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Structure activity relationships of peptidic analogs of MyoD for the development of Id1 inhibitors as antiproliferative agents

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Id proteins, inhibitors of DNA binding proteins, have highly conserved dimerization motif known as the helix-loop-helix (HLH) domain that acts as a negative regulator of basic HLH (bHLH) transcription factors. In signaling pathways, Id proteins play an important role in cellular development, proliferation, and differentiation. The mechanism of Id proteins is to antagonize bHLH proteins, thereby preventing them from binding to DNA and inhibiting transcription of cellular differentiation-associated genes in cancer. Recently, we reported an inhibitor of Id1, peptide 3C, which showed good affinity to Id1 protein and exhibited inhibitory effects in cancer cells. In this study, Ala (A)-substituted analogs of peptide 3C were synthesized by SPPS, purified by RP-HPLC, and characterized by MALDI-TOF MS. Binding of each peptide to Id1 or Id1-HLH (the HLH domain of Id1) was monitored by surface plasmon resonance (SPR)-based biosensor. Biological effect of each peptide in MCF-7 breast cancer cells was analyzed by MTT cell viability assay. The secondary structure of substituted analogs of peptide 3C was investigated by circular dichroism (CD) spectroscopy. SPR results revealed that A-substituted analogs of peptide 3C showed weaker binding to Id1 than that of peptide 3C, indicating that the six amino acid residues in the *N*-terminal of peptide 3C were all essential for binding to Id1 and the importance of amino acid residue was $I^2 > Q^6 > Y^1 > G^4 > L^5 > E^3$. In addition, substitution of E^3 in peptide 3C with D, Q, and R did not improve the binding potency of peptide 3C. MTT assay demonstrated that neither A-substituted nor position 3-substituted analogs of peptide 3C showed increased antiproliferative effect in MCF-7 cancer cells. CD results indicated that peptide 3C exhibited the highest content of α-helical structure (39.37%), suggesting that the α-helical structure may contribute to its binding potency for Id1 and Id1-HLH. SAR results provided important information for the development of peptidic inhibitors of Id1 as anticancer agents and demonstrated peptide 3C as a promising lead for further modifications.

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Keywords: Id1 protein; helix-loop-helix domain; MyoD protein; surface plasmon resonance; structure activity relationships; circular dichroism; anticancer agents

Introduction

Id proteins, the inhibitors of DNA-binding proteins, are dominant negative regulators within the basic helix-loop-helix (bHLH) transcription factors [1], which play important roles in cell growth [2–5], cell cycle control [6,7], differentiation [8,9], and tumorigenesis [10,11] in signaling pathway. BHLH proteins act as transcription factors to regulate the cell growth and differentiation in various cell lineages [8]. These proteins dimerize via their highly conserved HLH domain [12,13] and provide a common motif of a cluster of basic amino acids that was responsible for specific DNA binding.

The Id proteins contain a HLH domain but lack a basic DNA-binding motif and function as negative regulators of bHLH proteins by forming inactive heterodimers with bHLH proteins (such as MyoD and E proteins) that are unable to bind the DNA, thereby preventing bHLH proteins from binding to DNA and inhibiting the transcription of differentiation-associated genes [1,14]. Id proteins inactivation by expression of an engineered HLH dimerization partner has been shown to trigger human neuroblastoma cell differentiation and restrain tumorigenicity [15].

Id1 has been demonstrated to play the role of a mediator in several signaling pathways, such as PI3K/AKT signaling pathway, and has been implicated in regulating neoangiogenesis and metastasis [16–18]. In the present studies, overexpression of Id1 was found to be up-regulated in various human cancers, including cervical [19], breast [20], pancreas [21], prostate [22], and colorectal cancer cells [23]. These finding suggested that Id1 plays an important role in the development of tumors. Therefore, the inhibitors of Id1 are promising as anticancer agents for clinical therapy.

The use of synthetic polypeptides for therapy of certain human cancer is now well accepted in medical practice. Recently, we

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Abbreviations: *Id, inhibitor of DNA binding; bHLH, basic helix-loop-helix; SAR, structure activity relationships; SPR, surface plasmon resonance; CD, circular dichroism; TFE, 2,2,2-trifluoroethanol; MTT, 3-(4′,5′-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SPPS, solid-phase peptide synthesis; DIEA, N, N-*diisopropylethylamine; TFA, trifluoroacetic acid; DMF, dimethylformamide; RP-HPLC, reverse-phase high-performance liquid chromatography; hr, hours; min, minutes; HOBt, 1-hydroxybenzotriazole; HBTU, 2-(1H-benzotriazolyl)-tetramethyl-uronium hexafluorophosphate; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SPR, surface plasmon resonance; NHS, *N*-hydroxysulfosuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; RU, resonance unit; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; OD, optical density.

have reported on several potent small peptidic inhibitors of Grb2-SH2 that have antiproliferative effect on human breast cancer cells [24]. Pellegrino *et al*. have demonstrated that synthetic peptides based on a conserved 11-residue Id sequence have been shown to reduce cell growth and migration, increase expression of α-actin and decrease Id protein levels [25]. Furthermore, in breast cancer cells, a specific peptide aptamer targeting Id proteins induces E-box promoter activity, cell cycle arrest, and apoptosis [26].

Because increasing level of Id1 protein has been found in several types of human cancers, we attempted to develop inhibitors of Id1 and discovered peptide 3C, which showed good affinity to Id1 and induced the apoptosis of cancer cells [27]. We also found that the first six amino acid residues in the *N*-terminal of peptide 3C play an important role in binding to Id1 [28]. In this study, peptide 3C analogs were designed by substituting of its *N*-terminal amino acid with alanine. These analogs are synthesized by SPPS and then characterized by RP-HPLC and MALDI-TOF MS. Structure activity relationships (SAR) of the synthetic peptides were studied by surface plasmon resonance (SPR)-based biosensor, CD spectroscopy, and the MTT assay. SAR results should provide important information for the development of peptidic inhibitors of Id1 as anticancer agents.

Materials and Methods

Materials

Rink amide AM resin [4-(2′,4′-dimethoxyphenyl)-Fmoc-aminomethyl-phenoxy-acetamido-norleucylaminomethyl resin], all *N*α-Fmoc derivatives of standard amino acids, and the coupling reagents for solid-phase synthesis were purchased from AnaSpec Inc. (San Jose, CA, USA). Piperidine, DIEA, and trifluoroacetic acid (TFA) were purchased from Sigma (St Louis, MO, USA). DMF and acetonitrile (HPLC grade) were purchased from Tedia Company (Fairfield, OH, USA). TFE was purchased from Merck (Darmstadt, Germany). Purification of each peptide was performed by using semipreparative scale reverse-phase high-performance liquid chromatography (RP-HPLC) on a C18 column (244 × 10 mm, particle size 10 μm; Lichrospher 100 RP-18, Merck, Germany).

BIAcore 3000 biosensor, the BIAevaluation software, and all of the materials and reagents for performing BIAcore 3000 biosensor including the SPR, CM5 sensor chip, and HBS (HEPES-buffered saline; 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) were purchased from Biacore AB, GE Healthcare Company (Pollards Wood, UK). Human Id1 was provided by Biocheck Inc. (Foster City, CA, USA). The breast cancer cells (MCF-7) were obtained from the American Type Culture Collection. Culture medium (Dulbecco's modified Eagle's medium, DMEM), fetal bovine serum (FBS), and 1% penicillin and streptomycin were purchased from GIBCO/BRL (Grand Island, NY, USA). MTT, 3-(4′, 5′-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, cell proliferation kit was purchased from Boehringer Manheim (Indianapolis, IN, USA). The ELISA plate reader was purchased from Versamax (Sunnyvale, CA, USA).

Solid-Phase Peptide Synthesis of Analogs of Peptide 3C

Each peptide analogs of peptide 3C was synthesized in our laboratory by solid-phase peptide synthesis method, using Fmoc/tBu chemistry [29–31]. Briefly, the Rink amide AM resin was swollen in DMF for 10 min at room temperature, followed by the removal of the Fmoc protecting group from the resin by treatment with 20% piperidine in DMF for 15 min, repeated twice. The *N*α-Fmoc, side-chain protected amino acid, Fmoc-Cys (Trt)-OH, was activated by mixing with the coupling reagent, 1-hydroxybenzotriazole/2-(1H-benzotriazoelyl)-tetramethyl-uronium hexafluorophosphate/N, *N*-diisopropylethylamine (HOBt/HBTU/DIEA, 1 : 1 : 2), for 5 min and then added to the reaction vessel for coupling with resin at room temperature for 2 hr. Cycles of removing Fmoc and coupling with the subsequent amino acids were repeated to produce the desired peptide-bound resin. The crude peptide was removed from resin by TFA cleavage, lyophilized, and purified by RP-HPLC. After lyophilization, peptides were characterized by MALDI-TOF MS and RP-HPLC.

Analysis of Interactions of Each Peptide with the Immobilized Id1 and Id1-HLH by a Biosensor

The HLH domain of Id1 (Id1-HLH) was prepared as previously described [28]. The full-length Id1 or Id1-HLH was immobilized on the surface of biosensor chip, and their binding interactions with each synthetic peptide were analyzed to determine the binding potency of each peptide for Id1 and its HLH domain. The surface of CM5 chip was activated by injecting 35 μl of 0.1 M *N*-hydroxysulfosuccinimide (NHS)/0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (v/v = 1) onto the surface of chip at the flow rate of 5 μl/min. Id1 or Id1-HLH (30 μg/ml, 200 μl) was injected for immobilization on the activated surface of CM5 sensor chip. Finally, 35 μl of ethanolamine hydrochloride (0.1 M, pH 8.5) was injected for blocking the activated surface. An increased resonance unit (RU) shown in the sensorgram detected by BIAcore 3000 biosensor indicated that Id1-immobilized or Id1-HLH-immobilized CM5 chip was successfully prepared.

Purified peptides were diluted into various concentrations with PBS buffer, and each sample was introduced separately onto the Id1-immobilized or Id1-HLH-immobilized chip at the flow rate of 30 μl/min for 3 min. The binding interaction between each peptide and Id1 or Id1-HLH was detected and displayed as a sensorgram by plotting the RU against time, in triplicate. Detected changes of RU represent the association and dissociation of Id1 or Id1-HLH, and the data were analyzed using BIAevaluation software (Biacore AB, Pharmacia, Uppsala, Sweden) to determine the equilibrium constant of each peptide. The dissociation equilibrium constant (K_D) was calculated as the ratio of the dissociation rate constant (k_d) and the association rate constant (k_a). The K_D of the binding system could also be determined using the Scatchard analysis by plotting RU/(concentration of peptide) *versus* RU to yield a linear line with the slope equal to $-1/K_D$. The RU value is the maximal RU at a given peptide concentration.

Determination of the Cell Viability by Using the MTT Assay

The breast cancer cells (MCF-7) were maintained in DMEM with 10% FBS, 1% penicillin and streptomycin at 37 °C, and 5% CO_2. The inhibitory effect of each peptide on the proliferation of various cancer cells was determined using the MTT assay. Briefly, MCF-7 cells were loaded into 24-well culture plates. After 24 hr, cells were treated with fresh medium containing various concentrations of each peptide for 24 and 48 hr. The peptide-treated cancer cells and the control (cancer cells without treatment with any peptide) were washed once with phosphate-buffered saline (PBS) and reacted with the MTT solution at 37 °C for 1 hr to produce the formazan salt. Finally, the formazan salt formed in

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each cultured cells was dissolved in dimethylsulfoxide (DMSO), and the optical density (OD) value of each solution was measured at 540 nm using the VersaMax ELISA reader. The OD value detected for the control was plotted on the *x*-axis and considered as 100% of viable cancer cells. The OD value detected for the solution from the peptide-treated cells was also plotted on the *x*-axis, designated as proliferation (% control), to demonstrate the effect of each peptide on the viability of cancer cells. The IC_{50} value represents the concentration of a compound that caused 50% inhibition of certain reaction, such as the proliferation of cells. In the MTT assay, we recorded the concentration of peptide and the proliferation of cancer cells (% control) as the *x*-value and the *y*-value, respectively. Based on these known *x*-values and *y*-values, the IC_{50} value of each peptide can be calculated by using linear regression. The equation is $a + bx$, where $a = y - b\,x$ and $b = \Sigma(x - x)(y - y)/\Sigma(x - x)^2$ and where x and y are the means of the average of our known *x*-values and *y*-values, respectively. This formula can be used in Excel's built-in forecasting to calculate the IC_{50} value of each peptide by setting 50% as the *y*-value.

Analysis of the Secondary Structure of Each Peptide by Circular Dichroism Spectroscopy

Solutions of peptide analogs, 3C-($E^3 \rightarrow D^3$), 3C-($E^3 \rightarrow Q^3$), and 3C-($E^3 \rightarrow R^3$), were prepared at the same concentration (5.0 μM, in 0.1 M phosphate buffer, pH 7.2) and in 30% TFE for induction of the secondary structure. The secondary structure of peptides was analyzed by CD spectroscopy using Jasco-715 (Jasco Inc., Easton, MD, USA). The CD spectrum of each peptide solution was recorded at room temperature, and for each CD spectrum, two scans were accumulated using a step resolution of 1 nm, a bandwidth of 1 nm, a response time of 2 s, a scan speed of 100 nm/min, and a high sensitivity. The CD spectrum of the buffer was subtracted from that of each peptide to eliminate interferences from the cell, the solvent, or the optical equipment.

(A)

Sequence	Peptide
Y I E G L Q A L L R D Q C	3C
I E G L Q A L L R D Q C	3C-NtD1
E G L Q A L L R D Q C	3C-NtD2
G L Q A L L R D Q C	3C-NtD3
L Q A L L R D Q C	3C-NtD4
Q A L L R D Q C	3C-NtD5
A L L R D Q C	3C-NtD6
Y I E G L Q A L L R D Q	3C-CtD1
Y I E G L Q A L L R D	3C-CtD2
Y I E G L Q A L L R	3C-CtD3
Y I E G L Q A L L	3C-CtD4
Y I E G L Q A L	3C-CtD5
Y I E G L Q A	3C-CtD6

(B)

Sequence	Peptide
Y I E G L Q A L L R D Q C	3C
A I E G L Q A L L R D Q C	3C-($Y^1 \rightarrow A^1$)
Y A E G L Q A L L R D Q C	3C-($I^2 \rightarrow A^2$)
Y I A G L Q A L L R D Q C	3C-($E^3 \rightarrow A^3$)
Y I E A L Q A L L R D Q C	3C-($G^4 \rightarrow A^4$)
Y I E G A Q A L L R D Q C	3C-($L^5 \rightarrow A^5$)
Y I E G L A A L L R D Q C	3C-($Q^6 \rightarrow A^6$)

(C)

Sequence	Peptide
Y I E G L Q A L L R D Q C	3C
Y I D G L Q A L L R D Q C	3C-($E^3 \rightarrow D^3$)
Y I Q G L Q A L L R D Q C	3C-($E^3 \rightarrow Q^3$)
Y I R G L Q A L L R D Q C	3C-($E^3 \rightarrow R^3$)

Figure 1. The name and amino acid sequence of peptide analogs of peptide 3C. (A) *N*-terminal and *C*-terminal deleted analogs of peptide 3C reported in 2011 [28], (B) A (Ala)-substituted analogs of peptide 3C, and (C) position 3-substituted analogs of peptide 3C (substitution of E^3 with D, Q, and R).

Results

Design and Synthesis of Peptide Analogs of Peptide 3C

Recently, we have reported an inhibitor of Id1, peptide 3C, which exhibited inhibitory effects in cancer cells and induced the apoptosis of cancer cells [27]. To enhance its activity and selectivity in cancer cells, we designed a series of *N*-terminal and *C*-terminal deleted peptide analogs of peptide 3C (Figure 1A) and studied the SAR of the modified peptide 3C analogs [28]. SPR results demonstrated that peptide 3C and peptide 3C-CtD4 exhibited higher affinity to Id1-HLH, and the equilibrium dissociation constants (K_D) were 3.16 and 2.77 μM, respectively.

In the current study, we investigated the importance of six amino acid residues in the *N*-terminal of peptide 3C by Ala (A) substitution in each *N*-terminal position. The name and amino acid sequence of analogs of peptide 3C were shown in Figure 1B. Synthesized peptides were purified by RP-HPLC and characterized by MALDI-TOF MS (Table 1).

Analysis of Interactions of Each Peptide with the Immobilized Id1 and Id1-HLH by a Biosensor

The binding potency of each analog of peptide 3C to the full-length Id1 or the HLH domain of Id1 (Id1-HLH) was monitored by analyzing the sensorgrams obtained by interacting each peptide with the immobilized Id1 or Id1-HLH using the SPR technology developed with the biosensor BIAcore 3000. Because the helix 2 domain of MyoD has better binding affinity to the HLH domain of Id1 proteins [27], the HLH gene of Id1 was subcloned, expressed, and purified to produce the HLH domain of Id1 protein [28]. The Id1 was immobilized on the sensor chip

Table 1. Physicochemical characterization of the designed peptides

Peptide	Theoretical mass (Da)	MALDI-TOF MS (Da)	RP-HPLC (t_R, min)	Purity (%)
3C-($Y^1 \rightarrow A^1$)	1428.6	1429.3	13.63	95
3C-($I^2 \rightarrow A^2$)	1478.6	1479.3	13.63	97
3C-($E^3 \rightarrow A^3$)	1462.6	1463.3	13.63	96
3C-($G^4 \rightarrow A^4$)	1534.7	1535.5	13.63	96
3C-($L^5 \rightarrow A^5$)	1478.6	1479.3	14.60	95
3C-($Q^6 \rightarrow A^6$)	1463.6	1464.3	14.60	94
3C-($E^3 \rightarrow D^3$)	1506.6	1507.1	12.51	96
3C-($E^3 \rightarrow Q^3$)	1519.7	1520.3	12.21	95
3C-($E^3 \rightarrow R^3$)	1547.7	1548.3	11.41	95

separately for monitoring its interaction with each peptide in real time. The binding of each peptide to Id1 was determined by analyzing interactions of different concentrations of each peptide with immobilized Id1 or Id1-HLH (Figure 2A). The A (Ala)-substituted peptide 3C analogs interacted with Id1 in a dose-dependent manner, and the ⊿RU value detected by injection of 50 μM of each peptide onto the immobilized Id1 demonstrated that the order of binding potency is peptide 3C-($I^2 \rightarrow A^2$) < 3C-($Q^6 \rightarrow A^6$) < 3C-($Y^1 \rightarrow A^1$) < 3C-($G^4 \rightarrow A^4$) < 3C-($L^5 \rightarrow A^5$) < 3C-($E^3 \rightarrow A^3$) < peptide 3C. As amino acid residue at position 3 in peptide 3C showed the least

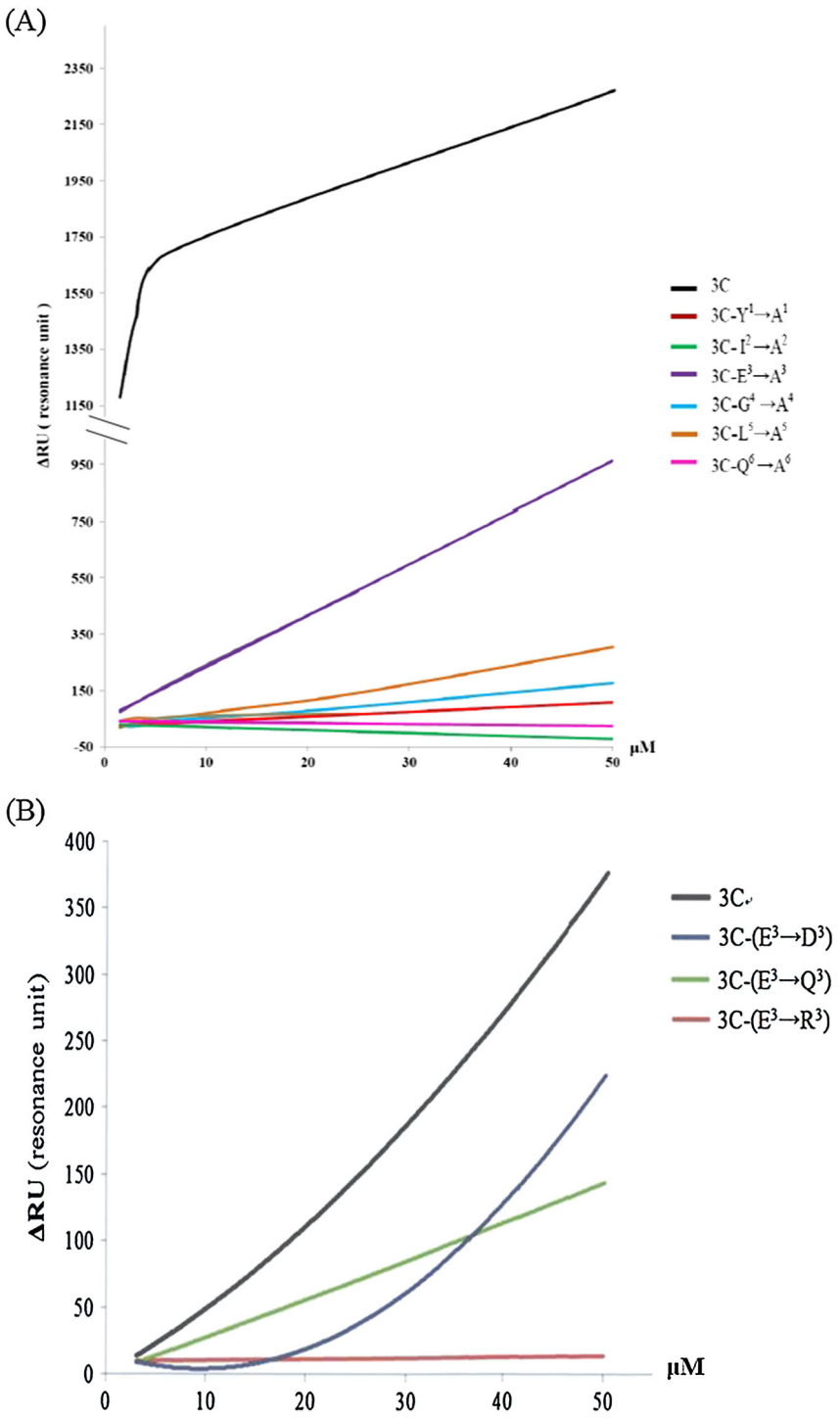

Figure 2. The binding of each peptide to the full-length Id1 or Id1-HLH determined by using SPR-based biosensor. (A) Interactions of various concentrations (3.13, 6.25, 12.5, 25.0, and 50.0 μM) of each peptide, including peptide 3C, 3C-($Y^1 \rightarrow A^1$), 3C-($I^2 \rightarrow A^2$), 3C-($E^3 \rightarrow A^3$), 3C-($G^4 \rightarrow A^4$), 3C-($L^5 \rightarrow A^5$), and 3C-($Q^6 \rightarrow A^6$), with the full-length Id1, and (B) interactions of various concentrations (3.13, 6.25, 12.5, 25.0, and 50.0 μM) of peptide 3C, 3C-($E^3 \rightarrow D^3$), 3C-($E^3 \rightarrow Q^3$), and 3C-($E^3 \rightarrow R^3$) with Id1-HLH were monitored and analyzed by using biosensor.

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importance for binding to the full-length Id1 ($K_D = 0.27$ mM, data not shown), E^3 in peptide 3C was substituted with D, Q, and R. All these analogs exhibit weaker binding than that of peptide 3C (Figure 2B). Among these three analogs, the K_D value of peptide 3C-($E^3 \rightarrow D^3$) is just 1.99 mM (data not shown). Furthermore, we demonstrated that peptide 3C exhibited higher affinity with the HLH domain of Id1 (Id1-HLH, 3.16 μM) than the full-length Id1 (12.50 μM).

Determination of the Viability of Cancer Cells by the MTT Assay

MCF-7 breast cancer cells were treated with 50 μM of each peptide for 24 and 48 hr and then analyzed by the MTT assay that detected the formazan product for determining the viability of cells. Results indicated that all the A-substituted peptides exhibited less inhibitory effect in the proliferation of cancer cells than that of peptide 3C; however, among the A-substituted peptides, peptide 3C-($E^3 \rightarrow A^3$) exhibited the highest inhibitory effect in 48 hr peptide-treated MCF-7 cells (Figure 3A). In addition, all the position 3-substituted analogs exhibited less inhibitory effect than that of peptide 3C, but the peptide 3C-($E^3 \rightarrow D^3$) exhibited the highest inhibitory effect in 24 hr peptide-treated MCF-7 cells, suggesting that the carboxyl group in the side chain of position 3 amino acid residue in peptide 3C was essential for antiproliferative effects in MCF-7 cancer cells (Figure 3B).

Analysis of the Secondary Structure of Peptides by Circular Dichroism Spectroscopy

The secondary structure of each peptide was analyzed by using CD spectroscopy. A maximum signal at 190 nm and two minima

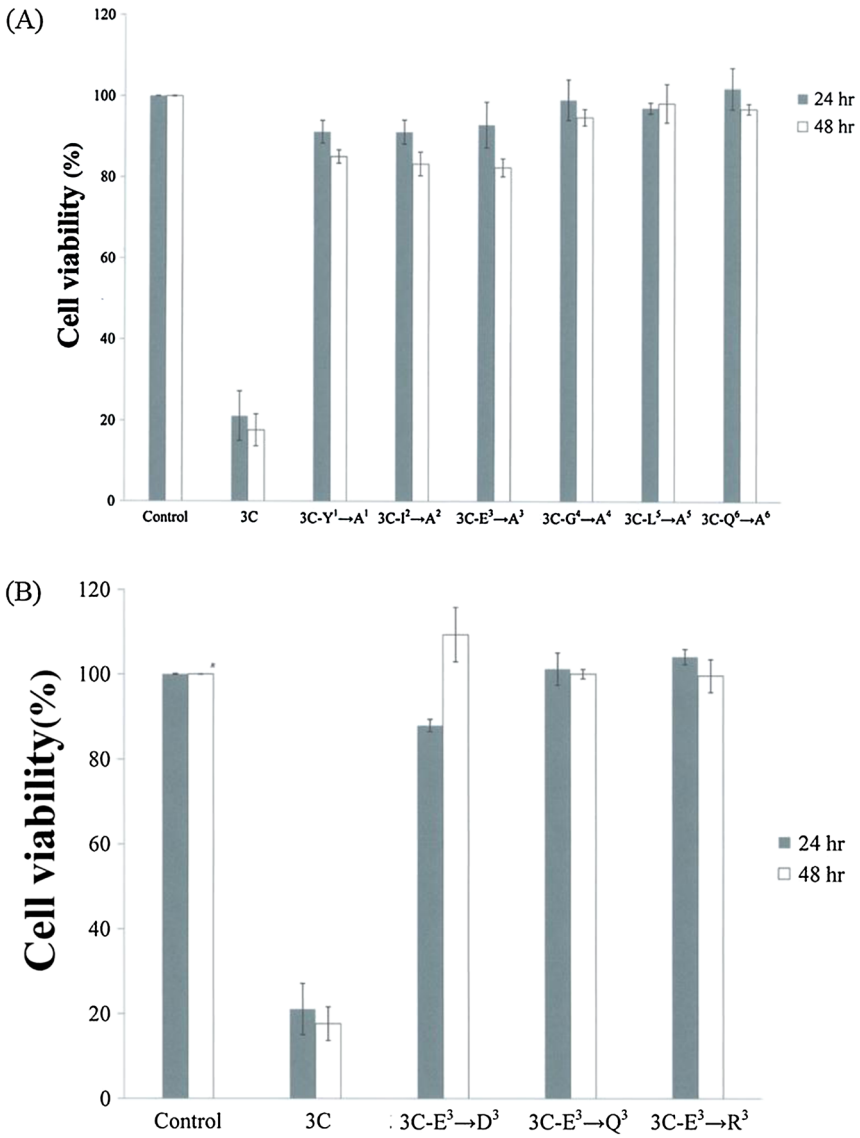

Figure 3. The cell viability of peptide-treated MCF-7 breast cancer cells. Breast cancer cells MCF-7 were treated with 50 μM of (A) A-substituted or (B) position 3-substituted analogs of peptide 3C for 24 and 48 hr, followed by determining the viability of cancer cells by using MTT proliferation assay. Data were reported as the mean value ± standard deviation of three independent experiments.

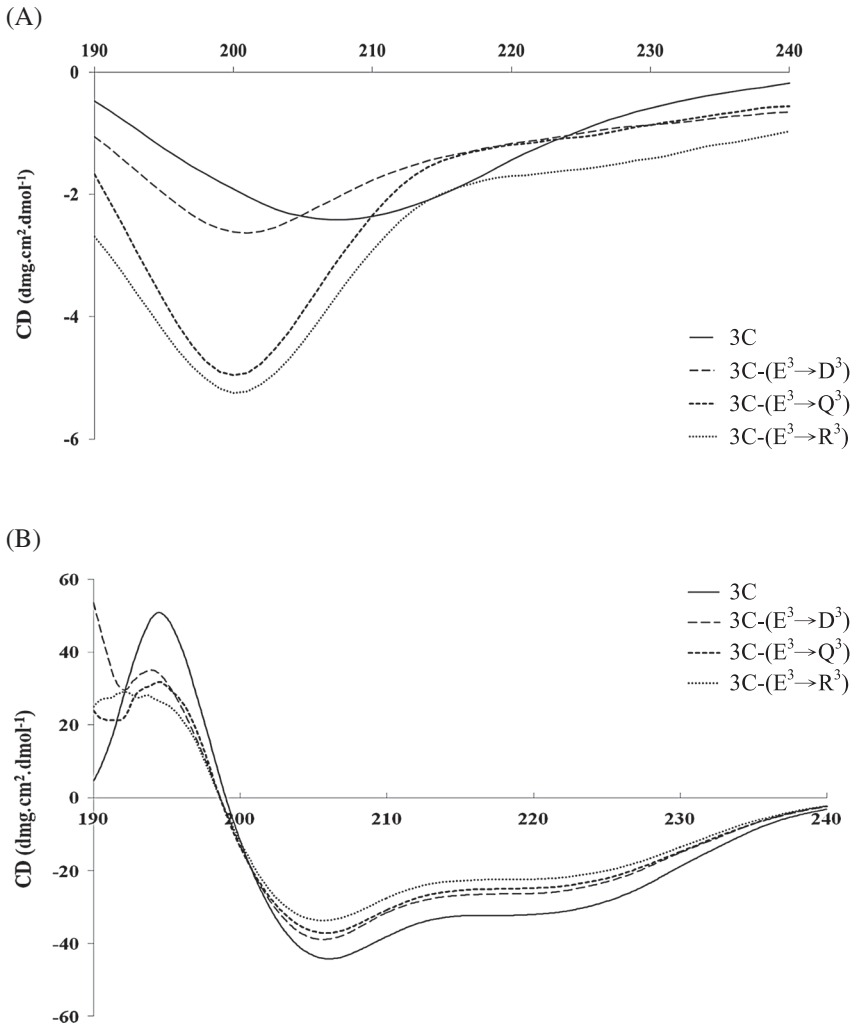

Figure 4. The secondary structure of each substituted analog of peptide 3C was analyzed by CD spectroscopy. Each substituted analog was dissolved in (A) 0.1% PBS and (B) 30% TFE.

signals at 203 and 221 nm were characteristics of these peptide analogs. Such, a CD spectra pattern has been generally attributed to a partially helical conformation, and the percentage of α-helix presented in peptides was estimated by using the K2D2 method [32] (Figure 4A). Peptides were dissolved in 30% TFE for the induction of secondary structure, and CD results indicated that comparing with the content of α-helix in peptide 3C (39.37%), the α-helix content in all of the substituted peptides was lower than that of peptide 3C, ranging from 10.98% to 18.08% (Figure 4B and Table 2).

Table 2. The secondary structure of these three substituted peptides was inducted in 30% TFE and analyzed by CD spectroscopy

Peptide	α-helix (%)	α-helix (%) (in 30% TFE induction)
3C	5.07	39.37
3C-($E^3 \rightarrow D^3$)	6.69	10.98
3C-($E^3 \rightarrow Q^3$)	7.93	18.08
3C-($E^3 \rightarrow R^3$)	7.93	18.08

Discussion

Peptide 3C and peptide 3C-CtD4 were found to exhibit good affinity to Id1-HLH [28], and it was demonstrated that the first six amino acid residues of the *N*-terminal of peptide 3C played an important role in binding to Id1 protein. In the current study, A-substitution of each of the first six amino acid residues in the *N*-terminal of peptide 3C (alanine-substituted peptide 3C) provided further insight into the importance of amino acids in peptide 3C for the binding potency to Id1 (Figure 2A). SPR results showed

Helix2 of Id1	KVEILQHVIDYIRDLQLELNSE
Helix2 of MyoD	KVEILRNAIRYIEGLQALLRDQ
Peptide 3C	YIEGLQALLRDQC
Peptide 3C-$E^3 \rightarrow D^3$	YIDGLQALLRDQC
Peptide 3C-$E^3 \rightarrow Q^3$	YIQGLQALLRDQC
Peptide 3C-$E^3 \rightarrow R^3$	YIRGLQALLRDQC

Figure 5. The sequence alignment of the helix 2 domain of Id1, the helix 2 domain of MyoD, and designed analogs of peptide 3C that contain a short fragment of MyoD.

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that all of the A-substituted analogs of peptide 3C exhibited weaker binding than that of peptide 3C. This result indicated that all of the first six amino acids were essential for binding to Id1, and their importance was $I^2 > Q^6 > Y^1 > G^4 > L^5 > E^3$. This finding correlated with previous result that the inhibitory effect of peptide 3C was higher than that of peptide 3C-NtD6 in which six amino acids were deleted from its *N*-terminal [27]. It was reported that hydrophobic interactions involved residues on the bHLH motif of MyoD and corresponding residues on Id1, via the branched amino acids: Val^{147}, Leu^{150}, and Ile^{154} [33]. These residues are located in the *N*-terminal part of MyoD helix 2 but are not contained in peptide 3C; Leu, which corresponds to position 5 in peptide 3C, could be involved in the interactions with Id1. The helical wheel projections for peptide 3C and Id1 were useful for explanation of our interpretation (Supplementary Figure). These observations were suggested that L^5 in peptide 3C is the most critical for forming of hydrophobic interactions with Id1.

According to the binding potency of A-substituted analogs, E^3 was the least important residue for binding to Id1; thus, the position 3 was chosen for further substitutions with D^3- (negative charge), Q^3- (neutral), and R^3- (positive charge) to study their SAR (Figure 1C). As shown in Figure 2B, the D^3-substituted analog exhibited higher binding to Id1-HLH than the other two analogs; however, all of the position 3-substituted analogs showed weaker binding to Id1-HLH than that of peptide 3C. Previously, we have demonstrated that the helix 2 domain of MyoD exhibited higher binding affinity to Id1 proteins [27]. Herein, we further analyzed the amino acid sequence of the helix 2 domain of Id1 and MyoD by sequence alignment (Figure 5) to identify regions of similarity in these proteins. It was suggested that the electrostatic interaction may contribute to their binding potency. SPR results showed decreased binding potency of 3C-($E^3 \rightarrow R^3$) for Id1-HLH, suggesting that the substitution of E^3 with R^3 may cause the repulsion between each other that disrupting the binding interactions between peptide and Id1. Although the neutral charge replacement in peptide 3C-($E^3 \rightarrow Q^3$) cannot afford the similar electrostatic interactions of E^3 or D^3 in peptide 3C or 3C-($E^3 \rightarrow D^3$) with Id1-HLH, the side-chain amide group of Q^3 provided additional possibility for hydrogen bonding with Id1-HLH; thus, these may be the reasons why 3C-($E^3 \rightarrow Q^3$) exhibited higher binding potency than peptide 3C-($E^3 \rightarrow R^3$). Among position 3-substituted analogs, peptide 3C-($E^3 \rightarrow D^3$) exhibited the higher binding potency (K_D value is 1.99 mM) but had lower binding potency than that of peptide 3C, demonstrating the importance of each amino acid residue in the *N*-terminal of peptide 3C.

MTT assay showed that treatment of MCF-7 with all the A-substituted analogs of peptide 3C did not enhanced antiproliferative effects in cancer cells, demonstrating that the composition and the sequence of amino acid residue in peptide 3C were important for antiproliferative activity. In Figure 3A, the cell viability observed with the A^2-analog is fully comparable with that observed with the A^3-analog, although the A^2-analog gives no RU response (Figure 2A). The K_D value of peptide 3C-($E^3 \rightarrow A^3$) is just 0.27 mM. It is possible that all A-analogs are not binding the Id1 comparing with peptide 3C. In addition, D^3-analog that shows a cell viability of about 85% after 24 hr but 100% after 48 hr incubation (Figure 3B). It seems that these peptide analogs showed no or very weak (less than 20%) inhibitory effect because of no binding of the HLH domain of Id1.

Peptide analogs were dissolved in 30% TFE for induction of their secondary structure, and CD results indicated that peptide 3C exhibited the highest content of α-helical structure (39.37%) than other substituted analogs, suggesting that in addition to the composition and sequence of amino acid residue in peptide 3C, the α-helical structure may also contribute to its good binding potency for the Id1-HLH (Figure 4B and Table 2).

In conclusion, SAR studies demonstrated the importance of the composition, the sequence, and the secondary structure of amino acid residue in peptide 3C for binding potency for Id1 and for antiproliferative effect in cancer cells. Peptide 3C is the promising target for further modifications for the development of peptidic inhibitors of Id1 as anticancer agents.

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