論文の内容の要旨

Abstract of Dissertation

論文題目　**Development and application of dynamic virtual bicyclic peptides as a prospective therapeutic scaffold**
(ダイナミック仮想２環ペプチドの開発と創薬への応用)

氏　　名　　エムディー　シャミン　レザ

（本文）

Background and Purpose of This Study:

There are numerous conceivable benefits as well as some downsides to the use of peptides as drugs. Many of these downsides (e.g., poor tissue penetration, proteolytic degradation, and quick elimination) were discovered during investigating the pharmacological properties of peptide-based drugs. However, more recently it has been shown that many of the downsides can be dealt with by the modification of peptides to achieve desired therapeutics properties. As a result, Novel peptide therapeutics are increasingly making their way into clinical application. In the past, due to lack of efficient screening methodology, stable and potent peptides had been difficult to discover from both the chemical and genetic/recombinant peptide libraries. However, with the use of more recent *in vitro* display technologies such as ribosome display, mRNA display, CIS or DNA display, it is now possible to find out high-affinity peptides against nearly all protein targets, including those involved in disease progressions. However, the poor bioavailability and limited cellular permeability of the proteinogenic peptides has restricted their use as therapeutics.

Considering the above-mentioned points, I planned to develop a novel class of constrained peptide scaffold, termed as Virtual Bicyclic (VB) scaffold, with an effort to expand peptide drugs as a popular therapeutics. VB peptide can be defined as a non-proteinogenic macrocyclic peptide having an intra-molecular hydrophobic interlocking arrangement scaffold (Fig. 1A) introduced by a non-proteinogenic amino acid, 2-Aminoheptonic acid (Ahep) (Fig. 1B). This hydrophobic interlocking arrangement scaffold provides a bicyclic like peptide structure but not the actual bicyclic structure formed by covalent disulfide bonding. I envisioned that VB peptide scaffold would provide a conformational and proteolytic stability similar to the macrocyclic compounds

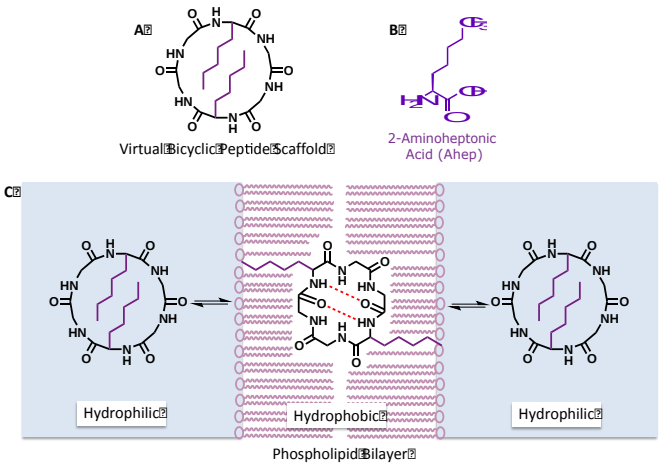

Figure 1: (A) Schematic presentation of VB peptide. (B) Structure of non-proteinogenic amino acid, Ahep. (C) Possible conformational switching hypothesis of VB peptides.

commonly found in nature and synthetic design. In addition, it would facilitate increased permeability across the cell membrane due to its flexible conformational structure. Based on the conformation switching of cyclosporin A, I hypothesized that in hydrophilic environment it would adopt a conformation in which all of its non-proteinogenic hydrophobic residues would be involved in the intra-molecular hydrophobic interactions resulting in a compact structure. On the other hand in hydrophobic environment, it would adopt another conformation in which most of its non-proteinogenic hydrophobic residues would point toward the hydrophobic environment, which in turn would facilitate internal hydrogen bonding in between other amino acid residues (Fig. 1C) resulting in a relative flexible structure. This hypothesized conformational switching of VB peptide would provide increased cellular permeability. Therefore, I anticipated that VB peptides would be able to solve the cell permeability issues in peptide–based drugs.

Design and Construction of Diverse mRNA Libraries for the Expression of VB Peptides:

FIT system based genetic code reprogramming is a unique technology for the ribosomal synthesis of non-standard peptides. It is a combination of two sophisticated catalytic systems, Flexizyme and custom-made reconstituted *in vitro* translation systems. Flexizymes are de novo tRNA acylation ribozymes capable of charging virtually any amino acids onto desired tRNAs with any body and anticodon sequences, and thus facilitate the preparation of desired tRNAs charged with non-proteinogenic amino (and hydroxy) acids. On the other hand, certain amino acids and cognate aminoacyl-tRNA synthetases can be omitted from the custom-made reconstituted *in vitro* translation systems, which results some vacant codon due to the unavailability of corresponding ‘‘aminoacyl-tRNAs’’. These vacant codons can be filled with non-proteinogenic amino acids by the addition of corresponding aminoacyl-tRNAs prepared by flexizymes. Thus, FIT system, facilitates expression of non-standard peptides from designed mRNA templates according to the newly designated genetic table.

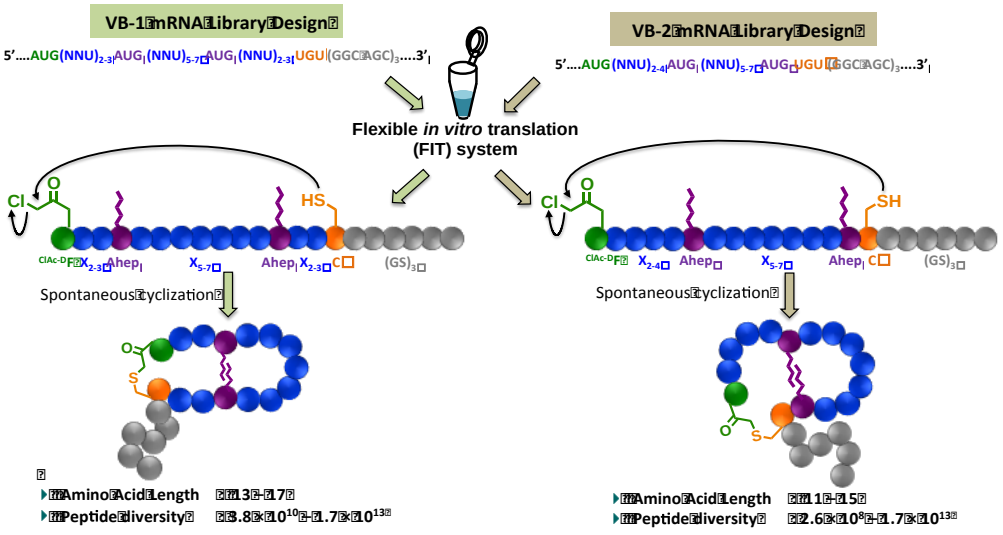

Figure 2: Design of VB1 and VB2 mRNA libraries to code peptides with VB scaffolds.

By using FIT system based genetic code reprogramming; I constructed two different mRNA libraries to express VB peptides with the prospect of *in vitro* screening of peptide inhibitors against various therapeutic targets. By using FIT system, it had already been reported the synthesis of head-to-tail circulized peptides containing thioether linkage generated by a spontaneous reaction

between a N-terminus chloroacetyl group and C-terminus sulfhydryl group of a cysteine residue. Therefore, this spontaneous macrocyclization strategy was used for the construction of two diverse mRNA libraries expressing VB scaffolds. Here, two non-proteinogenic amino acids, N^{α}-chloroacetyl-D-phenylalanine ($^{ClAc\text{-}D\text{-}}$F) and 2-Aminoheptonic acid (Ahep) were used to achieve VB like scaffolds. With the help of FIT system, one $^{ClAc\text{-}D\text{-}}$F and two Ahep residues were assigned to code by the initiation AUG and elongation AUG codon, respectively. As a result, the C-terminal chloroacetyl group of the $^{ClAc\text{-}D\text{-}}$F residue spontaneously reacted with the N-terminal cysteine residue to give a circulized thioether bond in the resulted peptides. On the other hand, in the translated peptides the incorporated Ahep residues interacted with each other to form a VB structure through their strong hydrophobic interactions. Upon successful translational incorporation of $^{Clac\text{-}D\text{-}}$F and Ahep by flexizyme, two different mRNA libraries, termed as VB-1 and VB-2 mRNA library, were constructed to code them in the translated peptides. Here, first double stranded DNA pools were constructed from the synthetic DNA templates, where random nucleotide sequences were introduced as $(NNT)_x$ codon, here N= A, T, G or C; X= 7, 8, 9, 10, 11, 12, or 13. These random sequences were placed in between an initiator ATG codon expressing $^{Clac\text{-}D\text{-}}$F and two elongation ATG codons expressing Ahep residues. The only difference between these two VB mRNA libraries is the position of elongation ATG codons expressing Ahep amino acids (Fig. 2), with the prospect of better VB scaffolds for the *in vitro* screening of peptide inhibitors against a particular therapeutic target. From these DNA libraries mRNA pools were prepared by *in vitro* transcription. In these NNU mRNA libraries, 15 proteinogenic amino acids are assigned in 16 active codons and for the intiation, D-form amino acid were chosen to increase the protease stability of VB peptides. Moreover, both these libraries did not have any stop codon in random regions, resulting in the generation of highly reliable VB peptide libraries.

***In Vitro* Screening of PAD4 Inhibitor Using VB Peptide Library:**

(i) RaPID display mediated *in vitro* selection of VB peptides against PAD4: To devise a robust drug discovery tool, FIT system was integrated with the mRNA display technology which is termed as the random non-standard peptide integrated discovery (RaPID) system. Therefore, as a therapeutic application of VB peptides, I performed RaPID display mediated *in vitro* selection of peptide inhibitors against peptidylarginine deiminase 4 (PAD4) using VB peptide libraries. It has been found that PAD4 has an active role in gene regulation as well as in the development of various diseases such as, rheumatoid arthritis and cancer. Therefore, PAD4 was chosen as a therapeutic target for this experiment. To perform selection of active VB species against PAD4, first the mRNA pools of VB1 and VB2 libraries were ligated with a Puromycin-CC-(PEG linker)-DNA fragment to install puromycin at the mRNA terminus and then the mRNA libraries were translated by FIT system supplemented with $ClAc^{D}F\text{-}tRNA^{fMet}_{CAU}$ and $Ahep\text{-}tRNA^{Asn\text{-}E2}_{CAU}$ instead of Met. In the first round selection, VB peptide libraries were mixed with immobilized PAD4 on His-Tag magnetic beads, followed by reverse transcription before recovery and amplification of cDNA. From the 2nd round,

A

VB1 Design	$^{ClAc\text{-}D}$F	X	X	X	Ahep	X	X	X	X	X	X	X	Ahep	X	X	X	C
VB1C12		N	A			Y	P	Y	R	P	P			T	S		
VB1C20		D	A			Y	P	F	R	P	P			A	H		
VB1C21		Y	R	C		H	P	V	P	V				P	T	P	
VB1C25		N	A			Y	P	F	R	P	P			T	T		
VB1C35		Y	R	C		Y	P	V	P	R				T	R	P	
VB1C36		Y	R	C		Y	P	L	P	S	P	P		T	P	H	

B

VB2 Design	$^{ClAc\text{-}D}$F	X	X	X	X	Ahep	X	X	X	X	X	X	X	Ahep	C
VB2C15		Y	R	C			Y	P	I	P	R	P	P		
VB2C35		Y	R	C			N	P	I	P	A	L	P		
VB2C37		Y	R	C			H	P	V	P	R	P	P		
VB2C11		V	S	R	S		F	D	A	L	P	N	N		
VB2C28		P	S	I	R		A	F	P	H	T	N	P		

Figure 3: Enriched VB1 and VB2 peptide sequences capable of binding to PAD4.

prior to the selection against PAD4 immobilized beads, the libraries were treated only with magnetic Dynabeads (up to 12 times) to remove undesired background binding peptide species in the pool, and the peptide fraction unbound to the beads was the applied to the selection against PAD4-immobilized beads. At the 8th round, an appreciable enrichment of active populations were observed from both the libraries. The enriched pool was cloned and individual colonies were arbitrarily picked for sequencing, yielding a total of 62 DNA sequences from which 11 high frequency clones were selected (Fig. 3) to evaluate the inhibitory properties against PAD4.

(ii) Evaluation of selected VB peptides to determine PAD4 inhibitory activity- To evaluate the binding affinity and inhibitory activities of obtained peptides, frequently appearing VB peptides were prepared by Fmoc solid phase peptide synthesis. Binding affinity analysis by surface plasmon resonance (SPR) revealed that VB1 peptides bound to the PAD4 with fast association ($9.21x10^4$ - $1.21x10^5$ M^{-1} s^{-1}) and slow dissociation (3.56 - 6.64 $x10^{-3}$ s^{-1}), resulting in high affinity of 38.7 - 54.9 nM (Fig. 4). To assess whether these macrocyclic peptides can inhibit PAD4 activity, *in vitro* PAD4 inhibition assay was performed using well-established colorimetric method. Cl-amidine, which is a known small molecule inhibitor of PAD4 was used in parallel with selected peptides as a standard of PAD4 inhibitory activity. Precise IC_{50} measurements for these peptides were not performed. However, it was found the both VB1C12 and VB1C20 peptide were able to inhibit PAD4 in a dose dependent manner. But they did not show strong inhibitory potency similar to known PAD4 inhibitor.

A

Name	Peptide Sequences	Scaffold	K_D (nM)	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})
VB1C12	AcID-F&N&&Ahep&R&&&&&&Ahep&&&&& S	Virtual Bicyclic	54.98	1.21×10^5	6.64×10^{-3}
VB1C20	AcID-F&D&&Ahep&R&&&&&&Ahep&&&&& S	Virtual Bicyclic	38.71	9.21×10^4	3.56×10^{-3}

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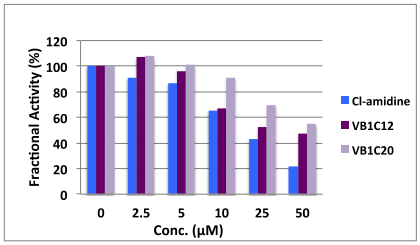

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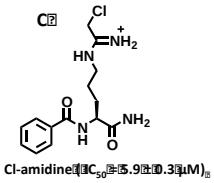

Cl-amidine (IC_{50} = 5.9 ± 0.3 μM)

Figure 4. (A) The K_D values of VB1C12 and VB1C20 measured from SPR experiments. (B) COLDER solution based colorimetric *in vitro* PAD4 inhibition assay. (C) Structure of Cl-amidine.

Conclusion:

I have successfully constructed VB peptides through FIT system mediated genetic code reprogramming technology and then implanted these peptides to the construction of diverse peptide library with the prospect of robust RaPID display mediated screening of peptide inhibitors against PAD4 enzyme. I have also become successful in the discovery of novel PAD4 peptide inhibitors using VB peptide scaffold and the discovered peptides are now under investigations of *in vitro* and *in vivo* biological studies to determine cellular permeability and precise PAD4 inhibitory activity.