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COMMUNICATION

A Comprehensive Study on the Effect of Backbone Stereochemistry of a Cyclic Hexapeptide on Membrane Permeability and Microsomal Stability

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Backbone stereochemistry of cyclic peptides has been reported to have a great influence on microsomal stability and membrane permeability, two important factors that determine oral bioavailability. Here, we comprehensively investigated the correlation between the backbone stereochemistry of cyclic hexapeptide diastereomers and their stability in liver microsomes, as well as passive membrane permeability.

Peptides are of interest as therapeutic molecules owing to their broad surfaces that allow them to bind to proteins with high specificity and selectivity. However, because of their low passive membrane permeability and low stability under physiological conditions, peptides are poorly orally bioavailable.[1,2] Macrocyclization is a promising strategy for increasing membrane permeability and stability of peptides. However, most cyclic peptides still exhibit low oral bioavailability.[3,4] Thus, it is highly demanded to understand the pharmacokinetic properties of cyclic peptides, such as membrane permeability and metabolic stability, because oral bioavailability is highly influenced by the gastrointestinal absorption rate and first-pass effects involving metabolic enzymes.

There have been studies on the passive membrane permeability of cyclic peptides and several reports on the successful development of highly membrane permeable cyclic peptides. For instance, it has been reported that the substitution of amino acids in cyclic peptides with D-amino acids,[5,6] *N*-methylated amino acids,[7–11] and α-hydroxy acids[12] changes membrane permeability. Interestingly, the substitution of L-amino acids with D-amino acids with the same side chains has been reported to largely change the permeability of cyclic peptides, although the substitution changes only the stereochemistry of the peptide backbone and does not change the molecular weight and atomic composition of the cyclic peptides. The large change in permeability is considered to be caused by the conformational change of the cyclic peptides upon the alteration of backbone stereochemistry. The effect of the backbone stereochemistry on the conformations and permeability of cyclic hexapeptides have been previously investigated for several cyclic peptides.[5,6,13,14] These reports revealed the importance of the solvent accessible surface area of cyclic peptides in a lipophilic environment for permeability, and provided several hypotheses on the permeation mechanism of cyclic peptides across membranes.[6]

There are also a few reports that evaluated the correlation of the backbone stereochemistry of cyclic peptides and stability in liver microsomes, which is another important factor determining oral bioavailability. Lokey and coworkers showed that two cyclic hexapeptide diastereomers exhibit significantly different metabolic stability in liver microsomes, even though their atomic compositions are the same.[11] In addition, Fairlie and coworkers evaluated the liver microsomal stability and oral bioavailability of three mirror image pairs of cyclic peptides. As a result, the enantiomers were shown to exhibit different

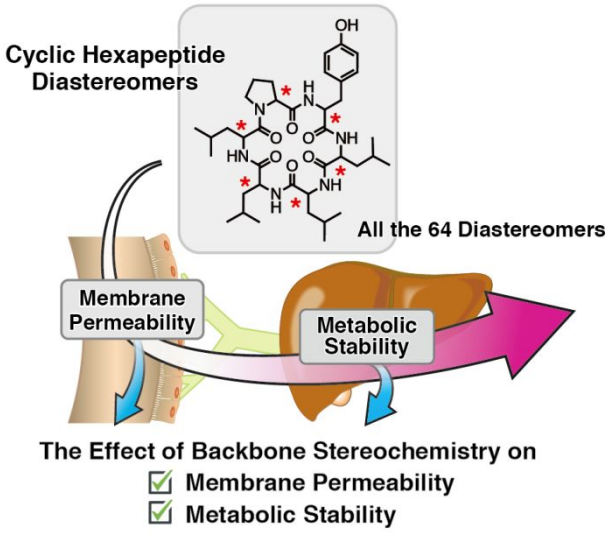

Fig. 1 Schematic illustration of this research.

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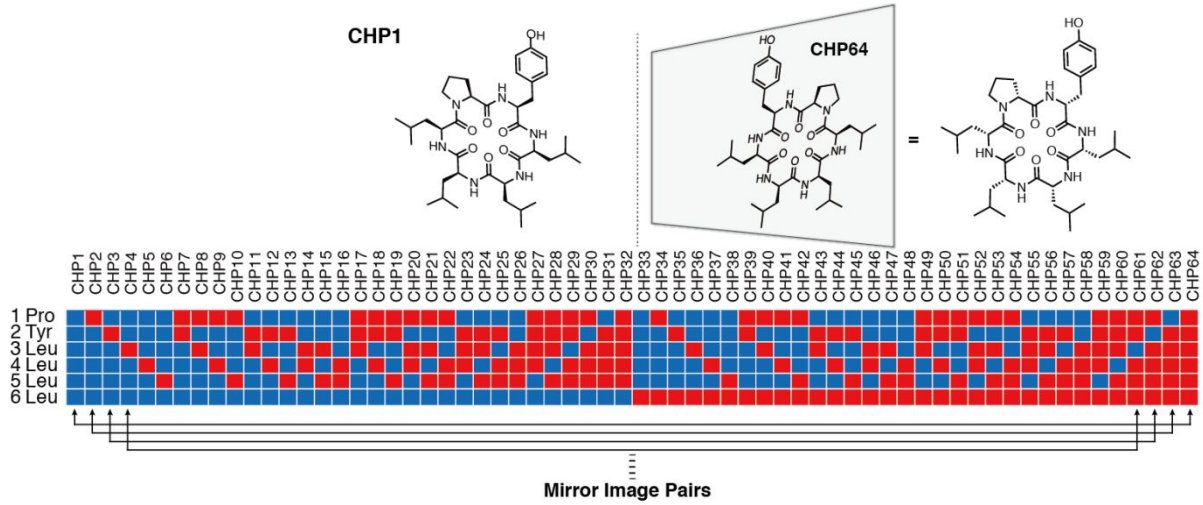

Fig. 2 The sequences of the synthesized peptides. Blue indicates L-amino acids and red indicates D-amino acids. The sequences are arranged so that mirror image pairs will be superposed when the paper is folded in the middle.

metabolic stability and the enantiomers with higher metabolic stability exhibited higher oral bioavailability than the enantiomers with lower metabolic stability.[15,16]

As described so far, there have been studies on the correlation of backbone stereochemistry, membrane permeability, and metabolic stability for a limited set of cyclic peptide diastereomers. However, it is not clear how general the large differences in permeability and microsomal stability among diastereomers are, because there are no reports on the investigation of membrane permeability and metabolic stability of a comprehensive set of cyclic peptide diastereomers. Here, we synthesized all-64 diastereomers of a cyclic hexapeptide and evaluated the membrane permeability and metabolic stability of all the diastereomers to obtain a comprehensive understanding of the relationships between the backbone stereochemistry and pharmacokinetic properties (Fig. 1).

Lokey's peptide was chosen as a model cyclic peptide to evaluate the effect of the backbone stereochemistry of cyclic peptides on the pharmacokinetic properties. This peptide is a cyclic hexapeptide; thus, there are 64 diastereomers in total. Previous reports evaluated the membrane permeability of several diastereomers of the peptide[5,13] and another report explored highly permeable peptides from a library of diastereomers of the peptide.[14] There is also a report about the metabolic stability of two diastereomers of the cyclic hexapeptide.[11] However, there are no reports on the systematic evaluation of membrane permeability and metabolic stability of all the 64 diastereomers.

We decided to synthesize and evaluate all the 64 diastereomers (**CHP1–CHP64**) individually (Fig. 2). First, linear precursors of the diastereomers were synthesized using standard solid phase peptide synthesis and, after cleaving the compounds from the solid support, we cyclized them in solution and deprotected their side chains. The crude products were purified by HPLC.

To examine the correlation between the backbone stereochemistry and membrane permeability of the peptide, the membrane permeability of all the diastereomers was evaluated using a parallel artificial membrane permeability assay (PAMPA) (Fig. 3a). As a result, the diastereomers showed significantly different permeabilities, with the effective permeability (P_e) values ranged from 0.05 to 6.7 × 10^{-6} cm/s. This is probably because the stable conformations of peptides differ depending on backbone stereochemistry, and the stable conformations affect the physicochemical properties of peptides, such as lipophilicity and the number of solvent-exposed amide hydrogens which act as hydrogen bonding donors.[5,17]

To examine whether the permeability correlates with the experimentally-determined lipophilicity, we measured the 1,9-decadiene–water distribution coefficients (Log$D_{dec/w}$) that were previously shown to have correlations with MDCK-II permeability of cyclic peptide diastereomers.[13] The result showed the good linear correlation (R^2 = 0.6022) between LogP_e and Log$D_{dec/w}$ (Fig. S1a). We also found that the permeability correlates with the UPLC retention time, which is another indicator of compound lipophilicity (R^2 = 0.4751) (Fig. S1b). These results indicate the good correlation between lipophilicity and permeability, which agrees with the suggestion from a previous report.[18]

We next examined whether there are any conformational features characteristic for highly membrane permeable peptides. For this purpose, we predicted low-energy conformations of representative diastereomers in hexane using a dielectric constant of 1.9 which mimics a lipophilic environment and in water using a dielectric constant of 74.4 using molecular mechanics (MM) calculations with OpenEye Scientific's OMEGA program in macrocyclic mode.[19] First, we determined the lowest-energy conformation of **CHP9** in hexane. The conformation and intramolecular hydrogen bonding (IMHB) network are consistent with the previously reported stable conformation determined by NMR (Fig. S1c),[5] supporting the reliability of the MM calculations. Therefore, using the MM calculations, we predicted low-energy conformations of all the diastereomers in hexane and estimated average IMHB of those conformers. Previously, the number of IMHB in lipophilic media was suggested to be an important factor for determining membrane permeability of peptides.[5] However, we found only weak linear correlation (R^2 = 0.2922) between the number of IMHB and permeability in our dataset (Fig. S1d). Previously, Ono

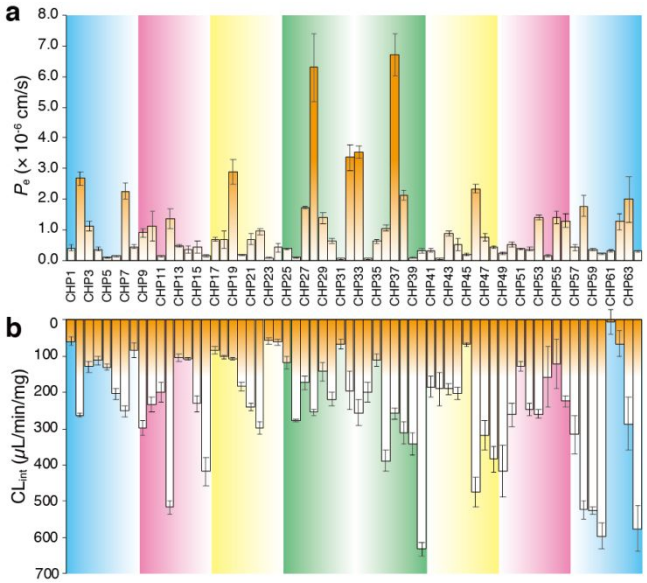

Fig. 3 Evaluation of pharmacokinetic properties of the 64 cyclic hexapeptide diastereomers. (a) Passive membrane permeability of the cyclic hexapeptides evaluated by PAMPA. (b) Metabolic stability of the cyclic peptides in rat liver microsomes. Cyclic hexapeptide diastereomers were arranged in the same manner with **Fig. 2**. Background of the bars are coloured to show enantiomer pairs. Each bar represents the mean value and the standard deviation from experiments carried out in triplicate.

and coworkers suggested that not only the exposed amide hydrogens in membrane but also the overlaps of the conformations in water and membrane are important for high membrane permeability.[6] Therefore, we calculated low-energy conformations of representative four diastereomers in hexane and water, and compared the conformations with each other. For this analysis, we chose **CHP19** and **CHP37** as peptides with high permeability and **CHP61** and **CHP64** as peptides with low membrane permeability (Fig. S2). As a result, **CHP19** and **CHP37** were found to stably form conformations **19-A** and **37-A**, respectively, which have 4 IMHB in hexane and the peptides were also found to form the same conformation in water as a major conformation. On the other hand, **CHP61** has a conformer **61-A** whose IMHB is 4 in membrane, but **61-A** was found to occupy only 8% of the conformations of **CHP61** in water. In the case of **CHP64**, conformer **64-A** is dominant in both membrane and water. However, the conformer **64-A** has three exposed amide bonds. The low permeability of **CHP64** is presumably due to a large desolvation energy required for the conformer **64-A** to cross membrane. The simulations suggest that the existence of a large number of IMHB conformers in membrane is important but insufficient for high permeability and the stable formation of the same conformers with a large number of IMHB in water is also important.

To examine the correlation between backbone stereochemistry and first-pass effects of the cyclic peptide diastereomers, the metabolic stability of all the 64 diastereomers in NADPH-supplied rat liver microsomes was evaluated. The intrinsic clearance (CL_{int}) values, which relate to the instability of compounds in microsomes, are calculated with the concentrations of intact peptides after 30 min incubation and shown in Fig. 3b. Microsomal stability was significantly different among the diastereomers. For example, **CHP61** exhibited high stability in that the peptide was not metabolized by metabolic enzymes in rat liver microsomes after 30 min incubation at 37°C. On the other hand, **CHP46** and **CHP60** almost completely disappeared after the same treatment. Interestingly, **CHP61** (cyclo[D-Pro–D-Tyr–Leu–D-Leu–D-Leu–D-Leu]) and **CHP64** (cyclo[D-Pro–D-Tyr–D-Leu–D-Leu–D-Leu–D-Leu]) have different stereochemistry only at a single residue, but their metabolic stabilities are significantly different from each other. In addition, mirror image pairs such as **CHP1** (cyclo[Pro–Tyr–Leu–Leu–Leu–Leu]) and **CHP64** (cyclo[D-Pro–D-Tyr–D-Leu–D-Leu–D-Leu–D-Leu]) showed significantly different clearance rates in rat liver microsomes, with CL_{int} values of 59 μL/min/mg and 576 μL/min/mg, respectively. As a control, we also measured the stability of selected 4 diastereomers (**CHP19**, **CHP37**, **CHP61** and **CHP64)** in rat liver microsomes without NADPH. All the compounds were found to be intact after 30 min incubation (Fig. S3). This result suggests that the degradation of the peptides observed in Fig. 3b is not from binding to microsomal proteins, but from oxidative modification by cytochrome P450 enzymes (CYPs). These results suggest that the conformations determined by backbone stereochemistry are important for recognition by metabolic enzymes in rat liver microsomes.

A previous paper suggests that the conformational flexibility plays an important role on metabolic stability.[20] To examine whether conformational flexibility is responsible for the metabolic stability in cyclic hexapeptide diastereomers, we calculated the conformations of 4 peptides with the lowest stability (**CHP40**, **CHP60**, **CHP64** and **CHP59**) and 4 peptides with the highest stability (**CHP24**, **CHP1**, **CHP23** and **CHP61**) in water using a dielectric constant of 74.4 by MM calculations. The 10 lowest energy conformers of each peptide were superposed to assess the conformational flexibility from the homogeneity/heterogeneity of the low energy conformers (Fig. S4). As a result, we did not find strong correlations between the conformational homogeneity/heterogeneity and metabolic stability in this data set. We also compared the metabolic stability with lipophilicity indicated from UPLC retention time and Log$D_{dec/w}$ (Fig. S5). However, we did not find any correlations between metabolic stability and the experimentally assessed lipophilicity.

We also evaluated the proteolytic stability of the peptide diastereomers to confirm that the cyclic peptides were proteolytically stable as generally considered (Fig. S6). As a result, none of the cyclic peptide diastereomers were found to be hydrolyzed after 1 h incubation in 50% rat serum, while a control linear peptide **LHP1** (H-Pro–Tyr–Leu–Leu–Leu–Leu-OH) was hydrolyzed and intact **LHP1** was not detected. This result demonstrates that all the cyclic hexapeptide diastereomers have high proteolytic stability. These results also suggests that the degradation of cyclic hexapeptides in rat liver microsomes is not mediated by proteolytic enzymes, but by other metabolic enzymes such as CYPs.

The results thus far show that cyclic peptide diastereomers exhibit different levels of permeability and metabolic stability. This suggests that both pharmacokinetic properties generally depend on the backbone conformations of the cyclic peptides. However, the side chains were unchanged during the study, and

it is unclear whether this trend is specific to the sequence of amino acid side chains.

To evaluate the dependence of the pharmacokinetic properties of cyclic peptides on side chain structures, we prepared cyclic hexapeptide libraries with randomized side chains and evaluated the membrane permeability and microsomal stability of the peptide libraries. Four diastereomers, **CHP37**, **CHP61**, **CHP19**, and **CHP64** were chosen for the evaluation based on the results so far: **CHP37** showed the highest passive membrane permeability with a P_e value of 6.7×10^{-6} cm/s; **CHP61** showed the highest microsomal stability with a CL_{int} value of 5 μL/min/mg; **CHP19** showed both high membrane permeability with a P_e value of 2.9×10^{-6} cm/s and high microsomal stability with a CL_{int} value of 108 μL/min/mg; and **CHP64** showed low membrane permeability with a P_e value of 0.3×10^{-6} cm/s and low microsomal stability with a CL_{int} value of 576 μL/min/mg. These four peptides were randomized at Leu-3, Leu-4, and Leu-5 using eight amino acids, including both aromatic and aliphatic amino acids (Fig. 4a). Pro-1 was not randomized because proline has a unique conformational preference that is different from the other 19 canonical amino acid residues, and substitution of the residue will largely change the backbone conformations of the peptides. Tyr-2 was also not randomized because it was used as a chromophore to determine the concentration of peptides and the number of hydrogen bonding donors will be changed by substituting the residue with other amino acid residues. The libraries were named CHP37L, CHP61L, CHP19L, and CHP64L, respectively, and were synthesized by the split and mix method.[21]

The membrane permeability of the peptides in the libraries was evaluated using PAMPA (Fig. 4b). The results showed that membrane permeability was dependent on the hydrophobicity of the side chains. On the other hand, focusing on the patterns of backbone stereochemistry, more peptides in CHP37L (56% of the peptides in the library) showed high membrane permeability, that is, P_e values of 1.0×10^{-6} cm/s or higher, than those in CHP64L (2% of the peptides in the library). This suggests that there is an optimal window of lipophilicity (ALogP) for high membrane permeability and inherent permeability determined by backbone stereochemistry modulates the window for good membrane permeability, which has been proposed by Lokey and coworkers using 18-membered libraries.[22] Our results using the larger 128-membered libraries support and strongly suggest the validity of the proposal.

Next, the microsomal stability was evaluated using the same libraries and plotted against the ALogP value (Fig. 4c). We incubated 16 library members each as a mixture with liver microsomes and measured the concentrations of intact peptides after 30 min incubation. The result indicated that, although the microsomal stability ranged broadly in each scaffold, more peptides in CHP61L (75% of the peptides in the library) had higher stability (80% or more intact peptide after 30 min of rat liver microsomal treatment) than peptides in CHP64L (50% of all the peptides in the library). In addition, microsomal stability linearly correlates with the hydrophobicity of the side chains in the range where ALogP is below 4. This is reasonable

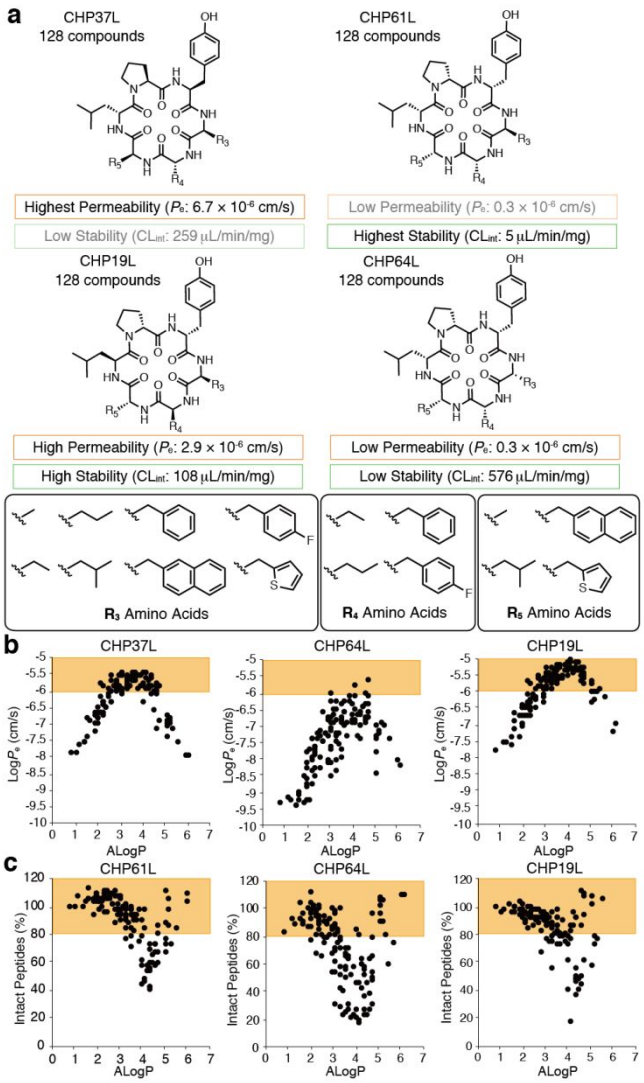

Fig. 4 Evaluation of pharmacokinetic properties of cyclic hexapeptide libraries with randomized side chains. (a) The design of the peptide libraries. Permeability and clearance rate in rat liver microsomes of the original cyclic hexapeptides are shown below the chemical structures. The results of (b) PAMPA and (c) rat liver microsomal stability assay. The obtained values (Log P_e and %intact peptide) are plotted against ALogP of each cyclic hexapeptides.

considering that metabolic enzymes in the liver modify hydrophobic compounds, thereby increasing the water solubility of the compounds to efficiently excrete them. On the other hand, some of the cyclic peptides with AlogP higher than 4 in each scaffold had high stability. This might be because peptides with high hydrophobicity escaped oxidation by CYPs via binding to other proteins in rat liver microsomes.

CHP19L is a library based on the cyclic peptide **CHP19**, which has both high membrane permeability and metabolic stability. 38% of the peptides in CHP19L showed both high membrane permeability (P_e value of 1.0×10^{-6} cm/s or higher) and metabolic stability (80% or more intact peptide after 30 min of rat liver microsomal treatment), whereas no peptides in CHP64L showed both high membrane permeability and metabolic stability. This result suggests that the backbone of a cyclic peptide with high membrane permeability and metabolic stability, such as **CHP19,** is potentially useful as a scaffold for orally bioavailable cyclic peptides.

In summary, we have obtained a comprehensive data set of membrane permeability and microsomal stability of 64 diastereomers of a cyclic hexapeptide. Regarding membrane permeability, lipophilicity and overlaps of stable conformations in membrane and in water were suggested to be important factors for high membrane permeability. On the other hand, we could not identify important factors that correlate with metabolic stability in this study. Further conformational analysis of the cyclic peptides is expected to provide more insights about structure-permeability/metabolic stability relationships in the future. The evaluation of membrane permeability and microsomal stability of cyclic peptide libraries with randomized side chains indicated that the trend of pharmacokinetic properties is determined by their backbone stereochemistry, although the hydrophobicity of the side chains affects their permeability and microsomal stability. We identified **CHP19** as a peptide with relatively high membrane permeability and high stability against metabolic enzymes in rat liver microsomes. In the future, investigation of peptides with different backbone structures such as different ring sizes, *N*-methyl amides, and esters, would lead to the discovery of other peptide scaffolds with high membrane permeability and microsomal stability, which would be useful as scaffolds for the development of orally bioavailable peptide-based drugs.

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Conflicts of interest

There are no conflicts to declare.

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