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Peptide fragmentation as an approach in modeling of an active peptide and designing a competitive inhibitory peptide for HMG-CoA reductase

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ABSTRACT

This study presents a simple method to design an active peptide based on a description of the structural preferences of peptide via its peptide fragments. In a previous design, while searching for lead peptide candidates, the efficacy of a design approach that was based on the use of a cyclic peptide as a model of linear analog was demonstrated. Analysis of the conformational behavior of the peptide models showed that an analogical approach could be applied in order to assess the conformational space that was occupied by a peptide by using peptide fragments. In order to assess the proposed method, a design of a competitive inhibitor for HMG-CoA reductase (HMGR) was performed. Two starting points were used in the design: (1) determined recognized residues and (2) the structural preference of a peptide, such as a β-turn conformation in the present design. Two sets of peptides were developed based on the different location of a β-turn structure relative to a recognized residue. Set 1 contains peptides in which a recognized residue is included in turn conformation. In Set 2, the turn structure is located distantly from the recognized residues. The design parameter '*V*' that was applied in previous studies was slightly modified for the purpose of the current research. The 17 previously and 8 newly designed peptides were estimated by this parameter. In each set, one sequence was selected as a lead peptide candidate for each set: GF(4-fluoro)PEGG for Set 1 and DFGYVAE for Set 2. The inhibitory activities improved in each set. The IC_{50} for the GF(4-fluoro)PEGG peptide was found to be 0.75 μM, while the linear DFGYVAE peptide (IC_{50} = 0.16 μM) showed a 3000-fold increase in inhibitory activity compared to the first isolated LPYP peptide (IC_{50} = 484 μM) from soybeans. The comparison of the structure–activity relationship (SAR) data between Set 1 and 2 provided an opportunity to design the peptides in terms of peptide selectivity. A structural analysis of the modeled peptides confirmed the appropriateness of the proposed method for the design of active peptides.

1. Introduction

3-Hydroxy-3-methylglutaryl CoA reductase (HMGR) is a major rate-limited enzyme in cholesterol biosynthesis. An elevated cholesterol level is well recognized as a major risk factor in atherosclerotic diseases and coronary heart diseases in many people.[1] Thus, HMGR is the primary target enzyme of many investigations that aim to treat high cholesterol and reduce the risk of hypercholesterolemia.[2,3]

In previous studies, the two hypocholesterolemic peptides (LPYP and IAVPGEVA) were found by analysing a digested soy glycinin by using trypsin and pepsin, respectively.[4,5] An alignment of the amino acid composition of soy 11S-globulin with the IAVPGEVA sequence revealed another IAVPTGVA peptide with inhibitory activity against HMGR.[6] Kinetic experiments elucidated a competitive inhibition of those peptides for HMGR. A structural analysis showed that a bioactive conformation is a 'turn' structure, which includes proline residue as a conformational constraint in the recognized motif.[7]

The effects of conformation constraints on the binding affinity of flexible molecules have been already examined in many studies.[8–11] The design of competitive inhibitory peptides for HMGR with a constrained structure based on the recognized VPTG sequence has previously been described (Table 1).[12] For those peptides, an active peptide conformation was identified as a type II of a β-turn structure.

In the next stage, the design approach for peptides, including the recognized motif as an unconstrained structure, was developed.[13] The FGYVAE, FPYVAE, and FFYVAE peptides were designed by using the YVAE sequence as a basis of the recognized residues for HMGR. The YVAE peptide was selected because of its strong ability to inhibit HMGR in a competitive manner among previously designed peptides.[14] A conformational analysis of YVAE derived peptides revealed a presence of a β-turn structure located in the N-terminus of those peptides.[13]

As was shown in previous studies, a β-turn conformation was found to be an important structural element for binding.[12,13] The different location of the turn structure relative to the recognized

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Table 1
Peptide sequence, peptide fragment, and inhibitory activities (IC_{50}) of the synthesized peptides

Peptide sequence	Peptide fragment					IC_{50} (μM)
	1	2	3	4	5	
Control[a]						
LPYP	LPYP					484.72
IAVPGEVA	IAVP	AVPG	VPGE	PGEV	GEVA	201.12
IAVPTGVA	IAVP	AVPT	VPTG	PTGV	TGVA	152.19
Set 1[b]						
GLPTGG	GLPT	LPTG	PTGG			19.43
GLPDGG	GLPD	LPDG	PDGG			22.31
GLPEGG	GLPE	LPEG	PEGG			27.28
GFPTGG	GFPT	FPTG	PTGG			16.94
GFPDGG	GFPD	FPDG	PDGG			1.52
GFPEGG	GFPE	FPEG	PEGG			1.78
GXPTGG[d]	GXPT[d]	XPTG[d]	PTGG			4.82
GXPDGG[d]	GXPD[d]	XPDG[d]	PDGG			6.95
GXPEGG[d]	GXPE[d]	XPEG[d]	PEGG			0.75
GXPGGG[d]	Negative control					Inactive
Set 2[c]						
IAVE			IAVE			75.23
IVAE			IVAE			52.67
YAVE			YAVE			44.81
YVAE			YVAE			41.21
FFYVAE	FFYV	FYVA	YVAE			2.56
FPYVAE	FPYV	PYVA	YVAE			1.47
FGYVAE	FGYV	GYVA	YVAE			0.41
XVAE[d]				XVAE[d]		3.84
FGXVAE[d]		FGXV[d]	GXVA[d]	XVAE[d]		8.52
GFGYVAE	GFGY	FGYV	GYVA	YVAE		0.27
TFGYVAE	TFGY	FGYV	GYVA	YVAE		0.26
DFGYVAE	DFGY	FGYV	GYVA	YVAE		0.16
EFGYVAE	EFGY	FGYV	GYVA	YVAE		0.24
DFGYVAG	Negative control					Inactive

[a] 'Control' contains the peptides isolated from soy protein.[4–6]
[b] 'Set 1' contains the peptides, which includes the recognized T, D or E residue as a corner residue of the β-turn structure.[12,17]
[c] 'Set 2' contains the peptides, where the E residue is a recognized residue for the HMG-binding pocket, and a β-turn structure is located in the N-terminus of the hexapeptides.[13]
[d] The substituted (4-fluoro)phenylalanine residue is indicated as 'X'.

residues proposed an opportunity to design active peptides with a focus on their conformational preferences that can be modeled in the peptide structures. Based on the previously isolated and designed peptides, this opportunity in designing peptides is discussed in the current study.

The correlation between the conformational flexibility and bioactivity was applied in the previous designs.[12,13] A Principle Component Analysis (PCA), which projects multidimensional data on low-dimensional subspace, was used to evaluate a head-to-tail peptide cycle as a model of linear analog in order to select a lead peptide candidate. A 6-, 8-, and 10-membered cyclic peptides were used as models of linear analogs while searching for less flexible sequences.[12,13] An analysis of a conformational space revealed a direct relation between the developed descriptor of the conformational behavior of the cyclic peptides and the volumes occupied in a conformational space by linear analogs.[12] The conformational behavior of the cyclic peptides showed that the 6-membered cyclic peptide was relatively stable compared to the 8-, and 10-membered cyclic peptides.[12] Taking into account all considerations, it was proposed that a less flexible site in peptides could be found by analyzing the conformational space that was occupied by the peptide fragments by using a six-membered cyclic peptide as a model.

In the current design, two binding site points were used to model the active peptides. The first was a binding of the models of the designed peptides inside the 'region bioactivity' through a common point/site in the previously and newly designed peptides. The second was a relative assessment of the 'region bioactivity', prescribed by the host molecule through an analysis of the bioactive structures of the previously designed peptides. A structure-functional analysis of the previously designed peptides revealed the importance of a 'turn' structure for binding. In the present design, a turn conformation was used to assess 'region bioactivity'.

The peptide fragmentation was applied in order to select a less flexible site in peptide and to estimate the 'region bioactivity'. Using a minimum length that is required to describe a β-turn structure, each peptide was divided by a fragment of the four residues (Table 1).[15] Based on the four residues, the peptide model performed a cyclic hexapeptide by adding two glycine residues to the N- and C-terminuses as a site for cyclization.

The experimentally determined coordinates and thermal parameters of cyclic hexaglycine peptide revealed that this ring consists of two β-turns that are stabilized by strong hydrogen bonds of the type 1←4. These hydrogen bonds connect NH and CO groups of glycine residues at positions of 1 and 4 according to the numbering of a β-turn structure.[16] In order to assess the conformational space occupied by peptides in terms of a space occupied by a 'turn' structure, a cyclic hexaglycine peptide was used as a basis for estimation. In accordance with that, the design parameter '*V*', which reflects an occupied volume in conformation space by an individual peptide, was slightly modified for the purpose of the current research.[12] The conformation space occupied by an individual peptide was assessed via its peptide fragments. The difference in the calculation of the design parameter was that the occupied volume in conformation space by a peptide fragment was adduced to the conformation space occupied by the cyclic hexaglycine peptide instead of the conformation space occupied by all of the peptide candidates from a library as it was used in the previous design.[12] Based on the procedure applied for the '*V*' parameter, the occupied volumes by the peptide candidates were estimated.[12,13] The biological activity and conformational preference of the designed peptides were assayed through the use of an in vitro test and a Circular Dichroism (CD) study.

2. Results and discussion

2.1. Peptide design

For testing the proposed design, 13 peptides that were designed in previous studies were used.[12–14,17] These peptides were divided into two sets in accordance with the location of a 'turn' structure relative to the recognized residue that is essential for binding. Set 1 was comprised six peptides: GLPTGG, GLPDGG, GLPEGG, GFPTGG, GFPDGG, and GFPEGG (Table 1). A common structural element of these peptides was a turn conformation, which included T, D, or E as a corner residue of the β-turn structure and was recognized by a HMG-binding pocket. All peptides inhibited HMGR in a competitive manner.[12,17]

Set 2 included seven peptides, in which E residue was also recognized residue for HMG-binding pocket. A conformational analysis of the IAVE, IVAE, YAVE, and YVAE peptides revealed no observable patterns that were related to a well-populated secondary structure conformation.[14] For the FFYVAE, FPYVAE, and FGYVAE peptides, a turn conformation was determined at the N-terminus of these peptides. All of these peptides presented an HMG-CoA competitive inhibitor.[13]

In previous studies, the structures of statin molecules that were extracted from the crystal structures of HMGR-statin complexes (PDB codes: 1HW8 (compactin), 1HW9 (simvastatin), 1HWI (fluvastatin), 1HWJ (cerivastatin), 1HWK (atorvastatin), and 1HWL (rosuvastatin)) were used. All of these structures were compared with the Set 1 peptides by their superposition.[12,17] The superposition of the bioactive conformations of statins and peptides were constructed by considering that the HMG-moiety of statins and a

side-chain of the recognized residues occupied the same narrow pocket in the active site, which is a common feature in statin-binding by HMGR. The analysis of the superposition of statins and peptide molecules showed a similar location of the iso-butyl (compactin and simvastatin) and the benzene ring of the 4-fluorophenyl radical of statins (fluvastatin, cerivastatin, atorvastatin, and rosuvastatin) with the side-chains of L (GLPTGG, GLPDGG, and GLPEGG) and F (GFPTGG, GFPDGG, and GFPEGG) residues, respectively.[17] In Set 2, the YVAE peptide was designed with an orientation of the side-chain of Y residue being close to the location of the 4-fluorophenyl radical of statins.[14] The FFYVAE, FPYVAE, and FGYVAE peptides were designed with a focus on the structural diversity of the rigid hydrophobic groups of statin molecules. The N-terminus of these peptides presents a relatively rigid structure of a peptide backbone with side-chains, which conform to the hydrophobic radicals of statins.[13] Figure 1 shows the superposition of atorvastatin with rosuvastatin and the linear FGYVAE peptide.

The analysis of the crystal structures of the catalytic domain of human HMGR complexed with statins showed additional binding interactions between fluorophenyl groups and the HMGR for fluvastatin, cerivastatin, atorvastatin, and rosuvastatin compared to compactin and simvastatin. In addition to these interactions, rosuvastatin and atorvastatin exhibited hydrogen bonds with the enzyme, which involved a sulfone oxygen atom in the case of rosuvastatin and a carbonyl oxygen atom in the case of atorvastatin (Fig. 1).[18]

After proposing an increase of the binding affinity due to an additional contribution of fluorophenyl group in binding, the side-chains of F and Y residues for the Set 1 and 2 peptides were replaced by a 4-fluorophenyl radical. Based on these considerations, the three peptides GF(4-fluoro)PTGG, GF(4-fluoro)PDGG, and GF(4-fluoro)PEGG were modeled on the Set 1 peptides, and the two peptides F(4-fluoro)VAE and FGF(4-fluoro)VAE were used by using the peptide sequences in Set 2.

In order to model an interaction between the oxygen atom of sulfone and carbonyl groups of rosuvastatin and atorvastatin, respectively, and the enzyme, TFGYVAE, DFGYVAE, and EFGYVAE peptides were designed by using the FGYVAE peptide as a basic sequence in Set 2. The FGYVAE peptide presented the most active peptide among previously designed peptides. In order to assess the functionality of the side-chains of T, D, and E residues in binding, the GFGYVAE peptide was additionally used in testing. The LPYP, IAVPGEVA, and IAVPTGVA peptides that were isolated from soy protein were included in each group as a control compounds.

Figure 2 presents all of the selected peptides in accordance with the parameter 'V_{fr}' for each peptide fragment in the peptide. The parameter 'V_{fr}' reflects a relative alteration of the occupied volume in conformation space by a model of the peptide fragment relative to the conformation space occupied by the cyclic hexaglycine peptide which was selected as a model of a β-turn structure. In order to assess the parameter 'V_{fr}', the 100 lowest energy conformers were used as the starting structures in the MD study for each pep-

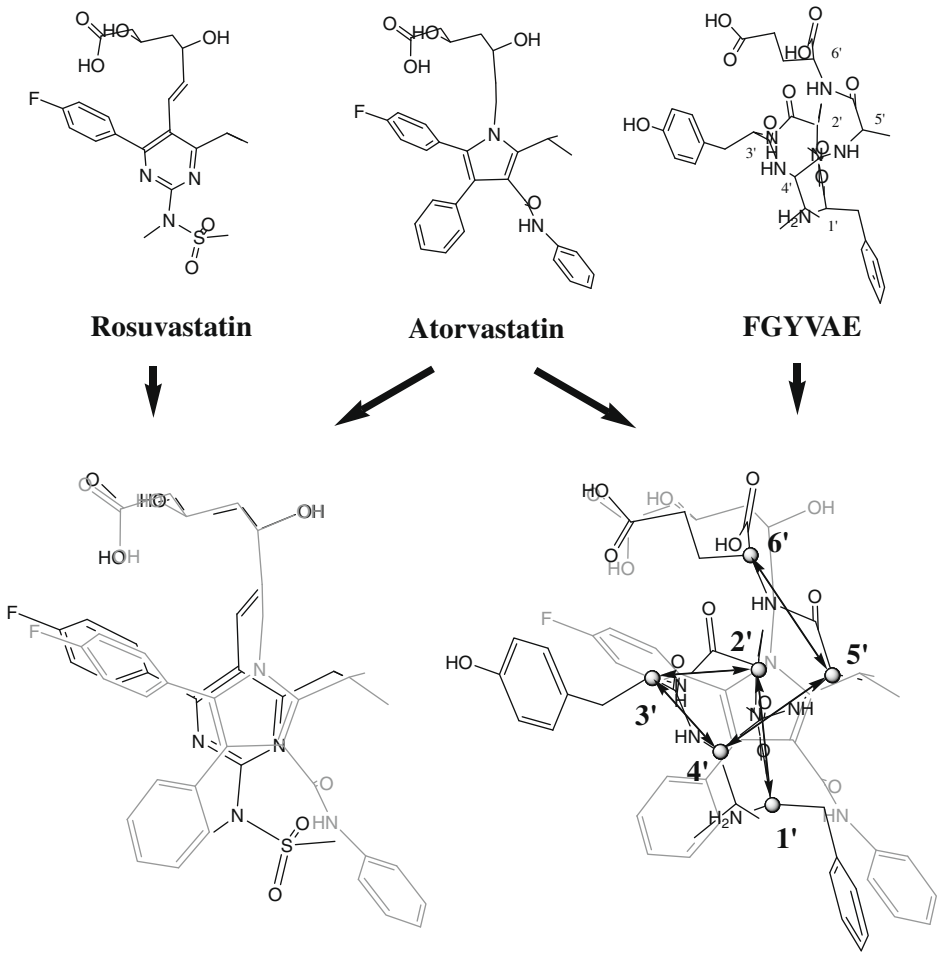

Figure 1. Spatial compatibility of atorvastatin with rosuvastatin and the linear FGYVAE peptide. The alpha-carbon atoms are indicated by marks from 1′ to 6′. The model of FGYVAE was built as type I of the β-turn on the basis of the fixed backbone dihedral angles adopted by the two corner glycine and tyrosine residues with values of $\varphi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\varphi_{i+2} = -90°$, and $\psi_{i+2} = 0°$, and the fixed dihedral angles obtained for the active backbone of the YVAE peptide and the optimized orientation of side-chains.[13]

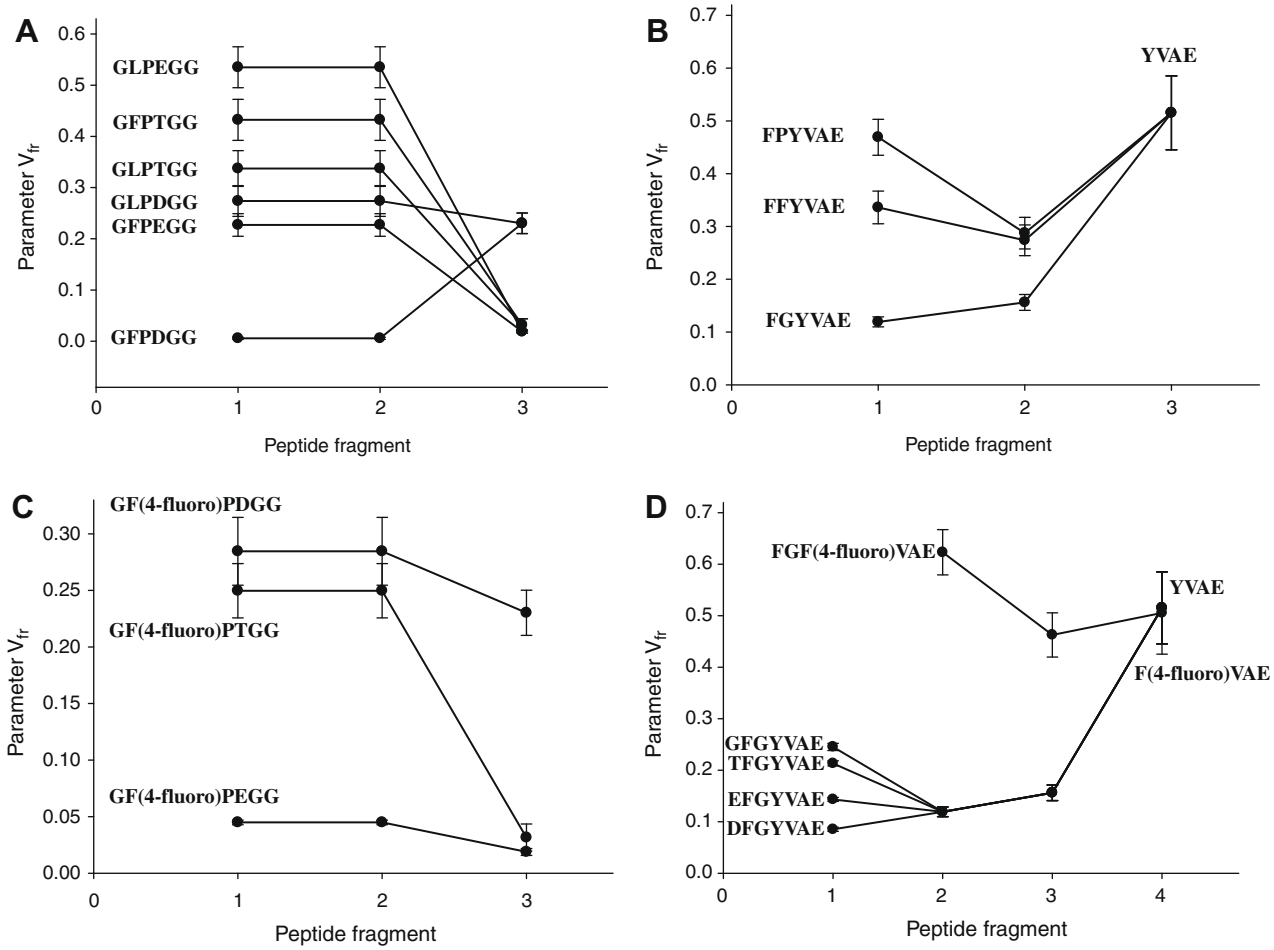

Figure 2. The calculated parameter 'V_{fr}' and standard deviation for each peptide fragment. The number of the peptide fragments started from the N-terminus of peptide based on the peptide length of the four amino acid residues (Table 1). (A) Set 1: the previously designed peptides, which include the recognized T, D or E residue as a corner residue of a β-turn structure. (B) Set 2: the previously designed peptides, in which the E residue is a recognized residue for the HMG-binding pocket and a β-turn structure is located in the N-terminus of the hexapeptides. (C) Set 1: the designed peptides, in which the phenyl ring of F residue was replaced by a 4-fluorophenyl radical. (D) Set 2: the peptides were designed by substituting 4-fluorophenyl radical instead of the 4-hydroxyphenyl radical of Y residue, or by adding the G, T, D or E residue to the N-terminus of FGYVAE peptide.

tide fragment. The comparison of the calculated values of the V_{fr} and the standard deviations showed that a relatively high value of the V_{fr} and the standard deviations belong to the peptide fragments that had a relatively high degree of flexibility, as in the case of IAVE, IVAE, YAVE, and YVAE peptides. This was confirmed by a conformational analysis of these peptides, in which a well-populated secondary structure was not determined.[14] The comparable values of V_{fr} and the small standard deviations that were observed in Fragments 1 and 2 for the GLPEGG and GFPTGG, and in Fragment 1 for the FPYVAE peptide, proposed a lower flexibility of these models compared to the IAVE, IVAE, YAVE, and YVAE peptides. The other peptide fragments exhibited relatively low values both in parameter V_{fr} and the standard deviations, indicating a restricted flexibility that can be interpreted in terms of a similarity in the conformational behavior that was observed for turn conformation (Fig. 2A and B). This is in agreement with the type II of the β-turn structure that was determined for the peptides in Set 1, and with types I and II of turn conformation that was observed in N-terminus for FPYVAE, FGYVAE, and FFYVAE peptides.[12,13] This finding proposes that the peptide fragmentation can be applied to a description of a structural feature of the peptide.

The same procedure was used for the designed peptides (Fig. 2C and D). According to the calculated data, the introduction of 4-fluorophenyl radical in peptides at Set 1 produced small changes in a structural preference relative to the defined structure of peptides in this set (Fig. 2C). In Set 2, some changes were observed in the peptide fragments with introduction of the 4-fluorophenyl group. This implies an alteration of the peptide flexibility and, consequently, the peptide's affinity compared to the basic peptide. According to the values of V_{fr}, the YVAE and F(4-fluoro)VAE peptides exhibit almost same peptide flexibility. This finding indicates that the peptide flexibility was not changed by introduction of 4-fluorophenyl group in the N-terminus amino acid. For the FGF(4-fluoro)VAE peptide, an increase of the peptide flexibility was observed at the first two fragments, probably, leading to the significant change in the peptide's affinity. For GFGYVAE, TFGYVAE, DFGYVAE, and EFGYVAE peptides, decreasing observed in the values of V_{fr} for the first fragments (GFGY, TFGY, DFGY, and EFGY) proposes a decrease in the flexibility in the N-terminus of these peptides and possibly an increase in the peptide's activity in accordance with the V_{fr} values (Fig. 2D).

In order to select a lead peptide candidate, a parameter 'V_{add}' was calculated as an equidistributed estimate using the means of the parameter 'V_{fr}' obtained for each fragment in peptide. The results of the conformational searches that involved the peptides of both sets are shown in Figure 3. According to the 'V_{add}' value, the GF(4-fluoro)PEGG and DFGYVAE sequences from the Sets 1 and 2, respectively, are the most rigid structures compared to the other peptides. This finding proposes that the peptides with GF(4-flu-

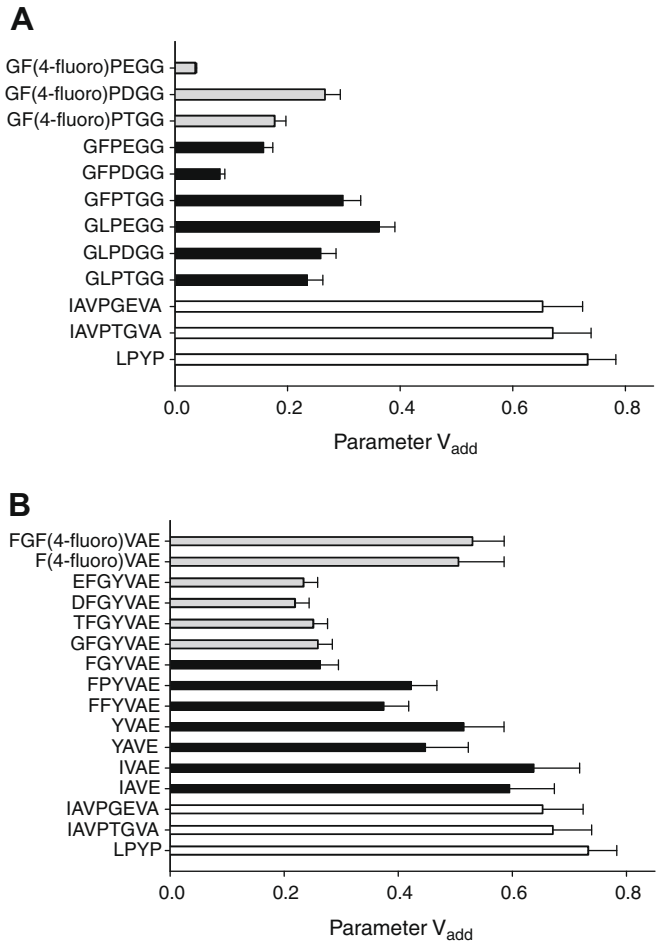

Figure 3. The calculated parameter 'V_{add}' and the standard deviation for the peptide models in each set. (A) Set 1: the peptides, which include the recognized T, D or E residue as a corner residue of a β-turn structure. (B) Set 2: the peptides, in which the E residue is a recognized residue for the HMG-binding pocket, and a β-turn structure is located in the N-terminus of the hexapeptides. The control, previously and newly designed peptides are marked as a white, black, and gray, respectively.

oro)PEGG and DFGYVAE amino acid sequences can be selected as lead peptide candidates.

Thus, all of the discussed peptides were synthesized and their biological activities were estimated. The LPYP, IAVPGEVA, and IAVPTGVA peptides that were isolated from soy protein were used as a control compound. In the GF(4-fluoro)PGGG and DFGYVAG peptides, the recognized glutamic residue was substituted with a glycine residue in order to provide a negative control for the binding study.

2.2. Inhibition activities of designed peptides

An assay of the in vitro susceptibility of HMGR to inhibition by the synthetic peptides was performed in a range of peptide concentrations from 0.1 to 1000 μM. Each of the synthesized peptides showed some ability to inhibit HMGR, with the exception of the GF(4-fluoro)PGGG and DFGYVAG peptides. No activity was detectable for these peptides when an upper boundary of the concentration range was used (Table 1). This finding supports the previously obtained data that showed that the active site of HMGR recognizes the glutamic residue as an essential component for the HMG-biding pocket.[12–14]

The most active peptides in sets were found as the GF(4-fluoro)PEGG and DFGYVAE peptides, which were selected as lead compounds. A threefold increase of the inhibitory activity for the GF(4-fluoro)PEGG, compared to the GFPEGG peptide proposed the contribution of the fluorine atom during binding. The same result was observed between the GF(4-fluoro)PTGG and GFPTGG peptides. As was expected, the inhibitory activity of the GF(4-fluoro)PDGG peptide was found to be low compared to the GFPDGG peptide in accordance with the value of the 'V_{add}' parameter. An interaction between the 4-fluorophenyl group and the side-chain of D residue probably led to an alteration in the arrangement of the 4-fluorophenyl group in the binding site compared to that observed for the phenyl group in GFPDGG peptide. The F(4-fluoro)VAE peptide showed a 10-fold increase of the inhibitory activity compared to the YVAE peptide. According to the close values of V_{add} for both peptides, this confirms the significant contribution of 4-fluorophenyl group in binding. A decrease in the inhibitory activity for the FGF(4-fluoro)VAE compared to the FGYVAE peptide can be explained by the structural preference described through the values of V_{fr}. A significant change was observed in the N-terminus when the 4-fluorophenyl group was introduced. A decrease in the binding affinity possibly occurred due to the altered location of the N-terminus in the binding site compared to that of the FGYVAE peptide.

The EFGYVAE, TFGYVAE, and GFGYVAE also showed an increase in the inhibitory activity compared to the FGYVAE peptide. The close values of IC_{50} that were observed for TFGYVAE and GFGYVAE peptides propose the contribution of the peptide backbone during binding. A difference between the values of IC_{50} that was observed for the DFGYVAE and GFGYVAE peptides indicates, probably, on an additional interaction of the oxygen atoms of the carbonyl group of the D side chain with the binding site.

In order to estimate the proposed approach, the dependence between the observed and predicted peptide potency was plotted by using the values of the 'V_{add}' parameters for the peptides in each set (Fig. 4). The highest value of the correlation coefficient that was obtained in Set 1 indicates that the better predictive ability can be obtained by using the peptides that have an equal peptide length. In this set, a relatively good correlation ($r^2 = 0.92$) was determined between the experimental bioactivity of the linear peptides, measured by log (IC_{50}), and the predicted bioactivity that was obtained through the 'V_{add}' parameter (Fig. 4A). For Set 2, the correlation coefficient is also good enough ($r^2 = 0.89$) in spite of the fact that a different length of peptides was used in this set.

The difference between the obtained values of the peptide bioactivities was checked with the help of the Fisher criterion.[19] An insignificant difference between these values was determined by using the Fisher criterion ($F_{exp} < F_{(0.05,f1,f2)}$). This finding suggests that the proposed approach is acceptable in the design of linear peptides.

Figure 5 presents the dependence between the calculated parameter 'V_{add}' and the observed peptide activity (log (IC_{50})) in each set. The difference in slopes that was observed for the correlation lines indicates a different arrangement of the designed conformation in the binding site. This finding proposes a possibility to design the peptides in terms of selectively determining the binding for peptides in the active site.

Statins are potent inhibitors of HMGR and function in a competitive manner with respect to HMG-CoA.[18] In order to clarify the properties of HMGR inhibition by the designed peptides, the enzyme activity was measured with HMGR in the absence and in the presence of GF(4-fluoro)PEGG or DFGYVAE, as these were the most potent inhibitors in the sets. A Dixon plot shows that both peptides inhibited HGMR in a competitive manner with respect to HMG-CoA (Fig. 6). The equilibrium constant of inhibitor binding (K_i) for the inhibition of HMGR by GF(4-fluoro)PEGG or DFGYVAE were estimated to be 0.26 ± 0.01 μM and 0.051 ± 0.004 μM, respectively.

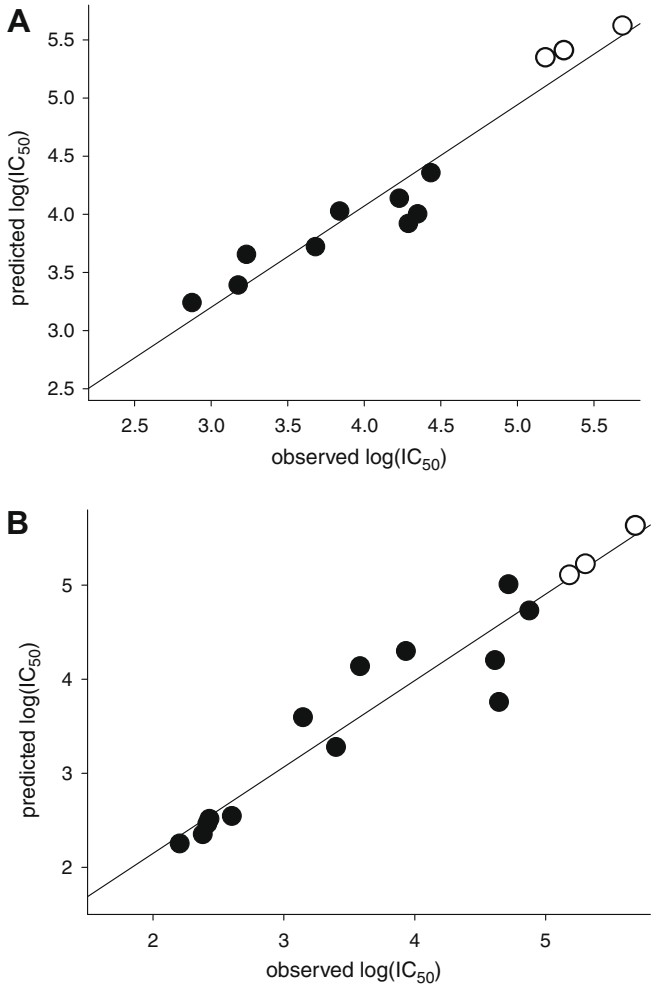

Figure 4. The correlation between the observed peptide potency and the predicted potency against HMGR for the designed and control peptides (LPYP, IAVPTGVA, and IAVPGEVA). (A) The peptides of Set 1. (B) The peptides of Set 2. The correlation coefficients (r^2) for the first and second groups are 0.92 and 0.89, respectively. The designed peptides are marked as (●); the control peptides are marked as (○).

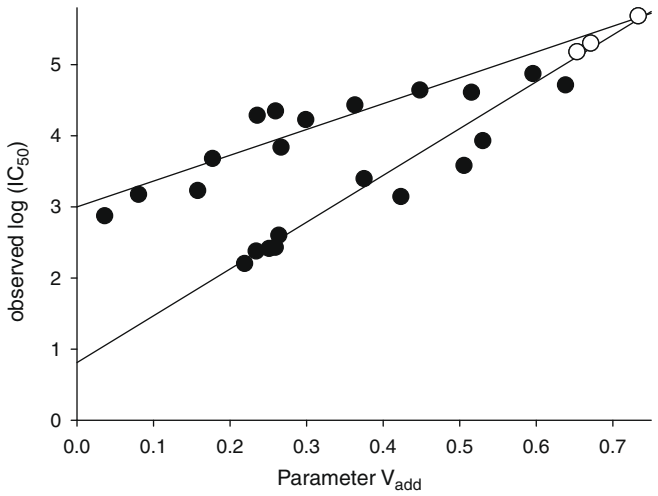

Figure 5. The dependence between the calculated parameter 'V_{add}' and the observed peptide activity (log (IC_{50})) in each set. The difference in slopes observed for the correlation lines indicates the different arrangement of the turn conformation in the binding site. The designed peptides are marked as (●); the control peptides are marked as (○).

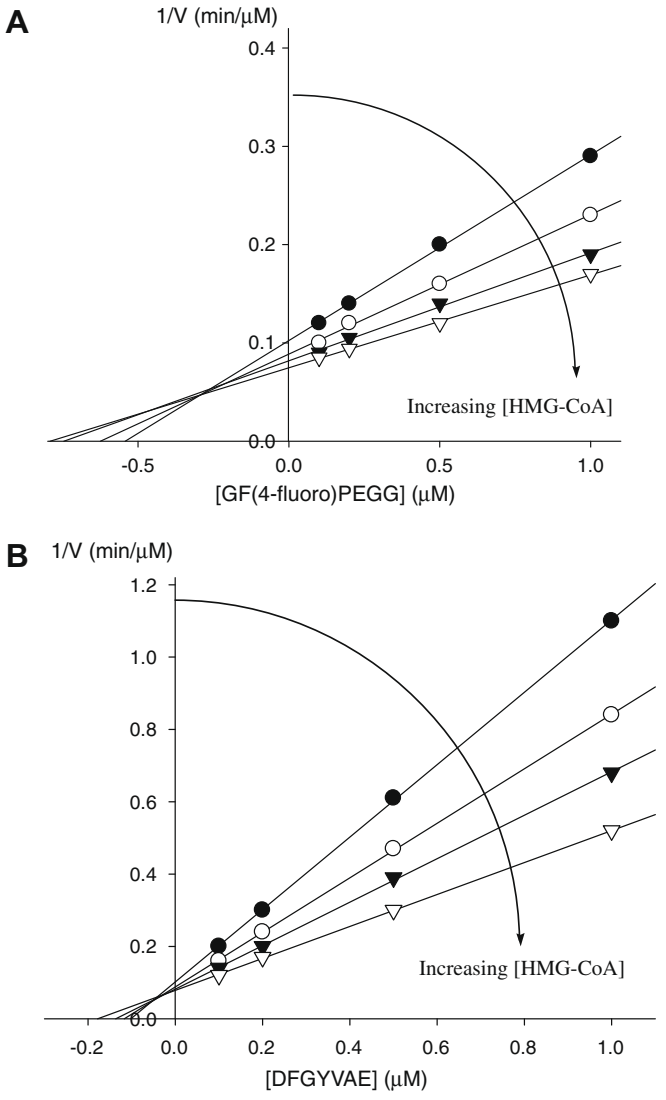

Figure 6. The kinetics of inhibition of HMGR by the most potent inhibitor designed at each group. (A) GF(4-fluoro)PEGG is the most active peptide of Set 1. (B) FGYVAE is the most active peptide of Set 2. The data are presented in a Dixon plot, 1/*V* against [GF(4-fluoro)PEGG] or [DFGYVAE], at a NADPH concentration of 120 μM and HMG-CoA concentrations of 72 μM (●), 96 μM (○), 120 μM (▼), and 144 μM (▽). The K_M value was found to be 68 ± 3.2 μM for HMG-CoA and the V_{Max} value was 19.4 ± 0.4 μM/min. Each straight line represents the results of triplicate experiments.

2.3. Peptide structure by circular dichroism study and modeling

The structure of the DFGYVAE peptide as the most potent inhibitor was investigated by CD spectroscopy in water and in trifluoroethanol (TFE). The spectrum of the DFGYVAE peptide in water is shown in Figure 7A. According to the spectrum profile observed, it is difficult to interpret what types of secondary structures are present.

In order to elucidate the structural features of the DFGYVAE peptide, the YVAE, FGYVAE, and GFYVAE peptides were additionally used in the CD study as the different segments of the peptide being studied. The spectra of YVAE, FGYVAE, and GFGYVAE in water are shown in Figure 7A. The spectrum of YVAE is characteristic of peptides that are in a random conformation. The shapes of the FGYVAE and GFGYVAE spectra are consistent with an ensemble of conformations rather than one or two unique conformations.

Figure 7B illustrates the effect of TFE on the secondary structures of DFGYVAE, GFYVAE, FGYVAE, and YVAE peptides. The disso-

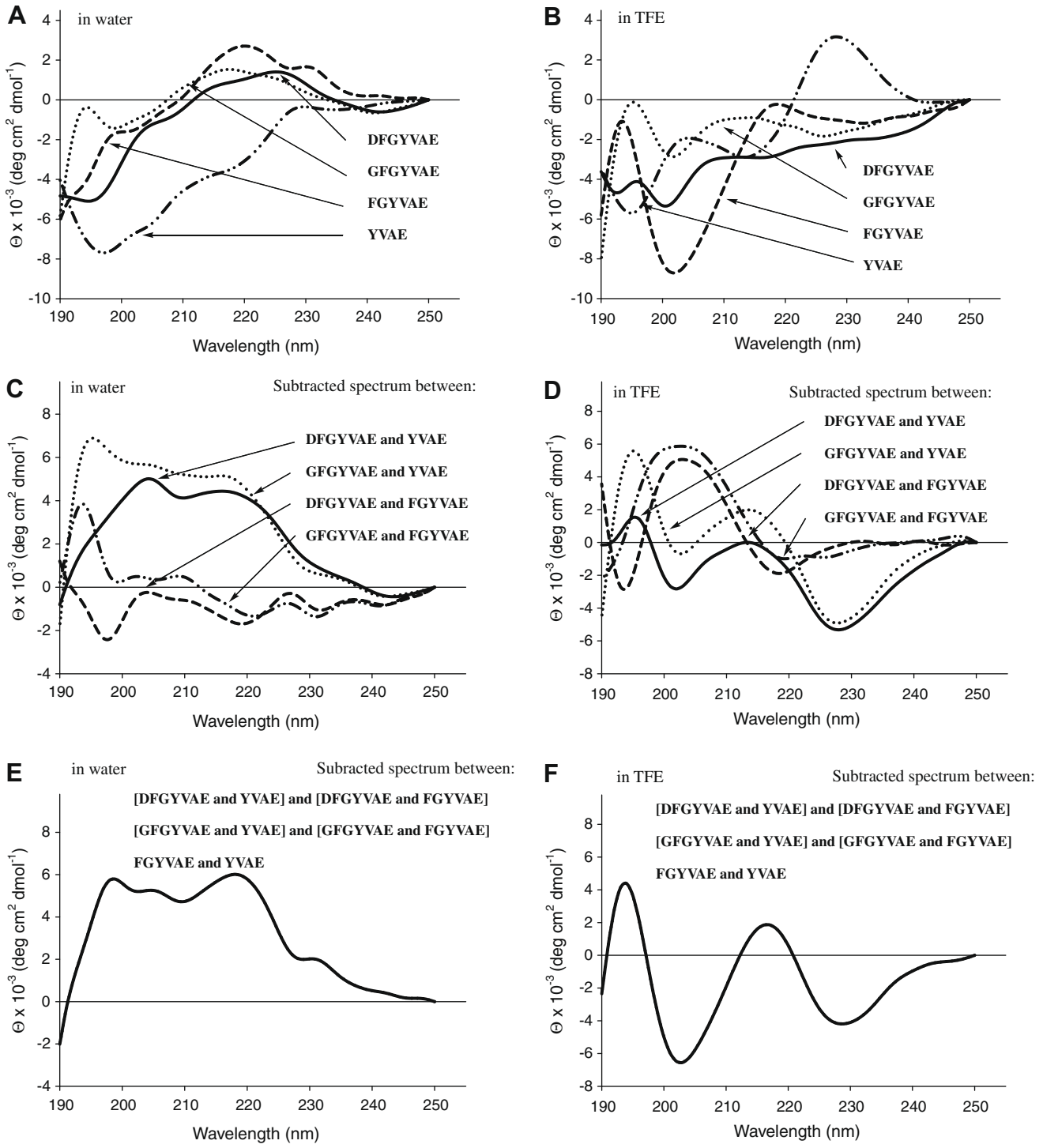

Figure 7. CD spectra of the peptides and N-terminus contribution in water and in TFE. (A) CD spectra of the DFGYVAE, GFGYVAE, FGYVAE, and YVAE peptides in water. (B) CD spectra of the DFGYVAE, GFGYVAE, FGYVAE, and YVAE peptides in TFE. (C) Contribution of the N-terminus of the DFGYVAE and GFGYVAE peptides compared to YAVE and FGYVAE in water. (D) Contribution of the N-terminus of the DFGYVAE and GFGYVAE peptides compared to YAVE and FGYVAE in TFE. (F) Contribution of the N-terminus of the FGYVAE peptide compared to YAVE and the resulting spectra obtained between the contributions of the N-terminus of the DFGYVAE and GFGYVAE with respect to YAVE and FGYVAE in water. (F) Contribution of the N-terminus of the FGYVAE peptide compared to YAVE and the resulting spectra obtained between the contributions of the N-terminus of the DFGYVAE and GFGYVAE with respect to YAVE and FGYVAE in TFE.

lution of these peptides in TFE results in significant changes in the CD spectra. The CD spectrum for DFGYVAE appears to have features that are contributed probably by the β-turn type I and the unordered structures due to the negative bands below 200 nm and at 205 nm, as well as the shoulder being between 210 and 220 nm. The spectrum of GFGYVAE displays a slightly different pattern compared to the DFGYVAE peptide due to a decrease of the intensity at the same absorption region. For FGYVAE, the shape of the spectrum transforms into that with strong and weak negative bands between 200–205 nm and 220–230 nm, respectively. The unordered YVAE in water shows the distinctive feature of the aromatic contribution with the maximum positive ellipticity maximum being 226 nm from tyrosine residue.[20] The CD spectra of these peptides indicate that, in all environments, no observable

patterns were displayed that was related to a well-populated secondary structure conformation.

In previous study, a structure that was close to a type I of β-turn was determined in the N-terminus of the FGYVAE peptide based on the analysis of N-terminus contribution.[13] In order to determine a structural preference in the N-terminus of the DFGYVAE peptide, three stage procedures was applied that used CD spectra in water and in TFE. The first was subtracting the spectrum of the YVAE peptide from that of peptides DFGYVAE and GFGYVAE in order to determine the N-terminus contribution. The second was subtracting the spectrum of the FGYVAE peptide from that of peptides DFGYVAE and GFGYVAE in order to elucidate an effect of the D and G residues. The final stage was subtracting the resulting spectra that were obtained at the first stage from that obtained at the second stage, and subtracting the spectrum of the YVAE from that of the FGYVAE peptide in order to estimate the N-terminus contribution between DFGYVAE, GFGYVAE, and FGYVAE peptides.

The resulting spectra that were obtained at the first and second stages are shown in Figure 7C and D. A similar shape in the resulting spectra that were obtained within each stage proposes a similarity in the structural preferences for DFGYVAE and GFGYVAE peptides. Figure 7E and F shows the resulting spectra obtained at the final stage. Here, the subtracted spectra exhibit the same profile obtained for DFGYVAE, GFGYVAE, and FGYVAE peptides in different environment. This indicates the same N-terminus contribution for these peptides and the location of the structure is close to a type I of β-turn in the N-terminus that is formed by the two corner glycine at position $i + 1$, and tyrosine residue at position $i + 2$, according to the numbering for the turn structure as it was defined for FGYVAE peptide. An analysis of the secondary structures in the proteins also supports that these residues have a high propensity to form a β-turn conformation.[21]

In the case of the designed GF(4-fluoro)PEGG peptide, which was the most active peptide in Set 1, CD analysis in TFE showed that the spectrum of this peptide, which was characterized by a weak negative band between 230 and 240 nm and a strong positive band around 200 nm, is associated with the presence of a folded conformation that is close to β-turn type II (Fig. 8).

Based on these considerations, an active conformation for GF(4-fluoro)PEGG and DFGYVAE peptides were constructed. The starting model of GF(4-fluoro)PEGG was modeled as a type II of the β-turn on the basis of the backbone dihedral angles that were adopted by the two corner proline and glutamic acid residues ($\varphi_{i+1} = -60°$, $\psi_{i+1} = 120°$, $\varphi_{i+2} = 80°$, and $\psi_{i+2} = 0°$). For the DFGYVAE peptide, the starting model was built as a type I of the β-turn by using the backbone dihedral angles that were adopted by the two corner glycine and tyrosine residues ($\varphi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\varphi_{i+2} = -90°$, and $\psi_{i+2} = 0°$). The orientations of the side-chains and other residues were determined by an optimization procedure. Figure 9 shows the superposition of the bioactive conformations of atorvastatin with the optimized structure of the GF(4-fluoro)PEGG and DFGYVAE peptides. As shown in Figure 9, the proposed contribution of the fluorine atom of GF(4-fluoro)PEGG peptide during binding led to an increase in the inhibitory activity for this peptide compared to the other members of Set 1. This was explained by a similar location of the 4-fluorophenyl group of atorvastatin and GF(4-fluoro)PEGG peptide. According to the constructed model of the DFGYVAE peptide, the oxygen atoms of the carbonyl groups of the side chain and the peptide backbone of the D residue occupied a space around the location of the oxygen atom of the phenylcarbamoyl group at position 4 of the pyrrolyl ring of atorvastatin. Furthermore, an orientation of the phenyl radical of the F residue close to that of atorvastatin at position 3 of pyrrolyl ring was also observed. Probably, the increase of inhibitory activity of the DFGYVAE peptide can be interpreted in terms of the contribution of the oxygen atom of the carbonyl group of the D side chain, while the increase of inhibitory activity of the GFGYVAE peptide by the contribution of the oxygen atom from the amide bond between N-terminus' G and F residues in binding. The modeled structures of these peptides exhibited a good enough approximation of the statin molecules, which confirms the appropriateness of the proposed method in the design of active peptides.

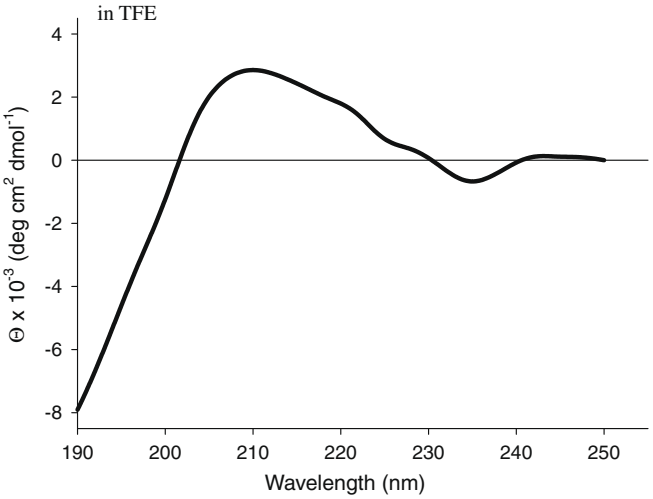

Figure 8. CD spectrum of the linear GF(4-fluoro)PEGG peptides in TFE. A weak negative band between 230 and 240 nm and a strong positive band around 200 nm indicates on the presence of a folded conformation that is close to β-turn type II.

3. Conclusion

The proposed method presents an approach in modeling an active peptide by using a design of a competitive inhibitory peptide for an HMGR. The two binding points were applied in the proposed design. The first was a recognized residue. The second was a structural element, such as β-turn conformation, that could be essential for binding. The two sets of peptides, with a different location of the β-turn structure relative to the recognized residue, were used in the proposed design. By using a peptide fragmentation based on the four residues, the conformational behavior of the peptide fragments were assessed in accordance with that of a β-turn structure by using the conformational behavior of the cyclic model of glycine hexapeptide as a basis in the MD study. A conformational analysis and the obtained results based on the peptide fragments confirmed a possibility in describing the peptide structure in terms of their structural preferences. This creates a good opportunity to select a prospective site for the peptide in order to design a more active peptide. The restriction of the peptide flexibility was used when searching for the lead compounds. By using the adduced means based on examining the conformational behavior of the peptide fragments for each peptide, the lead peptide candidates were selected in each set. The obtained correlations between the inhibitory activities and the design parameters in sets imply the design of a more tightly bound peptide by the HMGR active site. Furthermore, the difference in slopes observed between the two sets suggests an opportunity to create an active peptide with adjusted properties in terms of the peptide selectivity. In accordance of the two binding points that can be determined by the experimental procedure, the proposed design may be applied to ligands in order to investigate peptide–protein interactions, especially in the case, when the spatial information for the target is not available.

Thus, the present study shows the design of a more potent inhibitor for HMGR and a simple tool for designing active linear peptides.

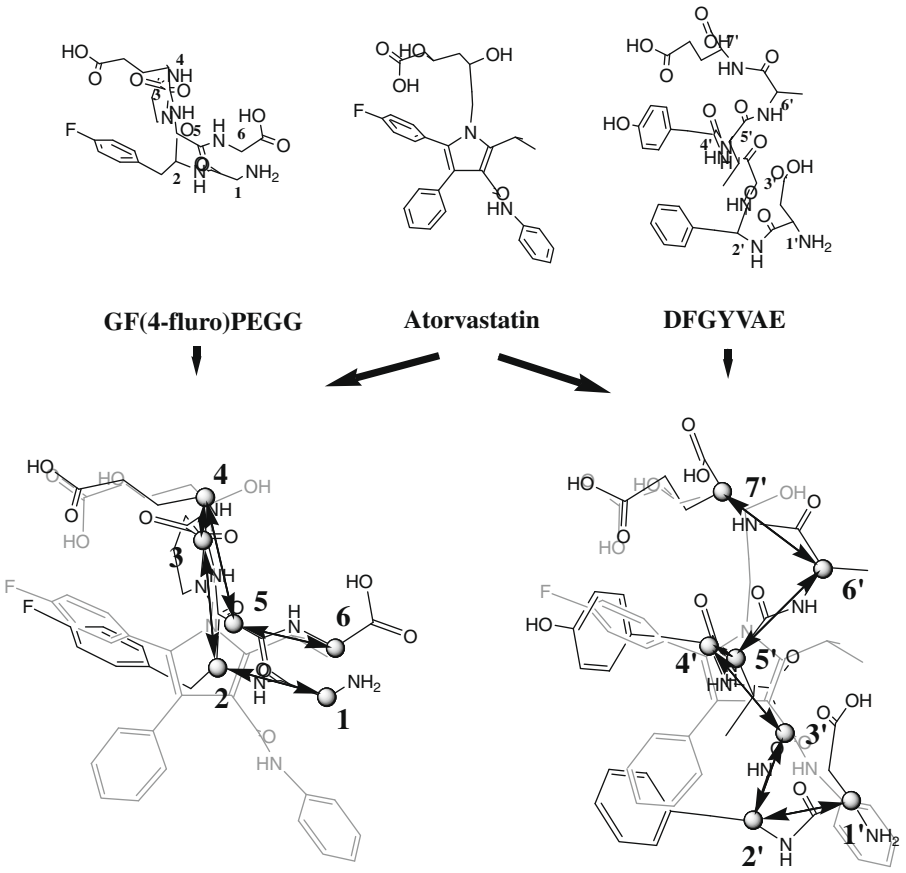

Figure 9. Spatial compatibility of the linear GF(4-fluoro)PEGG and DFGYVAE peptides with atorvastatin. The alpha-carbon atoms are indicated by marks from 1 to 6 for the GF(4-fluoro)PEGG peptide and from 1′ to 7′ for the DFGYVAE peptide. The model of GF(4-fluoro)PEGG was built as type II of the β-turn on the basis of the fixed backbone dihedral angles adopted by the two corner proline and glutamic acid residues ($\varphi_{i+1} = -60°$, $\psi_{i+1} = 120°$, $\varphi_{i+2} = 80°$, and $\psi_{i+2} = 0°$). For the DFGYVAE peptide, the model was constructed as type I of the β-turn by using the fixed backbone dihedral angles adopted by the two corner glycine and tyrosine residues ($\varphi_{i+1} = -60°$, $\psi_{i+1} = -30°$, $\varphi_{i+2} = -90°$, and $\psi_{i+2} = 0°$). The orientations of the side-chains and other residues were determined by an optimization procedure.

4. Experimental section

4.1. Materials

H-Gly-2-ClTrt resin (substituted at 0.5 meq/g), HGlu(Ot-Bu)-2-ClTrt resin (substituted at 0.55 meq/g), and Fmoc-amino acids were purchased from AnaSpec (San Jose, CA, USA). Chemicals for the peptide synthesis were obtained from Perkin–Elmer (Foster, CA, USA). Acetonitrile and methanol for HPLC were the products of Burdict and Jackson (Muskegon, MI, USA).

4.2. Peptides synthesis

Using standard Fmoc methodology, the solid phase synthesis of peptides was carried out on an automated Applied Biosystem Peptide Synthesizer (Model 433A, Perkin–Elmer, Foster, CA, USA).[22] Deprotection and cleavage were achieved by treatment with a mild trifluoroacetic acid (TFA).[23] Then peptides were precipitated in ether, followed by being filtered and dried under a vacuum. Purification and analysis of the synthetic peptides were done by using a Reversed-Phase High Pressure Liquid Chromatography (RP-HPLC) system (Waters, Milford, MA, USA). Synthetic peptides were analyzed by using a Vydac 218TP54 analytical column under the following gradient conditions: solvent A, 0.1% TFA in water, solvent B, 0.1% TFA in acetonitrile; initial condition 95% A, 25 min, final condition 65% A; flow rate, 1 mL/min. The purity of the synthetic peptides after purification was found to be above 99% by using a Vydac 218TP510 semi-preparative C18 column. Peptides were identified by an electrospray mass spectrometer (Platform II, Micromass, Manchester, UK) and an Applied Biosystems 491 Peptide Sequencer (Perkin–Elmer, Foster, CA, USA).

4.3. Assay of HMG-CoA reductase activity

The HMG-CoA-dependent oxidation of NADPH was monitored at 340 nm in a Jasco V-530 spectrophotometer (Model TUDC 1284, Japan Serco Co., Ltd, Japan). The assay conditions were as described in a previous study.[14] One unit (U) of HMGR was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per min. Protein concentration was determined by the method of Bradford.[24] The type of inhibition and Michaelis–Menten parameters were determined from a Dixon plot.[25]

4.4. Circular dichroism spectroscopy

Circular dichroism (CD) measurements were carried out on a Jasco J-710 spectropolarimeter (JASCO International Co., Tokyo, Japan). Measurements were taken by using a quartz cuvette with a path length of 1 mm. Single scans in a range between 190 and 250 nm were obtained at 18 °C with the following settings: a sensitivity of 10 millidegrees, a 0.5 s response time, a bandwidth of 1 nm, and a speed of 50 nm/min.[26] Three scans were collected for each sample. Each spectrum was corrected for sample cell and solvent contributions. All subsequent CD spectral values for peptides are expressed in units of molar residue ellipticity (deg cm^2 $dmol^{-1}$).

4.5. Computation methods

The structures of the peptides were built by using the program package ChemOffice Desktop 2004 for Windows (CambridgeSoft (CS) Corporation, MA, USA). The structures of the cyclic peptides used in the design of the peptide sequences were carried out by a molecular mechanics method (MM2) within Chem3D.[27] The calculations of the optimized structures for peptide models were carried out using the AM1 method within the CS MOPAC (Version 1.11) program package.[28] Quantum-chemical structures were optimized by gradient minimization to 0.001 kcal/mol Å.

Molecular Dynamics (MD) simulation was used for estimating of the peptide backbone behavior. This was performed by using ChemOffice Desktop 2004 for Windows.[29] The peptide structures were collected during simulation time of 300 ns in vacuum and were heated to 300 K.

The calculations of the design parameter 'V_{fr}' was slightly changed compared to that described in a previous study.[12] The difference was that the occupied volume in conformation space by a peptide model was adduced to the conformation space occupied by the cyclic hexaglycine peptide instead of the conformation space occupied by all of the peptide candidates from a library as it was used in the previous design.[12] Then, the calculated 'V_{fr}' parameter for each fragment in peptide was used to estimate a full peptide length via the design parameter 'V_{add}'. It was calculated as an equidistributed estimate based on the means of the parameter 'V_{fr}' obtained for each fragment in peptide. For each cyclic model, 100 geometry-optimized low-energy and MD conformers were collected by using a slightly modified method than the one in study.[30] The collection was based on differentiating the conformers by using the deviations of their internal coordinates.

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