce cell migration, invasion, and metastasis in MCF-7 breast cancer cells[26], there is no evidence to prove that the antiproliferative activity of MAP-04-03 in MCF-7 cells was correlated with cadherin or cell invasion.

Actin polymerization and remodeling plays a critical role in the morphologic and phenotypic events in cancer cells. Inhibition of the cytoskeleton proteins such as actin filaments and microtubules are potential targets for cancer chemotherapy.[10,27,28] To further investigate the effect of synthetic peptide MAP-04-03 on cytoskeleton alteration in breast cancer cells, the MCF-7 cells were treated with 1, 5, and 25 μM of MAP-04-03 for 24 h and 48 h, followed by fixing, staining and then observed by confocal microscopy. The distribution of two cytoskeleton proteins, α-tubulin and F-actin, were determined by staining with AlexaFluor 488 conjugated-goat anti-mouse IgG and tetramethylrhodamine B isothiocyanate (TRITC) conjugated phalloidin, respectively, and the nuclei of cells were stained with propidium iodide (PI).

As shown in Figure 4, in untreated MCF-7 cells, the cytoskeleton proteins α-tubulin and F-actin were concentrated at the periphery of cells, most staining was close to the outer edges of the cells, and there was clear definition between individual cells (Fig. 4A, Fig. 4B, and Fig. 4D). Compared to the untreated control, there is no significant change in cell morphology in the 1 μM peptid-treated group (Fig. 4E, Fig. 4F, and Fig. 4H). The morphology of cell has begun to shrink gradually by treatment of cells with 5 μM peptide (Fig. 4I and Fig. 4J), and the treatment with 25 μM of peptide causes collapse of cortical α-tubulin and F-actin, making it difficult to

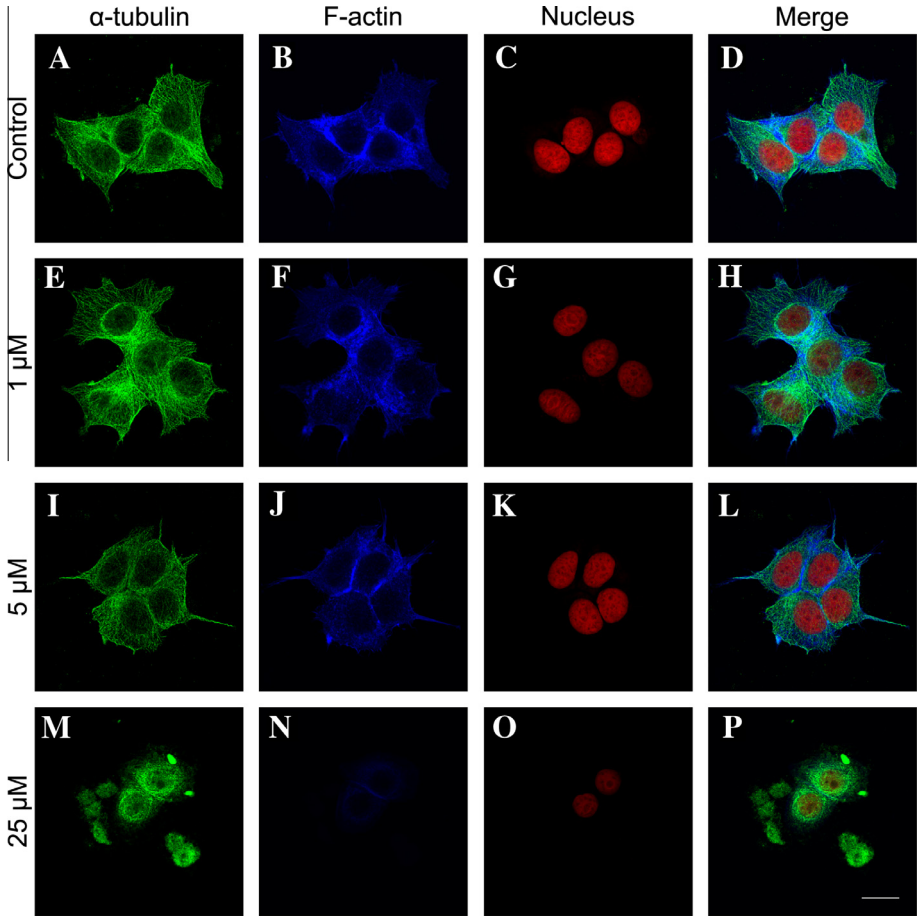


Figure 4. Effect of MAP-04-03 on the cytoskeleton alteration and cell morphology in MCF-7 breast cancer cells. MCF-7 cells were treated without (panels A–D) or with 1 (panels E–H), 5 (panels I–L), 25 μM (panels M–P) of MAP-04-03 peptide. F-actin, α-tubulin, and nuclei were stained with tetramethylrhodamine B isothiocyanate (TRITC) conjugated phalloidin, AlexaFluor 488 conjugated-goat anti-mouse IgG, and propidium iodide (PI), respectively. The treated or untreated MCF-7 cells were subjected to confocal microscopy analysis. The green color indicated the α-tubulin. α-tubulin. The blue color indicated the location of F-actin. The red color indicated the location of nuclei or chromosomes. The scale bar is 20 μm.

define the edge between individual cells (Fig. 4M, Fig. 4N, and Fig. 4P). In addition, the missing localization of α-tubulin was aggregated around the nucleus and the size of nucleus was smaller than untreated cells (Fig. 4C and Fig. 4O).

Based on the cell migration results (Fig. 3), 5 μM of MAP-04–03 was high enough to disturb the cell membranes of MCF-7 cells, suggesting that the staining of α-tubulin and F-actin might be caused by the potential damaging effects on the cell membrane by the amphipathic peptides through disrupting the integrity of cell membrane, however, the exact role of MAP-04–03 in regulating the cytoskeleton proteins dynamics is not clear.

In conclusion, anticancer activities of two peptide derivatives of Ixosin-B-amide were studied, and MAP-04-03 showed antiproliferative effects on breast cancer cells with the IC_{50} value of 61.5 μM and inhibited cancer cells migration at 5 μM, demonstrating it is a promising candidate for further development of anticancer agents.

Acknowledgments

This project was supported by the grants from the National Science Council (NSC99-2113-M-029-001) and Taichung Veterans General Hospital (TCVGH-997805), Taiwan, ROC.

References and notes

[1]. Hancock, R. E.; Sahl, H. G. *Nat. Biotechnol.* **2006**, *24*, 1551.
[2]. Boman, H. G. *J. Intern. Med.* **2003**, *254*, 197.
[3]. Bulet, P.; Stocklin, R.; Menin, L. *Immunol. Rev.* **2004**, *198*, 169.
[4]. Hancock, R. E.; Rozek, A. *FEMS Microbiol. Lett.* **2002**, *206*, 143.
[5]. Zasloff, M. *Nature* **2002**, *415*, 389.
[6]. Liu, Z.; Liu, H.; Liu, X.; Wu, X. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **2008**, *149*, 557.
[7]. Lung, F. D.; Wang, K. S.; Liao, Z. J.; Hsu, S. K.; Song, F. Y.; Liou, C. C.; Wu, Y. S. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4185.
[8]. Hoskin, D. W.; Ramamoorthy, A. *Biochim. Biophys. Acta* **2008**, *1778*, 357.
[9]. Mader, J. S.; Hoskin, D. W. *Expert. Opin. Investig. Drugs* **2006**, *15*, 933.
[10]. Jordan, M. A.; Wilson, L. *Curr. Opin. Cell Biol.* **1998**, *10*, 123.
[11]. Wilson, L.; Jordan, M. A. *Chem. Biol.* **1995**, *2*, 569.
[12]. Merrifield, R. B. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1969**, *32*, 221.
[13]. Chang, C. D.; Meienhofer, J. *Int. J. Pept. Protein Res.* **1978**, *11*, 246.
[14]. Lung, F. D.; Tsai, J. Y.; Wei, S. Y.; Cheng, J. W.; Chen, C.; Li, P.; Roller, P. P. *J. Pept. Res.* **2002**, *60*, 143.
[15]. Okamoto, T.; Atsumi, T.; Shimizu, C.; Yoshioka, N.; Koike, T. *J. Atheroscler. Thromb.* **2008**, *15*, 13.
[16]. Noels, H.; Bernhagen, J.; Weber, C. *Trends Cardiovasc. Med.* **2009**, *19*, 76.
[17]. Lester, R. D.; Jo, M.; Campana, W. M.; Gonias, S. L. *J. Biol. Chem.* **2005**, *280*, 39273.
[18]. Timoshenko, A. V.; Rastogi, S.; Lala, P. K. *Br. J. Cancer* **2007**, *97*, 1090.
[19]. Chao, W. T.; Daquinag, A. C.; Ashcroft, F.; Kunz, J. *J. Cell Biol.* **2010**, *190*, 247.
[20]. Chan, D. I.; Prenner, E. J.; Vogel, H. *J. Biochim. Biophys. Acta* **2006**, *1758*, 1184.
[21]. Nagafuchi, A.; Takeichi, M. *Embo. J.* **1988**, *7*, 3679.
[22]. Kemler, R. *Trends Genet.* **1993**, *9*, 317.
[23]. Behrens, J.; Mareel, M. M.; Van Roy, F. M.; Birchmeier, W. *J. Cell Biol.* **1989**, *108*, 2435.
[24]. Frixen, U. H.; Behrens, J.; Sachs, M.; Eberle, G.; Voss, B.; Warda, A.; Lochner, D.; Birchmeier, W. *J. Cell Biol.* **1991**, *113*, 173.
[25]. Thiery, J. P. *Nat. Rev. Cancer* **2002**, *2*, 442.
[26]. Hazan, R. B.; Phillips, G. R.; Qiao, R. F.; Norton, L.; Aaronson, S. A. *J. Cell Biol.* **2000**, *148*, 779.
[27]. Van Aelst, L.; D'Souza-Schorey, C. *Genes Dev.* **1997**, *11*, 2295.
[28]. Rao, J.; Li, N. *Curr. Cancer Drug Targets* **2004**, *4*, 345.