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Structure–activity relationship of a novel pentapeptide with cancer cell growth-inhibitory activity

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We previously reported that yamamarin, a pentapeptide with an amidated *C*-terminus (DILRG-NH_2) isolated from larvae of the silkmoth, and its palmitoylated analog (C16-DILRG-NH_2) suppressed proliferation of rat hepatoma (liver cancer) cells. In this study, we investigated the structure–activity relationship of yamamarin by *in vitro* assay and spectroscopic methods (CD and NMR) for various analogs. The *in vitro* assay results demonstrated that the chemical structure of the *C*-terminal part (-RG-NH_2) of yamamarin is essential for its activity. The CD and NMR results indicated that yamamarin and its analog adopt predominantly a random coil conformation. Moreover, a comparison of NMR spectra of DILRG-NH_2 and C16-DILRG-NH_2 revealed that the *N*-terminal palmitoyl group of C16-DILRG-NH_2 did not affect the conformation of the *C*-terminal part, which is essential for activity. Together, these results should assist in the design of more sophisticated anticancer drugs. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: insect pentapeptide; cell growth suppression; NMR; CD

Introduction

Throughout the course of evolution, peptides development has steadily continued in all living organisms, resulting in an enormous number of peptides with a great diversity of structures and biological functions, including hormonal and enzyme-regulating activities, intercellular communication, and participation in host defense. Today, large numbers of new biologically active peptides are still being discovered in an increasing variety of natural sources. Many of these novel peptides serve as research tools and have potential as diagnostic biomarkers and for the development of peptide and peptidometic drugs [1].

Insects have been shown to harbor peptides that can be developed into efficient chemotherapeutical agents [2]. Yamamarin, a novel insect pentapeptide with an amidated *C*-terminus (DILRG-NH_2), has been isolated from diapausing pharate first-instar larvae of the wild silkmoth *Antheraea yamamai* [3]. Yamamarin is thought to be responsible for the regulation of diapause, but its exact role in diapausing insects remains unclear. Recently, we have found that this peptide significantly suppresses the proliferation of rat hepatoma (liver cancer) cells but does not exert a suppressive effect on the lymphocytes of mouse spleen. Yamamarin does not possess any apoptotic/necrotic activity against rat hepatoma cells [4]. We have found further that its *N*-terminal palmitoylated derivative (C16-DILRG-NH_2) causes reversible growth arrest in rat hepatoma cells and inhibits embryonic development in the silkworm, *Bombyx mori*. In addition, its activity was over 20-fold higher than that of yamamarin [5]. Yamamarin also shows a strong cardioinhibitory effect [6]. These results strongly suggest that yamamarin and its derivatives are promising candidates for use as therapeutic agents or vermicides. However, its action mechanisms remain unknown. In this study, as a first step toward elucidating the mechanism of the growth suppression of rat hepatoma cells by yamamarin, we investigated the structure–activity relationship of yamamarin by *in vitro* assay and spectroscopic methods (CD and NMR) for various analogs of yamamarin, including those with alanine and *C*-terminal residue and group substitutions. The data obtained in this study will be useful for the design of peptides with pharmaceutical relevance.

Materials and Methods

Preparation of Peptides

The synthetic peptides listed in Table 1 were prepared for this study. All the peptides, except for C16-DILRG-NH_2, were purchased from SIGMA Genosys Japan K.K. (Hokkaido, Japan). These peptides

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Table 1. Peptides used in this study

Peptide	Residue length
DILRG-NH_2	5
AILRG-NH_2	5
DALRG-NH_2	5
DIARG-NH_2	5
DILAG-NH_2	5
DILRA-NH_2	5
DILRG-COOH	5
DILRGG-NH_2	6
DILRGG-COOH	6
DILR-NH_2	4
DILKG-NH_2	5
C16-DILRG-NH_2	5

-NH_2 and -COOH denote a *C*-terminal amide and carbonyl group, respectively; C16- denotes an *N*-terminal palmitoyl group.

were synthesized using the solid-phase method with standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. Purification was carried out by reverse-phase HPLC with a TSKgel ODS-80Ts column (TOSOH, Japan) to give final products of >95% purity. C16-DILRG-NH_2 was prepared as previously described [5].

Cell Growth Suppression Activity Assay

According to our previous papers [4,5], rat hepatoma cells (dRLh84) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn bovine serum (NBS) and supplemented with 4 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml kanamycin. The cells were cultured in the presence of 5% CO_2 at 37 °C under humidified conditions. The growth suppression activity was assessed by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [7]. The cells were suspended in a 0.25% trypsin solution and diluted with the culture medium to bring the cell concentration to 6×10^4 cells/ml. The diluted cell suspension was dispensed into each well of a 96-well microplate at a volume of 100 μl. After incubating the plate for 24 h, 11 μl of the extract sample, whose concentration was adjusted to 10-fold higher concentration than an appropriate assay concentration, was added to each well. The plate was incubated for another 48 h. The medium was then replaced with 100 μl of fresh medium containing 0.55 mg/ml of MTT and incubated for 4 h. After 4 h, the medium containing MTT was removed, and DMSO was added to each well at 200 μl to dissolve the formazan. The absorbance of each well at 590 and 620 nm was measured using a microplate reader (Immuno-Mini NJ-2300, Nippon InterMed KK, Tokyo, Japan) to assay for the growth suppression activity of the extracts.

CD Spectroscopy

CD data in the range of 200–250 nm were recorded on a Jasco J-725 spectropolarimeter using standard procedures. All measurements were taken using a 1-mm quartz cell at 25 °C. The concentrations of the peptides were estimated by amino acid analysis and were prepared to 100–140 μM in a 10 mM acetate buffer (pH 4.2). The ellipticity values obtained for the buffer were subtracted from the experimental values obtained for the peptide. Finally, the mean residue ellipticity is given as deg cm^2 $dmol^{-1}$.

NMR Spectroscopy

NMR experiments were carried out on a JEOL ECA600 spectrometer or a Bruker DRX500 spectrometer equipped with a cryo-probe. The spectra were measured at 10–35 °C using standard JEOL or Bruker pulse sequences. NMR samples were prepared with peptide concentrations of 1.0 mM and the pH was adjusted to 4.2 by the addition of HCl or NaOH. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate was used as a ^1H chemical shift reference. For all proton resonance assignments, the following experiments were performed: double quantum-filtered correlated spectroscopy (DQF-COSY) [8], TOCSY [9], NOESY [10], and ROESY [11]. All these data were acquired in the phase-sensitive mode using the States-time proportional phase incrementation method [12]. The water resonance was suppressed by the WATERGATE method [13]. TOCSY, NOESY, and ROESY spectra were obtained with a mixing time of 90, 300, and 300 ms, respectively. Data were processed using nmrPipe/nmrDraw software [14] and analyzed using the program Sparky [15].

Results and Discussion

Cell Growth Suppression Activity Assay for a Series of Yamamarin Analogs

Our previous paper demonstrated that the deletion of the first two residues or *C*-terminal amide group of yamamarin resulted in a loss of the cell growth suppression activity on dRLh84 cells [3]. To investigate the structure–activity relationship more systematically, alanine substitution mutagenesis was performed on the entire length of yamamarin. Figure 1(A) shows that yamamarin was active against dRLh84 cells and that there were structure–activity relationships, although the activity was much weaker than that of C16-DILRG-NH_2. The growth of dRLh84 cells cultured with DILRG-NH_2 was suppressed in a dose-dependent manner within the concentration range of 0–500 μM and decreased to 40% at a concentration of 500 μM. AILRG-NH_2 and DIARG-NH_2 exhibited activity comparable to that of DILRG-NH_2, and DALRG-NH_2 exhibited slightly lower activity, whereas DILAG-NH_2 and DILRA-NH_2 showed almost no activity. These results indicated that the *C*-terminal amino acid residues of yamamarin were more important for activity than the *N*-terminal ones, in agreement with our previous study [3]. In addition, I2 was found to be significant for activity.

Subsequently, we prepared various yamamarin analogs in which the *C*-terminal part was altered. To examine the effect of the *C*-terminal amide group on activity closely, DILRG-COOH, DILRGG-NH_2, and DILRGG-COOH were designed. DILRGG-NH_2 and DILRGG-COOH were each regarded as a substitution analog in which one amide proton of the amide group was replaced with a larger group. Figure 1(B) shows that none of the analogs exerted a suppressive effect on dRLh84 cells, even at 500 μM concentration. Each of the substitutions of the *C*-terminal amide group abolished activity. Interestingly, substitution of Arg to Lys in the fourth position (DILKG-NH_2) resulted in a complete loss of activity. This means that not only the electric charge but also the structure of the side chain of arginine is important for the activity. From the results shown in Figure 1, we conclude that the structure of -RG-NH_2 is essential for cell growth suppression activity on dRLh84 cells.

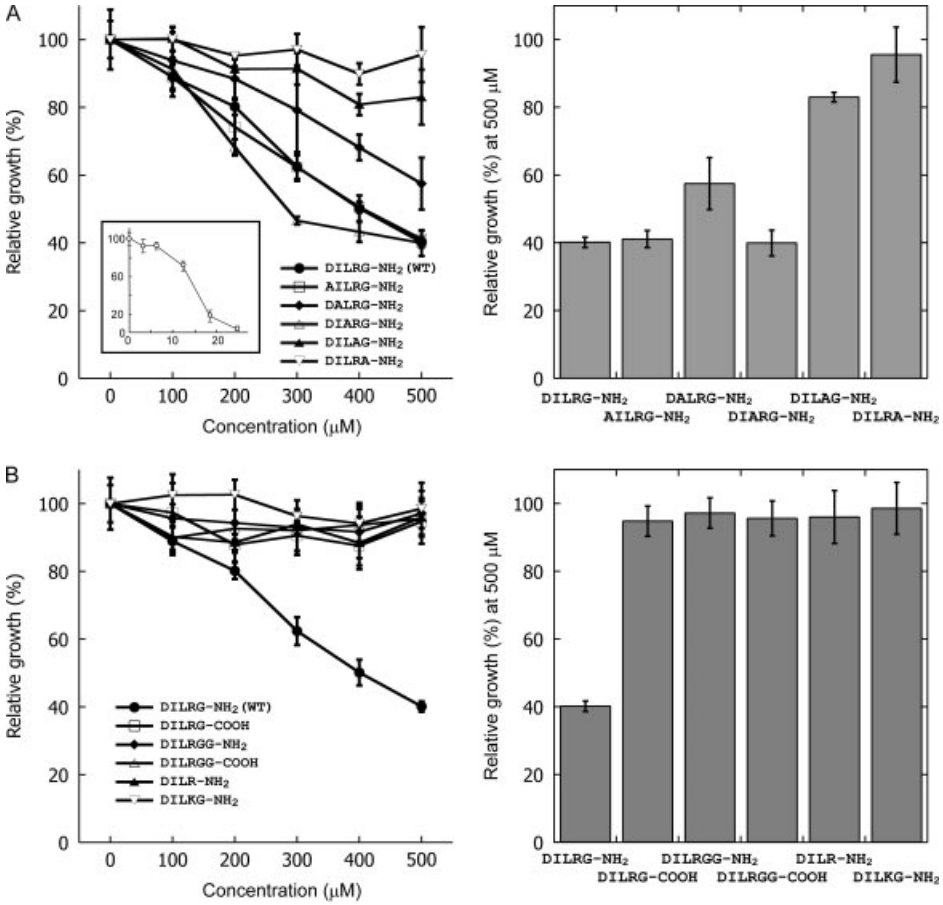

Figure 1. Cell growth suppressive assay on dRLh84 cells for alanine mutants (A) and *C*-terminal mutants (B). Left panels show dose-responsive curves of the relative growth of dRLh84 cells cultured with 0–500 μM peptide. The cell number was measured by MTT assay and the ratio of the percentage of the cells incubated with peptide to that incubated without peptide was expressed as relative growth. In the left panel of (A), the inset shows the dose-responsive curve of C16-DILRG-NH_2 generated based on the previously reported data [5]. Right panels show a bar graph of the relative growth value at 500 μM in the left panels. In the left panel in (B), the value of DILRG-NH_2 was taken from (A). Results are expressed as the mean ± SE of three independent experiments.

CD Analysis of Yamamarin and its Analogs in Various Environments

CD measurement was performed to obtain structural information on yamamarin and its analogs in various environments. Figure 2 shows the Far-UV CD spectra of yamamarin and its analogs including a palmitoyl-conjugated peptide, C16-DILRG-NH_2 in water, SDS, and dodecylphosphocholine (DPC) micelle solutions. As one can see, the CD spectra of each peptide showed differential spectral changes by SDS and DPC, respectively. However, the CD results indicated that all peptides are predominantly in random coil conformation independent of the solution environment. The random coil conformation of peptides was also independent of pH and temperature (data not shown). CD spectra indicated that the addition of a palmitoyl group at the *N*-terminus has almost no effect on the backbone conformation of C16-DILRG-NH_2.

NMR Analysis of DILRG-NH_2 and C16-DILRG-NH_2 in Water, SDS and DPC

To obtain residue-specific information on the structural nature of yamamarin, DQF-COSY, TOCSY and ROESY experiments for DILRG-NH_2 were carried out in water. Using the spectra obtained from these experiments, the full sequential assignment for all proton resonances was unambiguously completed according to the standard procedure described by Wüthrich [16] (Figure 3(A)). In ROESY spectra, the H^N–H^N ROE cross-peaks only between sequential residues were observed and other ROE cross-peaks were also observed only between adjacent residues (data not shown). These results suggested that DILRG-NH_2 did not adopt a certain secondary structure. This was supported by other NMR parameters obtained by DQF-COSY and TOCSY spectra: the chemical shift index (CSI), $^3J_{H^N-H^\alpha}$ coupling constant, and temperature coefficient of amide proton (data not shown). Figure 3(B) shows the TOCSY spectrum of DILRA-NH_2 which exhibited no activity. The spectrum of DILRA-NH_2 was similar to that of DILRG-NH_2. The spectra of DILRG-COOH and DILAG-NH_2, neither of which exhibited activity, were also similar to that of DILRG-NH_2 (data not shown). Therefore, the comparison of TOCSY spectra suggested that the alteration in the *C*-terminal part did not perturb the backbone conformation.

Next, to investigate the effect of the *N*-terminal addition of a palmitoyl group in C16-DILRG-NH_2 on the structural properties of peptides, the NMR spectra of DILRG-NH_2 and C16-DILRG-NH_2 were acquired in DPC and SDS micelle solutions, respectively, because C16-DILRG-NH_2 is insoluble in water. Figure 3(C–F) shows the TOCSY spectra of DILRG-NH_2 and C16-DILRG-NH_2 in DPC or SDS micelle solutions. The proton assignment of all the TOCSY spectra was completed unambiguously in DPC and SDS micelle solutions.

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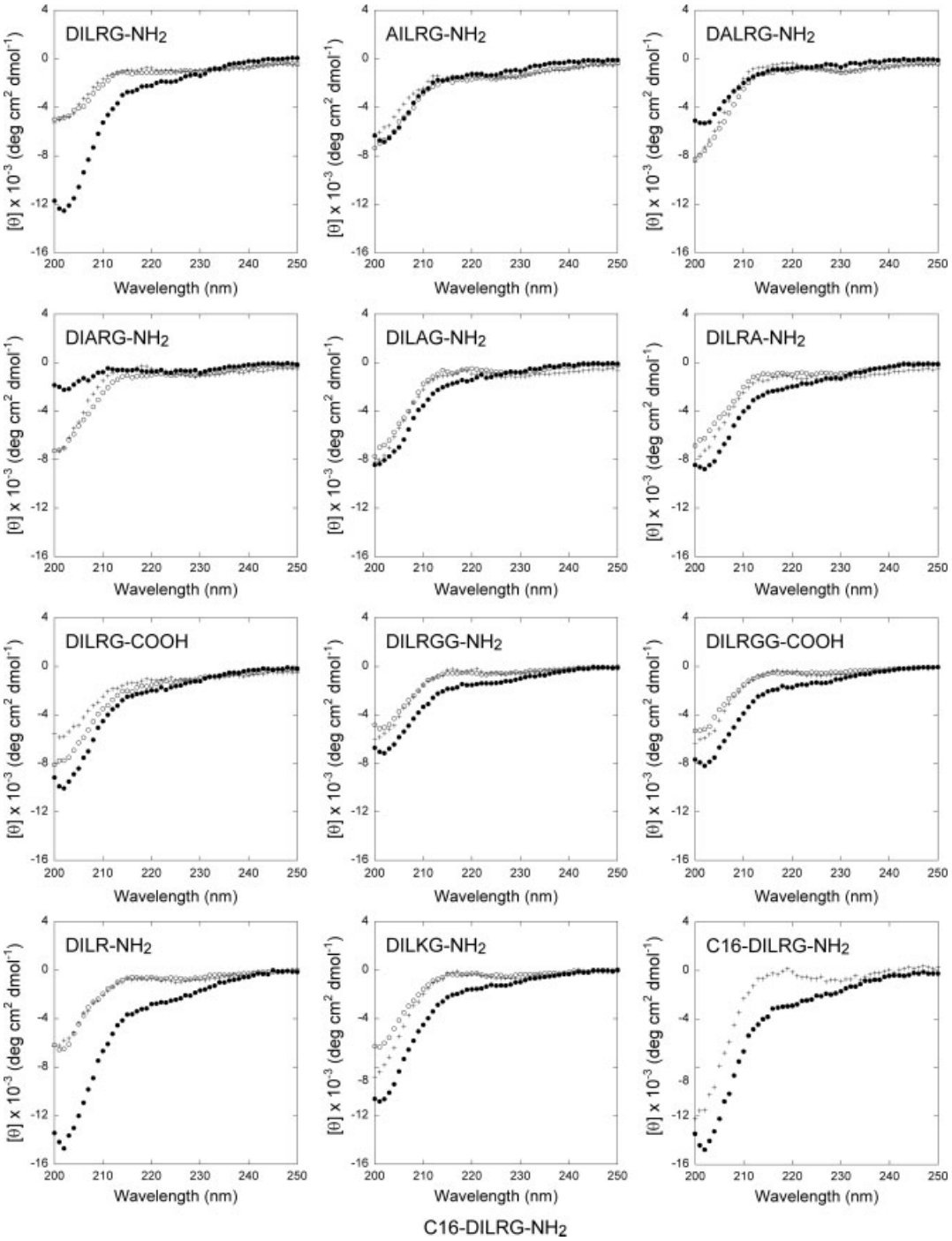

Figure 2. Far-UV CD spectra of yamamarin and its analogs in various environments. The spectra were recorded at a 100–120 μM peptide concentration in water (●), SDS (●), and DPC (+) micelle solutions.

DPC and SDS micelle solutions caused different effects on the chemical shift of the proton resonances in either peptides. The chemical shift difference between C16-DILRG-NH_2 and DILRG-NH_2 was calculated according to $\Delta\delta = \mathrm{sqrt}(\Delta\delta(H^{\alpha})^2 + \Delta\delta(H^{N})^2)$ (Figure 3(G)). In the DPC micelle solution, the values of $\Delta\delta$ of I2, L3, and R4 were larger than that of G5, whereas in the SDS micelle solution, only the value of I2 was larger than that of G5, and those of L3 and R4 were small and comparable to that of G5. DPC micelles are zwitterionic, while SDS micelles are anionic. It has been reported that the different surface charge nature of the two micelles has different effects on peptide conformation and peptide/micelle interactions [17–19]. Therefore, the charge difference between the micelles might result in the difference in $\Delta\delta$. Although there are differences in $\Delta\delta$ in both micelle solutions, the value became small toward the *C*-terminus, suggesting that the palmitoyl group of C16-DILRG-NH_2 was not located in close proximity to the *C*-terminus.

This was also supported by NOESY analysis of C16-DILRG-NH_2. Figure 4 shows NOE cross-peaks for C16-DILRG-NH_2 in DPC or SDS micelle solution. As can be seen, the NOE patterns in both micelle

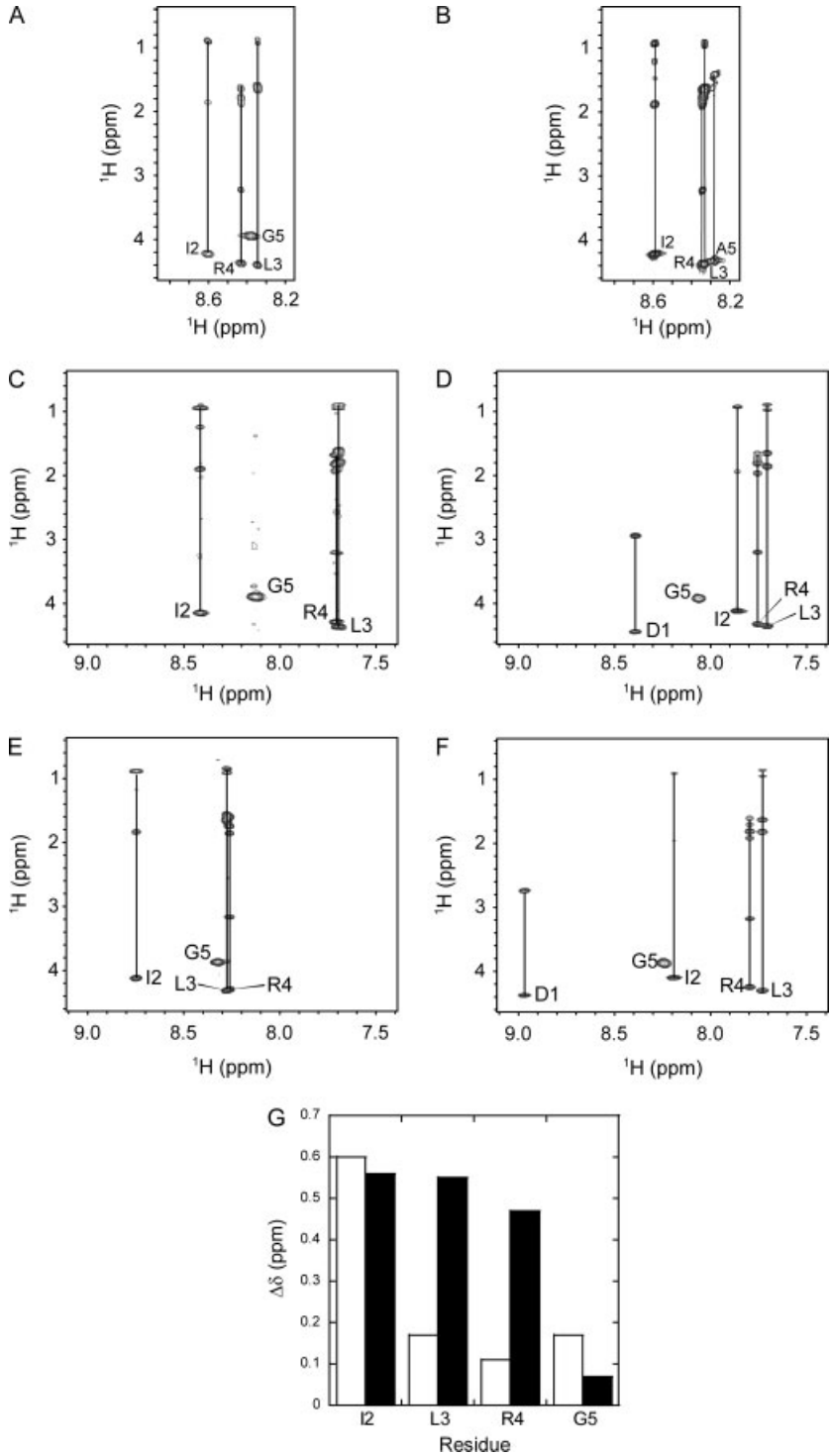

Figure 3. TOCSY spectra of DILRG-NH_2 in water (A), SDS (C), and DPC (E); DILRA-NH_2 in water (B); C16-DILRG-NH_2 in SDS (D), and DPC (F) and the chemical shift difference between C16-DILRG-NH_2 and DILRG-NH_2 in SDS (white bar) and DPC (gray bar) (G). The chemical shift difference was calculated as $\Delta\delta = \mathrm{sqrt}(\Delta\delta(H^{\alpha})^2 + \Delta\delta(H^{N})^2)$.

solutions were similar, and the inter-residue NOE cross-peaks were observed only between sequential residues (Figure 4(A) and (B)). A number of NOE cross-peaks between a palmitoyl group and an *N*-terminal residue, Asp[1], were observed (Figure 4(A), inset). The NOE analysis indicated that an *N*-terminal palmitoyl group of C16-DILRG-NH_2 did not interact specifically with its *C*-terminal part.

Relationship between Structure and Activity

It is unknown how yamamarin and its palmitoylated analog (C16-DILRG-NH_2) cause reversible growth arrest in rat hepatoma cells. The original role of yamamarin in diapause also remains unclear, but its mode of action resembles that of the diapause hormone (DH), which induces embryonic diapause in the silkworm, *B. mori*

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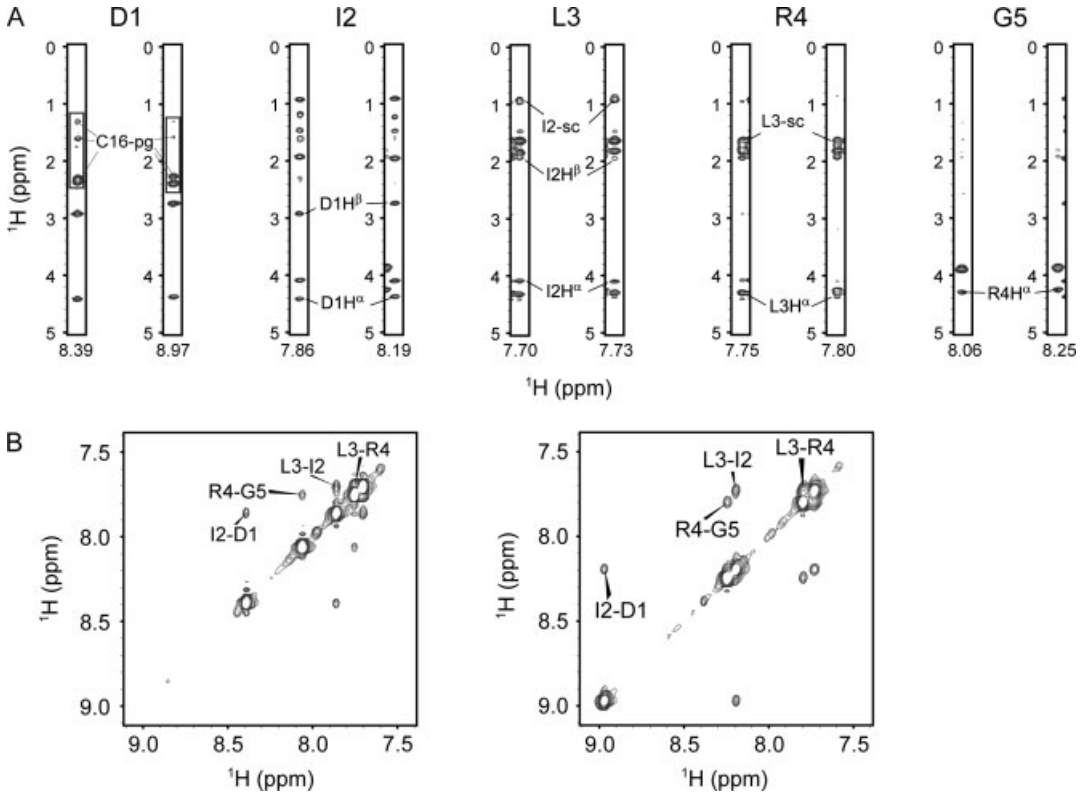

Figure 4. NOESY spectra of C16-DILRG-NH_2 in SDS and DPC. (A) The strip plots of NOESY spectra in SDS (left) and DPC (right) for each residue. 'C16-pg' denotes the protons belonging to an *N*-terminal palmitoyl group of C16-DILRG-NH_2; 'sc' denotes the side-chain protons. (B) The H^N–H^N region of NOESY spectra of C16-DILRG-NH_2 in SDS (left panel) and DPC (right panel).

[20,21]. Yamamarin can inhibit embryonic development in the silkworm, whereas a palmitoylated derivative of DH can also suppress the proliferation of rat hepatoma cells [5]. These findings may be a clue to elucidating the molecular mechanisms of action.

Recently, we have identified a 37-kDa protein as a molecule that interacts with yamamarin from extract of S2 cells of *Drosophila* (Koichi Suzuki, Shin-ichi Ishiguro, Yoji Ishida, Kunio Imai, unpublished data). The CD and NMR results indicated that yamamarin and its analog adopt predominantly a random coil conformation in aqueous or micelle solutions. The *in vitro* assay results suggested that the structure -RG-NH_2 of yamamarin, which is essential for its activity, may be responsible for binding to a 37-kDa protein. Therefore, the structure of *C*-terminal part would need to change to acquire specific binding to the protein.

As seen in the literature [22,23], the modification of fatty chains such as palmitoyl groups of peptides has been used to increase the membrane permeability. Similarly, the *N*-terminal palmitoyl group of C16-DILRG-NH_2 is thought to contribute to an increase in membrane permeability, resulting in a more than 20-fold increase in activity compared with yamamarin. The NMR results of C16-DILRG-NH_2 indicated that the *N*-terminal palmitoyl group did not significantly affect the peptide conformation. Therefore, it is speculated that modification of the palmitoyl group of yamamarin would not interrupt the binding to the receptor.

Conclusion

We identified the key element for cell growth suppression activity and obtained the structural information of yamamarin. Although the mechanism of the cell growth suppression remains to be elucidated, the results of this study should contribute to the design of more sophisticated anticancer agents.

Acknowledgement

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References

1 Adermann K, John H, Ständker L, Forssmann WG. Exploiting natural peptide diversity: novel research tools and drug leads. *Curr. Opin. Biotechnol.* 2004; **15**: 599–606.
2 Chernysh S, Kim SI, Bekker G, Pleskach VA, Filatova NA, Anikin VB, Platonov VG, Bulet P. Antiviral and antitumor peptides from insects. *Proc. Natl. Acad. Sci. U.S.A.* 2002; **99**: 12628–12632.
3 Suzuki K, Minagawa T, Minekawa T, Kumagai T, Naya S, Endo Y, Osanai M, Kuwano E. Control mechanism of diapause of the pharate first-instar larvae of the silkmoth, *Antheraea yamamai*. *J. Insect Physiol.* 1990; **36**: 855–860.
4 Yang P, Abe S, Zhao Y, An Y, Suzuki K. Growth suppression of rat hepatoma cells by a pentapeptide from *Antheraea yamamai*. *J. Insect Biotech. Sericology* 2004; **73**: 7–13.
5 Yang P, Abe S, Sato Y, Yamashita T, Matsuda F, Hamayasu T, Imai K, Suzuki K. A palmitonyl conjugate of an insect pentapeptide causes growth arrest in mammalian cells and mimics the action of diapause hormone. *J. Insect Biotech. Sericology* 2007; **76**: 63–69.
6 Szymanowska-Dziubasik K, Marciniak P, Rosinski G, Konopinska D. Synthesis, cardiostimulatory, and cardioinhibitory effects of selected insect peptides on *Tenebrio molitor*. *J. Pept. Sci.* 2008; **14**: 708–713.
7 Oka M, Maeda S, Koga N, Kato K, Saito T. A modified colorimetric MTT assay adapted for primary cultured hepatocytes: application

to proliferation and cytotoxicity assays. *Biosci. Biotechnol. Biochem.* 1992; **15**: 1472–1473.
8 Rance M, Sørensen OW, Bodenhausen G, Wagnera G, Ernst RR, Wüthrich K. Improved spectral resolution in COSY ^{1}H NMR spectra of proteins via double quantum filtering. *Biochem. Biophys. Res. Commun.* 1983; **117**: 479–485.
9 Braunschweiler L, Ernst RR. Coherence transfer by isotropic mixing application to proton correlation spectroscopy. *J. Magn. Reson.* 1983; **53**: 521–528.
10 Jeener J, Meier BH, Bachmann P, Ernst RR. Investigation of exchange processes by two-dimensional NMR spectroscopy. *J. Chem. Phys.* 1979; **71**: 4546–4553.
11 Bax A, Davis DG. Practical aspects of two-dimensional transverse NOE spectroscopy. *J. Magn. Reson.* 1985; **63**: 207–213.
12 Marion D, Ikura M, Tschudin R, Bax A. Rapid recording of 2D NMR spectra without phase cycling, application to the study of hydrogen exchange in proteins. *J. Magn. Reson.* 1989; **85**: 393–399.
13 Piotto M, Saudek V, Sklenár V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J. Biomol. NMR* 1992; **2**: 661–665.
14 Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 1995; **6**: 277–293.
15 Goddard TD, Kneller DG. *SPARKY 3*. University of California: San Francisco, 2006.
16 Wüthrich K. *NMR of Proteins and Nucleic Acids*. John Wiley & Sons, Inc.: New York, 1986.
17 Khandelia H, Kaznessis YN. Molecular dynamics investigation of the influence of anionic and zwitterionic interfaces on antimicrobial peptides' structure: implications for peptide toxicity and activity. *Peptides* 2006; **27**: 1192–1200.
18 Tinoco LW, Gomes-Neto F, Valente AP, Almeida FC. Effect of micelle interface on the binding of anticoccidial PW2 peptide. *J. Biomol. NMR* 2007; **39**: 315–322.
19 Bourbigot S, Dodd E, Horwood C, Cumby N, Fardy L, Welch WH, Ramjan Z, Sharma S, Waring AJ, Yeaman MR, Booth V. Antimicrobial peptide RP-1 structure and interactions with anionic versus zwitterionic micelles. *Biopolymers* 2009; **91**: 1–13.
20 Imai K, Konno T, Nakazawa Y, Komiya T, Isobe M, Koga K, Goto T, Yaginuma T, Sakakibara K, Hasegawa K, Yamashita O. Isolation and structure of diapause hormone of the silkworm, *Bombix mori*. *Proc. Jpn. Acad. Ser.* 1991; **B67**: 98–101.
21 Yamashita O. Diapause hormone of the silkworm, *Bombix mori*: structure, gene expression and function. *J. Insect Physiol.* 1996; **42**: 669–679.
22 Niidome T, Kawakami R, Okamoto K, Ohmori N, Mihara H, Aoyagi H. Interaction of lipophilic peptides derived from mastoparan with phospholipids vesicles. *J. Peptide Res.* 1997; **50**: 458–464.
23 Nachman RJ, Teal PE, Ujvary I. Comparative topical pheromonotropic activity of insect pyrokinin/PBAN amphiphilic analogs incorporating different fatty and/or cholic acid components. *Peptides* 2001; **22**: 279–285.

