Research Article

Journal of PeptideScience

Received: 29 December 2010 Revised: 16 May 2011 Accepted: 16 May 2011 Published online in Wiley Online Library: 1 August 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1386

Structure–activity relationships of a peptidic antagonist of Id1 studied by biosensor method, circular dichroism spectroscopy, and bioassay

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Id1 proteins, inhibitors of differentiation or DNA binding, act as dominant negative antagonists of the bHLH family of transcription factors, which play an important role in cellular development, proliferation, and differentiation. The mechanism of Id proteins is to antagonize bHLH proteins by forming high-affinity heterodimers with other bHLH proteins, thereby preventing them from binding to DNA and inhibiting transcription of differentiation-associated genes. Our goal is to study the SARs of a peptidic antagonist of Id1, peptide 3C, which exhibits high affinity for Id1 and inhibitory effect on the proliferation of cancer cells. A series of N-terminal- and C-terminal-deleted analogs of peptide 3C were designed, synthesized, and characterized. Affinity of each peptide for Id1 or Id1-HLH domain was determined by SPR-based biosensor. The secondary structure of each peptide was studied by CD spectroscopy. Biological effect of each peptide in breast cancer cell (MCF-7) was analyzed by the MTT cell viability assay. Results demonstrated that peptide 3C and peptide 3C-CtD4 exhibited higher affinity for Id1-HLH and the equilibrium dissociation constants (K_D) were 3.16 and 2.77 μM, respectively. CD results indicated that the percentage of α-helix (%) in the secondary structure of deleted peptides were different, ranging from 7.93 to 10.45%. Although MTT results showed that treatment of MCF-7 with these peptides did not cause antiproliferative effects in cancer cells, SPR results demonstrated that the high-affinity peptides 3C and 3C-CtD4 are promising for further modifications to enhance their affinity for Id1-HLH and antiproliferative effects in cancer cells and for the development of peptidic antagonists as anticancer agents.

Keywords: Id1 protein; MyoD protein; surface plasmon resonance; SAR; CD; anticancer agents

Introduction

Id proteins, the inhibitors of DNA-binding proteins or the inhibitors of differentiation, act as negative regulators of transcription factors within the bHLH family [1], which play an important role in cell growth [2–7], cell cycle control [8,9], differentiation [10–14], and tumorigenesis [12–15].

The bHLH proteins such as Max, MyoD, and E proteins contain a DNA-binding motif which contain a cluster of amino acids rich in basic residues and a dimerization motif comprised of the HLH domain. Heterodimers regulate the expression of cell cycle regulatory proteins and tissue-specific genes formed between tissue-specific and ubiquitous bHLH transcription factors which bind to the DNA sequence 'E-box'. Id proteins, which contain an HLH dimerization motif but lack a basic DNA-binding motif, negatively regulate transcriptional activation by sequestering ubiquitous bHLH transcription factors in heterodimers that are unable to bind the DNA, thereby preventing bHLH proteins from binding to DNA and inhibiting the transcription of differentiation-associated genes [1,16,17].

Id1 promotes tumorigenicity and metastasis of several cancer cells through the activation of PI3K/AKT signaling pathway [18–21]. Over-expression of the Id proteins has been observed in several tumor types and favors tumor neo-angiogenesis and metastasis. Thus, the Id proteins and the antagonists of Id1 have been the potential targets for cancer therapy [22–28].

Recently, we reported a potent peptide fragment of MyoD, peptide 3C, which showed high affinity for Id1, exhibited inhibitory effects on cancer cells and induced the apoptosis of cancer cells [28]. In this study, we used peptide 3C as the lead peptide and designed a series of N-terminal- and C-terminal-deleted analogs of peptide 3C to study the SARs by SPR technology, CD spectroscopy, and the MTT cell viability assay. SAR results should provide important information for further modifications of peptides as potent antagonists of Id1 and anticancer agents.

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Abbreviations used: *bHLH, basic helix–loop–helix; CaCl, calcium chloride; DMEM, Dulbecco's modified Eagle's medium; EDT, 1,2-ethanedithiol; FBS, fetal bovine serum; Id1, inhibitors of differentiation/DNA binding; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaCl, sodium chloride; NHS, N-hydroxysulfosuccinimide; Ni, nickel; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RU, resonance unit; SPR, surface plasmon resonance; TCEP, tris(2-carboxyethyl)phosphine; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol.*

Materials and Methods

Materials

All Nα-Fmoc derivatives of standard amino acids, Rink amide AM resin [4-(2′,4′-dimethoxyphenyl)-Fmoc-aminomethyl-phenoxy-acetamido-norleucylaminomethyl resin], and the coupling reagents for solid-phase synthesis were purchased from Anaspec Inc. (San Jose, CA, USA). DIEA, piperidine, TFA, EDT, and thioanisole were purchased from Sigma (St Louis, MO, USA). DMF and acetonitrile (HPLC grade) were purchased from Tedia Company (Fairfield, OH, USA). Purification of each peptide was performed by using semi-preparative scale RP-HPLC on a C18 column (244 × 10 mm, particle size 10 μm; Lichrospher 100 RP-18, Merck (Darmstadt, DEU)).

The pET32 vector, Factor-Xa protease, Factor-Xa cleavage buffer, and Ni affinity column were purchased from Novagen-Merck Chemicals Taiwan (Taipei, Taiwan, ROC). Isopropyl-D-thiogalactopyranoside was purchased from Invitrogen (Carlsbad, CA, USA). Lysis buffer and gel filtration buffer were purchased from Sigma (St. Louis, MO, USA). Amicon Ultra device was purchased from Millipore (Billerica, MA, USA).

BIAcore 3000 biosensor, the BIAevaluation software, and all 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 P-20) were purchased from Biacore AB (Pharmacia, Uppsala, Sweden), GE Healthcare company (Pollards Wood, UK).

The breast cancer cells (MCF-7) were obtained from the American-Type Culture Collection. Culture medium (DMEM), FBS, and 1% penicillin and streptomycin were purchased from GIBCO/BRL (Grand Island, NY, USA). MTT, cell proliferation kit was purchased from Boehringer Manheim (Indianapolis, IN, USA). The ELISA plate reader was purchased from Versamax (Sunnyvale, CA, USA). Apoptotic cells were detected by FACS analysis, using FACS Calibur flow cytometer, Becton Dickinson Immunocytometry Systems (Mountain View, CA, USA).

Solid-Phase Peptide Synthesis of Peptide Analogs of Peptide 3C

Each peptide analogs of peptide 3C were 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 Nα-Fmoc, side-chain protected amino acid, Fmoc-Cys(Trt)-OH (2 equiv.), was activated by mixing with the coupling reagent, ·1 : 1 : 2), for 5 min and then added to the reaction vessel for coupling with resin (1 equiv.) at room temperature for 2 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 removed from resin by treatment with the TFA cleavage mixtures A, B, or C according to the amino acid sequence. TFA cleavage mixture A: 95% TFA solution (used for synthesizing peptides without using Arg, Met, Trp, and the Trt as protecting group of amino acid). TFA cleavage mixture B: 10 ml of TFA, 0.25 ml of EDT, 0.5 ml of thioanisole, and 0.5 ml of ddH_2O (used for synthesizing peptides containing Arg or Met in the sequence). TFA cleavage mixture C: 9.5 ml of TFA, 0.25 ml of EDT, and 0.25 ml of ddH_2O (used for synthesizing peptides containing Trp or the Trt-protecting group). The crude peptide solution was lyophilized and then purified by RP-HPLC. After purification, peptide was characterized by MALDI-TOF MS and RP-HPLC.

Preparation of the HLH Domain of Id1

The Id1-HLH gene was subcloned into pET32 vector. Thioredoxin-Id1 fusion protein with a His6 tag was expressed in *Escherichia coli* BL21 (DE3). To facilitate the removal of Trx tag by a Factor-Xa protease, protein expression was induced by the addition of 1.0 mM isopropyl-d-thiogalactopyranoside at OD (OD595) of 0.6 and cell growth was continued for another 4 h at 310 K. The cells were harvested by centrifugation and the cell pellet was resuspended in lysis buffer (50 mM Tris ± HCl 500 mM NaCl, 0.5 mM PMSF, 10% glycerol, and 5 mM mercaptoethanol, pH 8.0). Following cell disruption and centrifugation, the clarified call lysate was loaded onto an immobilized Ni affinity column. The bound Id1-HLH was eluted with buffer containing 150 mM imidazole. The elutes were collected and dialyzed against Factor-Xa cleavage buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM CaCl, pH 8.0) followed by Factor-Xa cleavage at 16 °C for 4 days. The Trx tag was separated from Id1-HLH by a second Ni-NTA His-bind resin chromatography and Id1-HLH was obtained. Fractions containing Id1-HLH were pooled concentrated by ultrafiltration using an Amicon Ultra device (3.5 kDa cutoff) and loaded onto a Hi-Load 16/60 Superdex-200 (Uppsala, SWE) size-exclusion column equilibrated in gel filtration buffer (20 mM Tris ± HCl, 100 mM NaCl, 0.5 mM EDTA, 2mM TCEP, pH 8.0). The peak fractions containing Id1 were pooled and concentrated by ultrafiltration to 30 mg/ml.

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

Id1-HLH was immobilized on the surface of biosensor chip and then the binding interaction of Id1-HLH with each synthetic peptide was analyzed to determine the binding affinity of each peptide for Id1-HLH [29,32,33]. The surface of CM5 chip was activated by injecting 35 μl of 0.1 M NHS/0.4 M EDC (v/v = 1) onto the surface of chip at a flow rate of 5 μl/min. Id1-HLH (30 μg/ml, 200 μl) was injected for immobilization on the activated surface of sensor chip. Finally, 35 μl of ethanolamine hydrochloride (0.1 M, pH 8.5) was injected for blocking the activated surface. An increased RU shown in the sensorgram detected by BIAcore 3000 biosensor indicated that the Id1-HLH-immobilized CM5 chip was successfully prepared.

Purified peptides were diluted into various concentrations with HBS buffer, and each sample was introduced separately onto the Id1-HLH-immobilized chip at athe flow rate of 30 μl/min for 3 min. The binding interaction between each peptide and the Id1-HLH was detected and displayed as a sensorgram by plotting the RU against time, at least, in triplicate. Detected changes of RU represent the association and dissociation of Id1-HLH, and the data were analyzed using BIA evaluation software (Biacore AB) to determine the equilibrium constant of each peptide. The K_D was calculated as the ratio of the dissociation rate constant (k_d) and the association rate constant (k_a). 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.

Analysis of the Secondary Structure of Each Peptide by CD Spectroscopy

Solutions of peptide analogs (3C, 3C-NtD1, 3C-NtD2, 3C-NtD3, 3C-NtD4, 3C-NtD5, 3C-CtD1, 3C-CtD2, 3C-CtD3, 3C-CtD4, 3C-CtD5,

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and 3C-CtD6) were prepared at the same concentration (3.7 μM in 0.1 M phosphate buffer, pH 7.2). The secondary structure of these peptides was analyzed by CD spectroscopy using Jasco-715 (Jasco Inc., Easton, MD, USA). The CD spectrum of each peptide solution was recorded by measuring the mean residue molar ellipticity (degree cm^2/dmol) of each peptide solution at room temperature, and for each CD spectrum, three 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.

Determination of the Cell Viability by Using the Cell Viability Assay

The breast cancer cells (MCF-7) were maintained in DMEM with 10% FBS, 1% penicillin, and streptomycin at 37 °C, 5% CO_2. The inhibitory effect of each peptide on the proliferation of various cancer cells was determined using the MTT cell viability assay. Briefly, MCF-7 cells were loaded into 24-well culture plates. After 24 h, cells were treated with fresh medium containing various concentrations of each peptide for 24 and 48 h. The peptide-treated cancer cells and the control (cancer cells without treatment with any peptide) were washed once with PBS and reacted with the MTT solution at 37 °C for 2 h to produce the formazan salt. Finally, the formazan salt formed in each cultured cells was dissolved in DMSO and the OD value of each solution was measured at 570 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. On the basis of 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 $\alpha = \overline{y} - b\overline{x}$ and $b = \sum(x - \overline{x})(y - \overline{y}) / \sum(x - \overline{x})^2$ and where $\overline{x}$ and $\overline{y}$ are the means of the average of our known *x*-value and *y*-value, respectively. This formula can be used in Excel's built-in forecasting to calculate the IC_{50} value of our peptide by setting 50% as the *y*-value.

Results

Design and Synthesis of Peptide Analogs of Peptide 3C

Recently, we reported a potent antagonist of Id1, peptide 3C, which exhibited inhibitory effects in cancer cells and induced the apoptosis of cancer cells [28]. To enhance its activity and selectivity in cancer cells, it is important to investigate the SARs of peptide 3C for further modifications. Thus, a series of N-terminal- and C-terminal-deleted peptide analogs of peptide 3C (Table 1) were designed and synthesized by solid-phase method.

Synthesized peptides were purified by RP-HPLC and characterized by MALDI-TOF MS (Table 2).

The thioredoxin-Id1-HLH fusion protein was produced and purified by liquid chromatography using Ni-NTA His-bind resin, and then characterized by PAGE analysis (Figure 1).

Table 1. The name and amino acid sequence of peptide analogs of peptide 3C

Name of peptides	Amino acid sequence of peptide
Peptide 3C	H-Tyr-Ile-Glu-Gly-Leu-Gln-Ala-Leu-Leu-Arg-Asp-Gln-Cys-NH_2
Peptide 3C-NtD1	H-Ile-Glu-Gly-Leu-Gln-Ala-Leu-Leu-Arg-Asp-Gln-Cys-NH_2
Peptide 3C-NtD2	H-Glu-Gly-Leu-Gln-Ala-Leu-Leu-Arg-Asp-Gln-Cys-NH_2
Peptide 3C-NtD3	H-Gly-Leu-Gln-Ala-Leu-Leu-Arg-Asp-Gln-Cys-NH_2
Peptide 3C-NtD4	H-Leu-Gln-Ala-Leu-Leu-Arg-Asp-Gln-Cys-NH_2
Peptide 3C-NtD5	H-Gln-Ala-Leu-Leu-Arg-Asp-Gln-Cys-NH_2
Peptide 3C-CtD1	H-Tyr-Ile-Glu-Gly-Leu-Gln-Ala-Leu-Leu-Arg-Asp-Gln-NH_2
Peptide 3C-CtD2	H-Tyr-Ile-Glu-Gly-Leu-Gln-Ala-Leu-Leu-Arg-Asp-NH_2
Peptide 3C-CtD3	H-Tyr-Ile-Glu-Gly-Leu-Gln-Ala-Leu-Leu-Arg-NH_2
Peptide 3C-CtD4	H-Tyr-Ile-Glu-Gly-Leu-Gln-Ala-Leu-Leu-NH_2
Peptide 3C-CtD5	H-Tyr-Ile-Glu-Gly-Leu-Gln-Ala-Leu-NH_2
Peptide 3C-CtD6	H-Tyr-Ile-Glu-Gly-Leu-Gln-Ala-NH_2

Table 2. Physicochemical characterization of the designed peptides

Peptide	Theoretical mass (Da)	MALDI-TOF MS (Da)	RP-HPLC (t_R, min)	Purity (%)
3C	1520.7	1521.2	14.2	95
3C-NtD1	1357.72	1357.1	11.09	96
3C-NtD2	1244.64	1244.1	10.67	96
3C-NtD3	1115.59	1115.4	8.97	95
3C-NtD4	1058.57	1058.1	8.97	95
3C-NtD5	945.49	945.0	9.48	94
3C-CtD1	1417.78	1417.7	11.01	95
3C-CtD2	1289.72	1289.3	12.37	96
3C-CtD3	1174.69	1174.5	12.16	96
3C-CtD4	1018.59	1018.60	13.47	95
3C-CtD5	905.50	905.49	10.72	95
3C-CtD6	792.42	791.9	8.31	96

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

The affinity (or binding potency) of peptide 3C for the full length or the HLH domain of Id1 was determined by analyzing the sensorgrams obtained by interacting peptide with the immobilized Id1 or Id1-HLH domain using the SPR technology developed with the biosensor-BIAcore 3000.

The full length Id1 or the HLH domain of Id1 was immobilized on the sensor chip separately for monitoring the interaction between each peptide with Id1 or Id1-HLH in real time. The affinity of each peptide for Id1 or Id1-HLH was determined by analyzing the interactions of different concentrations of each peptide with immobilized Id1 or Id1-HLH. SPR results indicated that peptide 3C-CtD4 has the highest affinity for Id1-HLH at higher concentration (Figure 2).

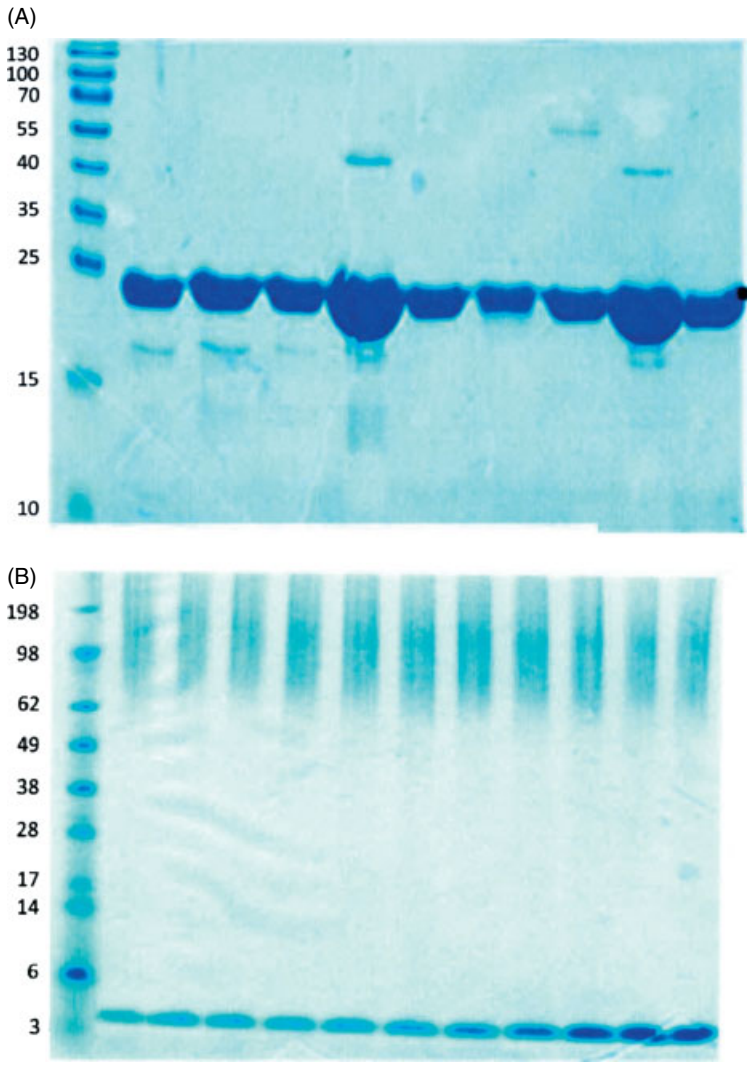

Figure 1. (A) PAGE analysis of thioredoxin-Id1 fusion protein which was purified by liquid chromatography using Ni-NTA His-bind resin. (B) PAGE analysis of the Trx tag which was separated from Id1-HLH by liquid chromatography using Ni-NTA His-bind resin.

The equilibrium dissociation constants of peptide 3C and peptide 3C-CtD4 for Id1 or Id1-HLH domain were calculated and summarized in Table 3.

Analysis of the Secondary Structure of Each Peptide by CD Spectroscopy

CD spectroscopy was applied to analyze the secondary structure of each synthetic peptide (Figure 3). A maximum signal at 190 nm and two minima signals at 203 and 221 nm are characteristics of the peptide analogs. Such a CD spectra pattern has been generally attributed to a partially helical conformation. The percentage of α-helix presented in these peptides was estimated by using the K2D2 method [34] (Table 4). CD results indicated that the percentage of α-helix (%) in the secondary structure of deleted peptides were different, ranging from 7.93 to 10.45%.

Determination of the Viability of Cancer Cells by the MTT Cell Viability Assay

MCF-7 breast cancer cells were treated with various concentrations (0, 25, 50, 60, and 80 μM) of each peptide for 24 (Table 5) and 48 h (Table 6) and then analyzed by the MTT cell viability assay that detects the formazan product of MTT assay for determining the viability of cells. Results indicated that peptide 3C-CtD4 exhibited inhibitory effect in MCF-7 cells at 25 μM, whereas other peptides did not exhibit significant inhibitory effect in MCF-7 cells at 80 μM.

Discussion

The Id1 is a selective mediator of lung metastatic colonization in the triple-negative subgroup of human breast cancer, i.e. cells lacking expression of estrogen receptor, progesterone receptor, and Her2 (human epidermal growth factor receptor 2) amplification. Thus, antagonists of Id1 are promising as anticancer agents. Recently, we reported a peptide fragment of MyoD, peptide 3C, which has moderate affinity for Id1, exhibited inhibitory effects on cancer cells (IC_{50} = 25 μM), and induced the apoptosis of cancer cells. We demonstrated that peptide 3C is a promising lead for the development of antiproliferative agents; thus, it is important to understand the SARs of peptide 3C for further modifications to enhance its activity and selectivity in cancer cells. The N-terminal-

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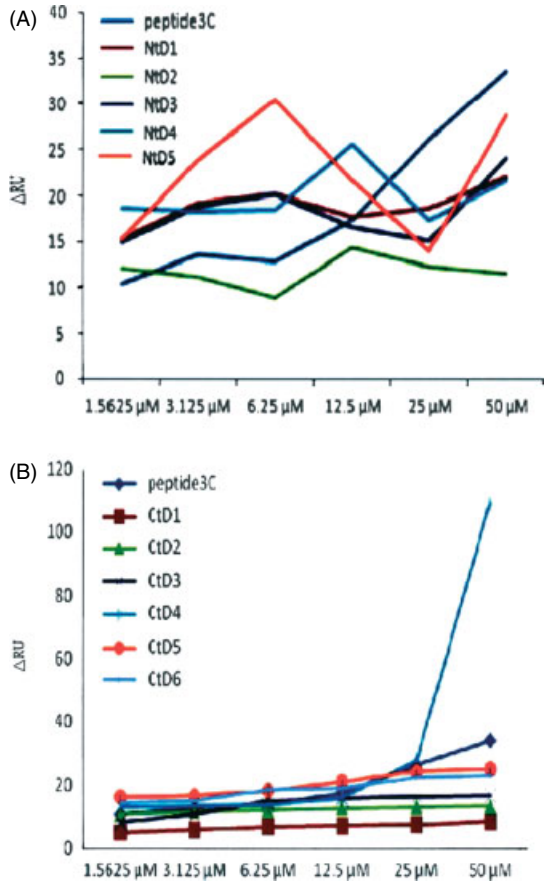

Figure 2. The affinity of each peptide for HLH domain of Id1 was determined by the interaction of different concentration of each peptide at the same time range. (A) Various concentrations (50, 25, 12.5, 6.25, 3.125, and 1.5625 μM) of each peptide (peptide 3C, peptide 3C-NtD1, peptide 3C-NtD2, peptide 3C-NtD3, peptide 3C-NtD4, or peptide 3C-NtD5) were interacted with the HLH domain of Id1 separately. (B) Various concentrations (50, 25, 12.5, 6.25, 3.125, and 1.5625 μM) of each peptide (peptide 3C, peptide 3C-CtD1, peptide 3C-CtD2, peptide 3C-CtD3, peptide 3C-CtD4, peptide 3C-CtD5, or peptide 3C-CtD6) were interacted with the HLH domain Id1 separately.

Table 3. The equilibrium dissociation constant of each peptide

Name of peptides	K_D (μM)[a]
Peptide 3C[b]	3.16
Peptide 3C[c]	12.50
Peptide 3C-CtD[b]	2.77

[a] The equilibrium dissociation constant.
[b] The HLH domain of Id1 was immobilized on the surface of biosensor chip.
[c] The full length of Id1 was immobilized on the surface of biosensor chip.

and C-terminal-deleted peptide analogs of peptide 3C (Table 1) were designed to identify the important amino acid residue in peptide 3C for high affinity of Id1 and for the antiproliferative effects in cancer cells.

The characterized peptides (Table 2) were analyzed for their binding potency to the HLH domain of Id1 by the SPR technology developed with the BIACORE biosensor (Figure 1). SPR results

Table 4. The percentage of α-helix in each peptide

Name of peptides	α-helix (%)
Peptide 3C	8.41
Peptide 3C-NtD1	7.93
Peptide 3C-NtD2	8.02
Peptide 3C-NtD3	8.42
Peptide 3C-NtD4	10.45
Peptide 3C-NtD5	8.02
Peptide 3C-CtD1	7.93
Peptide 3C-CtD2	8.07
Peptide 3C-CtD3	8.42
Peptide 3C-CtD4	10.04
Peptide 3C-CtD5	8.16
Peptide 3C-CtD6	7.93

showed that the N-terminal-deleted peptide 3C analogs interacted with Id1-HLH in a dose-independent manner and the ΔRU value was changed slightly within a very small region (from near 10 to 35) by interacting different concentrations of each peptide with the immobilized Id1-HLH, indicating that the interactions among these analogs and the Id1-HLH are non-specific interaction. Most of the C-terminal-deleted peptide 3C analogs interacted with Id1-HLH in a dose-independent manner, with the exception of peptide 3C and 3C-CtD4. The K_D of peptide 3C and peptide 3C-CtD4 were determined as 3.16 and 2.77 μM, respectively, which indicated that the two peptides are comparable in binding affinity (Table 3).

CD results indicated that peptide 3C-CtD4 exhibited higher percentage of (α-helical structure, 10.04%) than that of Peptide 3C (8.41%); however, a change in the helical content from 8.41 to 10.04% is not significant and these short peptides are obviously disordered in buffer solution. These results suggest that the α-helical structure in peptide 3C-CtD4 may not be the major factor that contributed to its affinity for Id1-HLH, but the amino acid residues in the C-terminal of peptide 3C are very important for affinity.

MTT assay demonstrated that the treatment of MCF-7 with all the terminal-deleted analogs of peptide 3C, except for the peptide 3C-CtD4, did not caused antiproliferative effects in cancer cells, suggesting that most of the amino acid residues in peptide 3C are important for antiproliferative activity. Peptide 3C-CtD4 exhibited higher antiproliferative effect than other analogs, suggesting that the helical structure and/or the hydrophobic amino acid residue in peptide 3C-CtD4 are important for the antiproliferative activity. Taken together, we demonstrate that peptide 3C and peptide 3C-CtD4 are promising for further modifications to enhance their antiproliferative effects in cancer cells. Results of this SAR study are very useful for the development of peptidic antagonists of Id1 as anticancer agents.

Acknowledgements

This work was supported by the grants from the National Science Council and Taichung Veterans General Hospital, Taiwan, ROC (NSC99-2113-M-029-002 and TCVGH-977801). SPR assays were performed by using Biosensor-BIAcore 3000 at the Institute of Biochemistry in the National Chung Hsing University (Taichung, Taiwan, ROC).

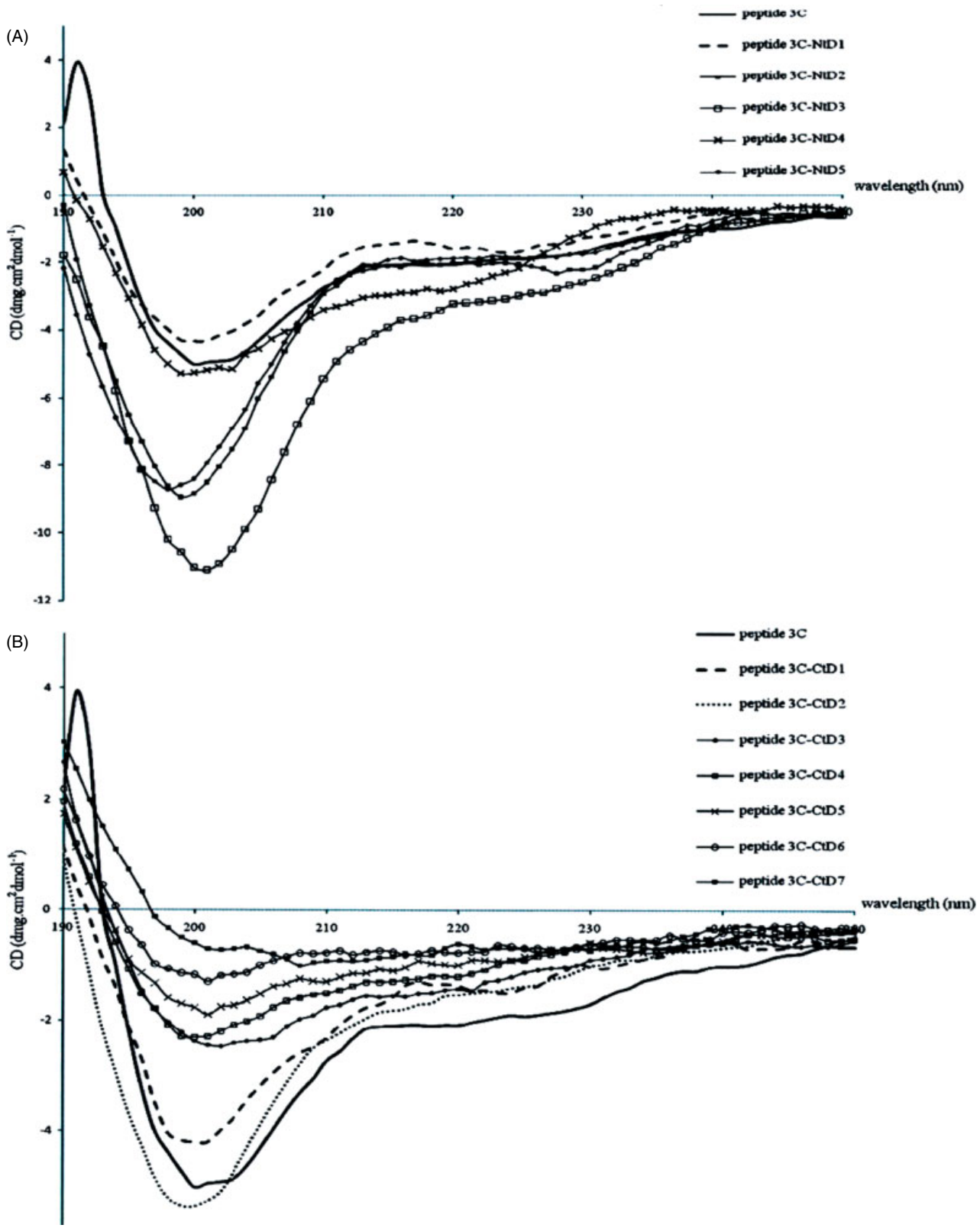

Figure 3. The spectra of terminal-deleted peptide analogs of peptide 3C detected by CD spectroscopy. (A) The spectra of the N-terminal-deleted analogs of peptide 3C detected by CD spectroscopy, measuring the mean residue molar ellipticity (degree cm^2/dmol). (B) The spectra of the C-terminal-deleted analogs of peptide 3C detected by CD spectroscopy, measuring the mean residue molar ellipticity (degree cm^2/dmol).

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Table 5. The cell viability of human breast cancer cells treated with each peptide for 24 h

Name of peptides	Control	25 μM	50 μM	60 μM	80 μM
Peptide 3C-NtD1	100 ± 0.0	95.7 ± 0.6*	95.3 ± 2.6*	92.8 ± 3.5*	93.8 ± 2.2*
Peptide 3C-NtD2	100 ± 0.0	101.9 ± 1.5	102.2 ± 0.8	103.4 ± 3.8	105.4 ± 1.6
Peptide 3C-NtD3	100 ± 0.0	97.8 ± 2.5	96.78 ± 1.5	96.4 ± 0.8*	99.0 ± 0.9
Peptide 3C-NtD4	100 ± 0.0	99.2 ± 2.2	97.0 ± 0.3	95.5 ± 1.8*	94.8 ± 0.2*
Peptide 3C-NtD5	100 ± 0.0	101.1 ± 3.1	101.6 ± 2.8	100.3 ± 2.3	102.0 ± 1.2
Peptide 3C-CtD1	100 ± 0.0	99.1 ± 0.6	96.3 ± 3.4	94.8 ± 3.9	95.4 ± 4.5
Peptide 3C-CtD2	100 ± 0.0	96.2 ± 3.0	97.9 ± 1.7	97.9 ± 2.9	100.3 ± 1.3
Peptide 3C-CtD3	100 ± 0.0	97.6 ± 3.1	93.1 ± 1.5*	93.6 ± 1.5*	94.0 ± 1.3*
Peptide 3C-CtD4	100 ± 0.0	86.5 ± 1.5*	84.7 ± 2.5*	83.2 ± 2.7*	82.9 ± 3.4*
Peptide 3C-CtD5	100 ± 0.0	100.6 ± 0.8	95.9 ± 0.8*	94.9 ± 1.4*	94.8 ± 2.6*
Peptide 3C-CtD6	100 ± 0.0	96.0 ± 1.7*	91.9 ± 3.5*	94.6 ± 4.1	99.5 ± 2.7

Data are the mean value ± SD of three independent experiments.
* $p < 0.05$ compared with control.

Table 6. The cell viability of human breast cancer cells treated with each peptide for 48 h

Name of peptides	Control	25 μM	50 μM	60 μM	80 μM
Peptide 3C-NtD1	100 ± 0.0	100.9 ± 0.6	103.2 ± 0.5	103.2 ± 0.4	99.4 ± 3.4
Peptide 3C-NtD2	100 ± 0.0	96.7 ± 2.7	97.8 ± 0.4	100.4 ± 1.8	105.8 ± 1.8
Peptide 3C-NtD3	100 ± 0.0	99.1 ± 0.6	97.9 ± 2.1	97.6 ± 1.5	95.5 ± 2.6
Peptide 3C-NtD4	100 ± 0.0	98.1 ± 0.4	97.8 ± 0.9	96.8.1 ± 0.5	97.7 ± 1.3
Peptide 3C-NtD5	100 ± 0.0	100.6 ± 0.7	100.7 ± 1.1	102.3 ± 0.8	103.7 ± 3.0
Peptide 3C-CtD1	100 ± 0.0	96.2 ± 0.5*	96.61 ± 1.2	97.7 ± 1.9	99.5 ± 0.9
Peptide 3C-CtD2	100 ± 0.0	98.6 ± 1.3	97.6 ± 1.1	96.1 ± 1.8	96.9 ± 1.2
Peptide 3C-CtD3	100 ± 0.0	99.4 ± 4.0	98.8 ± 3.0	100.0 ± 3.1	103.6 ± 2.6
Peptide 3C-CtD4	100 ± 0.0	93.81 ± 1.3*	91.64 ± 1.4*	90.93 ± 0.5*	90.18 ± 2.8*
Peptide 3C-CtD5	100 ± 0.0	96.1 ± 1.2*	96.2 ± 1.3*	95.3 ± 0.3*	101.6 ± 3.4
Peptide 3C-CtD6	100 ± 0.0	101.4 ± 2.1	98.4 ± 0.3	96.7 ± 0.5*	98.5 ± 2.7

Data are the mean value ± SD of three independent experiments.
* $p < 0.05$ compared with control.

References

1 Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* 1990; **61**(1): 49–59.
2 Lasorella A, Uo T, Iavarone A. Id proteins at the cross-road of development and cancer. *Oncogene* 2001; **20**(58): 8326–8333.
3 Mori S, Nishikawa SI, Yokota Y. Lactation defect in mice lacking the helix-loop-helix inhibitor Id2. *Embo J.* 2000; **19**(21): 5772–5781.
4 Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benezra R. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* 1999; **401**(6754): 670–677.
5 Iavarone A, Garg P, Lasorella A, Hsu J, Israel MA. The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. *Genes Dev.* 1994; **8**(11): 1270–1284.
6 Hara E, Yamaguchi T, Nojima H, Ide T, Campisi J, Okayama H, Oda K. Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. *J. Biol. Chem.* 1994; **269**(3): 2139–2145.
7 Barone MV, Pepperkok R, Peverali FA, Philipson L. Id proteins control growth induction in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 1994; **91**(11): 4985–4988.
8 Ruzinova MB, Benezra R. Id proteins in development, cell cycle and cancer. *Trends Cell Biol.* 2003; **13**(8): 410–418.
9 Zebedee Z, Hara E. Id proteins in cell cycle control and cellular senescence. *Oncogene* 2001; **20**(58): 8317–8325.
10 Kee BL. E and ID proteins branch out. *Nat. Rev. Immunol.* 2009; **9**(3): 175–184.
11 Ciarapica R, Annibali D, Raimondi L, Savino M, Nasi S, Rota R. Targeting Id protein interactions by an engineered HLH domain induces human neuroblastoma cell differentiation. *Oncogene* 2009; **28**(17): 1881–1891.
12 Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM. Id proteins in cell growth and tumorigenesis. *Cancer Cell* 2003; **3**(6): 525–530.
13 Coppe JP, Smith AP, Desprez PY. Id proteins in epithelial cells. *Exp. Cell Res.* 2003; **285**(1): 131–145.
14 Norton JD. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J. Cell. Sci.* 2000; **113**(Pt 22): 3897–3905.
15 Alani RM, Hasskarl J, Grace M, Hernandez MC, Israel MA, Munger K. Immortalization of primary human keratinocytes by the helix-loop-helix protein, Id-1. *Proc. Natl. Acad. Sci. U.S.A.* 1999; **96**(17): 9637–9641.
16 Christy BA, Sanders LK, Lau LF, Copeland NG, Jenkins NA, Nathans D. An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene. *Proc. Natl. Acad. Sci. U.S.A.* 1991; **88**(5): 1815–1819.
17 Mern DS, Hoppe-Seyler K, Hoppe-Seyler F, Hasskarl J, Burwinkel B. Targeting Id1 and Id3 by a specific peptide aptamer induces E-box promoter activity, cell cycle arrest, and apoptosis in breast cancer cells. *Breast Cancer Res. Treat.* 2010; **124**(3): 623–633.
18 Lee JY, Kang MB, Jang SH, Qian T, Kim HJ, Kim CH, Kim Y, Kong G. Id-1 activates Akt-mediated Wnt signaling and p27(Kip1) phosphorylation through PTEN inhibition. *Oncogene* 2009; **28**(6): 824–831.
19 Li B, Cheung PY, Wang X, Tsao SW, Ling MT, Wong YC, Cheung AL. Id-1 activation of PI3K/Akt/NFkappaB signaling pathway and its significance in promoting survival of esophageal cancer cells. *Carcinogenesis* 2007; **28**(11): 2313–2320.

20 Lopez-Carballo G, Moreno L, Masia S, Perez P, Barettino D. Activation of the phosphatidylinositol 3-kinase/Akt signaling pathway by retinoic acid is required for neural differentiation of SH-SY5Y human neuroblastoma cells. *J. Biol. Chem.* 2002; **277**(28): 25297–25304.
21 Su Y, Zheng L, Wang Q, Bao J, Cai Z, Liu A. The PI3K/Akt pathway upregulates Id1 and integrin alpha4 to enhance recruitment of human ovarian cancer endothelial progenitor cells. *BMC Cancer* 2010; **10**: 459.
22 Pellegrino S, Ferri N, Colombo N, Cremona E, Corsini A, Fanelli R, Gelmi ML, Cabrele C. Synthetic peptides containing a conserved sequence motif of the Id protein family modulate vascular smooth muscle cell phenotype. *Bioorg. Med. Chem. Lett.* 2009; **19**(22): 6298–6302.
23 Zhao ZR, Zhang ZY, Zhang H, Jiang L, Wang MW, Sun XF. Overexpression of Id-1 protein is a marker in colorectal cancer progression. *Oncol. Rep.* 2008; **19**(2): 419–424.
24 Ling MT, Lau TC, Zhou C, Chua CW, Kwok WK, Wang Q, Wang X, Wong YC. Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF). *Carcinogenesis* 2005; **26**(10): 1668–1676.
25 Lee KT, Lee YW, Lee JK, Choi SH, Rhee JC, Paik SS, Kong G. Overexpression of Id-1 is significantly associated with tumour angiogenesis in human pancreas cancers. *Br. J. Cancer* 2004; **90**(6): 1198–1203.
26 Schoppmann SF, Schindl M, Bayer G, Aumayr K, Dienes J, Horvat R, Rudas M, Gnant M, Jakesz R, Birner P. Overexpression of Id-1 is associated with poor clinical outcome in node negative breast cancer. *Int. J. Cancer* 2003; **104**(6): 677–682.
27 Schindl M, Oberhuber G, Obermair A, Schoppmann SF, Karner B, Birner P. Overexpression of Id-1 protein is a marker for unfavorable prognosis in early-stage cervical cancer. *Cancer Res.* 2001; **61**(15): 5703–5706.
28 Chen CH, Kuo SC, Huang LJ, Hsu MH, Lung FD. Affinity of synthetic peptide fragments of MyoD for Id1 protein and their biological effects in several cancer cells. *J. Pept. Sci.* 2010; **16**(5): 231–241.
29 Lung FD, Tsai JY, Wei SY, Cheng JW, Chen C, Li P, Roller PP. Novel peptide inhibitors for Grb2 SH2 domain and their detection by surface plasmon resonance. *J. Pept. Res.* 2002; **60**(3): 143–149.
30 Chang CD, Meienhofer J. Solid-phase peptide synthesis using mild base cleavage of N alpha-fluorenylmethyloxycarbonylamino acids, exemplified by a synthesis of dihydrosomatostatin. *Int. J. Pept. Protein Res.* 1978; **11**(3): 246–249.
31 Merrifield RB. Solid-phase peptide synthesis. *Adv. Enzymol. Relat. Areas Mol. Biol.* 1969; **32**: 221–296.
32 Lung FD, Tsai JY. Grb2 SH2 domain-binding peptide analogs as potential anticancer agents. *Biopolymers* 2003; **71**(2): 132–140.
33 Lung FD, Chen HY, Lin HT. Monitoring bone loss using ELISA and surface plasmon resonance (SPR) technology. *Protein Pept. Lett.* 2003; **10**(3): 313–319.
34 Perez-Iratxeta C, Andrade-Navarro MA. K2D2: estimation of protein secondary structure from circular dichroism spectra. *BMC Struct. Biol.* 2008; **8**: 25.

