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Potent angiotensin-converting enzyme inhibitory tripeptides identified by a computer-based approach

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ABSTRACT

Currently, peptides and peptidomimetics are the main focus in attempts to identify inhibitors of angiotensin-converting enzyme (ACE), the dipeptidyl carboxypeptidase that causes blood vessels to constrict and blood pressure to increase. This study was conducted to identify the most potent ACE-inhibitory tripeptides with a proline C-terminus, using a novel three-step (tautomerization-docking-ADME simulation) virtual screening process and *in vitro* assays. Sixteen candidates were identified, and their IC_{50} values ranged from 5.6 to 274.4 μM. ACE inhibition activity for 14 of the 16 tripeptides was reported for the first time. We also found that changing from the L-form to the D-form of the amino acid at the amino and carboxyl termini resulted in a decrease of inhibition, but a greater decrease was observed for C-terminal changes. With low IC_{50} values and high-predicted bioavailability, the peptides identified by our protocol are comparable in terms of ACE-inhibition to those derived from costly and time-consuming wet screening. Our *in vitro* and docking results showed that the configuration of the C-terminus is a critical parameter contributing to the inhibitory activity of tripeptides with proline at this position. These findings will contribute to the use of simulation tools for rational drug design, especially for ACE inhibitors.

1. Introduction

One of the most commonly used antihypertensive therapies is the inhibition of angiotensin-converting enzyme (ACE), the dipeptidyl carboxypeptidase that causes blood vessels to constrict and blood pressure to increase [1]. Since the discovery of the first ACE peptide inhibitor (from a viper's venom), significant efforts have been made to develop ACE inhibitors by both synthetic and nature-derived approaches. The identification of ACE inhibitor candidates has involved a costly and time-consuming process of screening and activity testing. In this context, high-throughput screening (HTS), in which large libraries of synthetic and natural compounds are subjected to bioassays [2], seems to be a solution. However, the two drawbacks of the HTS approach are the low hit rates of active compounds and incomplete information about the biological target features [3].

Recently, the use of computers for evaluating the bioactivities of large compound libraries against a target structure, known as "virtual screening" [4], has been facilitated by the elucidation of protein structures by X-ray crystallography and the development of simulation software. In virtual screening, docking is a helpful tool for predicting the optimal pose and binding affinity of small molecules (ligands) when they bind to a target protein (receptor) to form a stable complex. Docking tools can also be used to identify potential candidates at a specified activity from a library of thousands of (or even more) compounds or to determine a lead structure by optimizing the potential candidates in the library [5].

In the field of ACE inhibition, peptides and peptidomimetics are the dominant structures, as most of the drugs currently used for ACE inhibition therapy are peptide-like compounds. Peptides have been reported with low oral bioavailability and intestinal absorption [6], but many studies (both *in vivo* and placebo-controlled trials) have shown the hypotensive effect of different peptides on rats and humans [7–13]. For hypotensive peptides, proline at the C-terminus of peptides was proven to be more potent than other naturally occurring amino acids [14]. The presence of proline and proline-like moieties in the structures of antihypertensive drugs and many ACE inhibitor peptides demonstrates the vital role of this moiety in ACE-inhibitory agents.

There is virtually no absorption for peptide sequences with more than four amino acids, whereas di- and tripeptides have been demonstrated to be actively transported by many oligopeptide

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transporters such as PepT1 and PepT2 [15]. The objective of the present study was to identify potent ACE inhibitor tripeptides with proline at the C-terminal position, using docking-guided experiments. Unlike previous virtual screening studies, which focused mostly on docking without considering the tautomers' form at the pH used in the assay (an important factor influencing screening results) or the bioavailability of the obtained candidates, we used a three-step screening process to identify potent peptides. The process includes compound tautomerization, virtual docking and ADME (absorption, distribution, metabolism, excretion) prediction. With this method, we sought to identify candidates with characteristics of a true drug with high potential for practical application.

2. Materials and methods

2.1. Materials

The protein structures were obtained from the Protein Data Bank (http://www.pdb.org), whereas the structures of peptides were created with the use of MarvinSketch (ChemAxon, Budapest, Hungary). ACE test kits were purchased from Dojindo (Dojindo Laboratories, Kumamoto, Japan), and synthetic peptides (>95% of purity) were obtained from CS Bio (Shanghai, China).

2.2. Comparison of the drugs' structures

We used four commonly prescribed anti-hypertensive drugs (enalaprilat, lisinopril, trandolaprilat and perindoprilat) for the comparison, and we used them to identify the most common parts of an ACE inhibitor and to test whether the proline moiety is an important part of ACE inhibitors.

2.3. Peptide-structures design and tautomer selection

We created 529 C-terminal proline-containing tripeptides (from the 20 standard amino acids and three phosphorylated amino acids, pTyr, pSer and pThr), and we optimized their 3D-structures and calculated the tautomer forms at pH 8.0 (the pH of the ACE inhibitory assay) using MarvinSketch. Only the two most dominant tautomers were selected and saved in a .mol2 format for further investigation.

2.4. Virtual screening

2.4.1. Template docking (ligand-based or pharmacophore)

To create the docking template, we used the lisinopril structure extracted from the ACE-lisinopril complex obtained from the Protein Data Bank (PDB code 1O86) [16]. We created the pharmacophore of the structure using the template-docking function of Molegro Virtual Docker 6.0 software – MVD (Molegro ApS, Aarhus, Denmark) in which the effective radius of each contributing factor was set at 1.8 Å and the charge threshold was set at 0.0; the default setting of other parameters were used. The binding site coordinates were the same as those of lisinopril ($x = 41.31$; $y = 33.82$; $z = 46.21$), and the docking was run inside a sphere with a 15-Å radius centered at the active site. Peptide structures were stored in separated folder and docking software was directed to take the structures from the folder for docking.

We set the overall strength of the template scoring at −500. For each docking, five runs were done with each peptide structure and a maximum of five poses were retuned for each run. Only one pose, which had a SimilarityScore lower (lower is better) than that of IPP (isoleucine-proline-proline, a well-known ACE inhibitory peptide from sour milk), was used for further investigation. SimilarityScore reflects a reward or penalty from similarity of structural and chemical features (steric, hydrogen donor, hydrogen acceptor, ring. . .) of ligands and the template's. Therefore, the score can, in part, reflect the drug-like properties of studied tripeptides.

2.4.2. Docking (structure-based)

To prepare for docking, we saved the protein structure downloaded from the Protein Data Bank in .pdb format. We chose ACE-lisinopril complex (PDB code 1O86) for the extraction of the protein structure used in the docking experiments, and we imported the extracted protein file into the docking program MVD 6.0 with the default setting of the software. Except for cofactor GLY_2000, all components of the complex (include crystalized water) subjected to the docking experiment were imported to the workspace of the MVD program without any structural modification. The docking space was restricted within a 15-Å radius sphere centered at the observed binding site of lisinopril (as mentioned above).

The docking was run with the MolDock SE docking algorithm [17] incorporated in the MVD program. We set the maximum iteration at 1500, the population size as 50, and the energy threshold at 100. For every structure, 50 runs were conducted, and five docking poses with the lowest MolDock score were retained. We then filtered the poses by RerankScore and used only the one pose with the lowest RerankScore lower than that of IPP for further investigation. The more computationally sophisticated RerankScore combines additional energy terms (such as steric self-interaction energy of the pose, protein steric interaction energy. . .), which are not used by the MolDock Score, generally producing better estimation of pose and interaction strength.

2.5. ADME properties evaluation

For our evaluation of some ADME properties, we imported SMILES strings of potential candidates to ACD/Percepta (Advanced Chemistry Development, Inc. Ontario, Canada). Lipinski's rule-of-five violations were also evaluated. Only structures with "moderate" or "good" agreement with Lipinski's rules and no significant first-pass metabolism (evaluated by Percepta) were selected and subjected to ACE inhibitor assays.

2.6. Angiotensin-converting enzyme inhibition assay

We performed inhibition activity assays using ACE kits in strict accordance with the manufacturer's instructions, using a 96-well plate. Principles of the method were described elsewhere by Lam et al. [18]. We calculated the inhibition rates based on the comparison of the optical absorbance of sample-treated wells (A_s), control wells (A_c) and blank wells (A_b) as shown in Eq. (1) below. Absorbance was measured at 450 nm using a microplate reader ELX800 (BioTek, Winooski, VT, USA).

$$\text{Inhibition rate}(\%) = \frac{A_c - A_s}{A_c - A_b} \times 100 \quad (1)$$

To determine the IC_{50} values (the half maximal (50%) inhibitory concentration), we assayed five concentrations of each peptide in triplicate against ACE and plotted dose-response curves for calculation of the values.

2.7. Stereochemical effect evaluation

To evaluate the stereochemical effect of peptides on the inhibitory activity, we selected the four most active peptides *in vitro* with IC_{50} values <20 μM (*i.e.*, WQP, WTP, IYP and WMP) and subjected them to the structural change. Only the middle residue of the tripeptides was kept original; the N-terminus and C-terminus were replaced by the D-form of the corresponding amino acids.

We then evaluated the modified peptides for ACE inhibition activity and subjected them to docking studies to clarify the effects of the configurational changes.

2.8. Statistical analysis

The *in vitro* experiments were performed in triplicate, and the results are expressed as the mean ± SD. The correlation coefficient between docking score and IC_{50} values was determined by a least-squares linear regression analysis using Microsoft Excel 2010.

3. Results and discussion

3.1. Structural comparison

Dozens of ACE inhibitors are currently used for hypertension medication, but we chose only four compounds for a comparison of their structural similarity and to identify the most common parts that play a role in ACE inhibitor activity. We did this for three reasons. First, the X-ray data of these compounds (in complex with ACE or an ACE homologue; 1UZE – enalaprilat, 1O86 – lisinopril, 2X93 – trandolaprilat and 2X94 – perindoprilat) have been deposited in the Brookhaven Protein Data Bank and could be accessed freely when needed. Second, these compounds have molecular weights similar to those of tripeptides, and their structures can be divided into parts corresponding to the three parts of tripeptides, the main target of this study. Third, these four compounds contain three structural changes that can be used to demonstrate the role of each part of tripeptides.

In Fig. 1, the common parts of all compounds are covered by a dashed line; all four compounds contain the proline moiety connecting to an alanine-like chain by a peptide bond. Changing these compounds' structures without touching the common parts retains the inhibitory activity, although small changes in IC_{50} values were reported [19]. The similarity in molecular weight and structure between proline-C-terminal tripeptides and these antihypertensive substances suggest the existence of peptides with high ACE inhibitor potency.

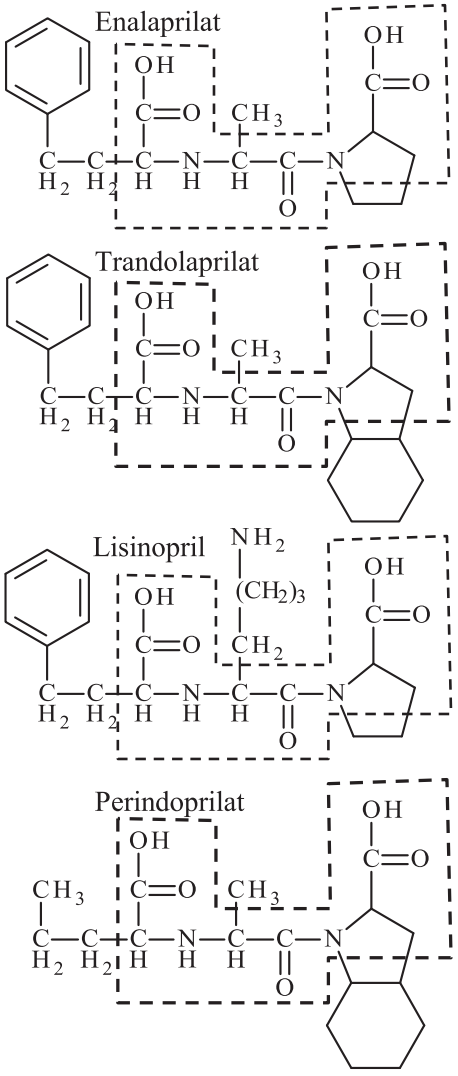

Fig. 1. Structure of selected antihypertensive drug and their common part.

3.2. Virtual screening

Virtual screening is being used more and more in drug design as a complement to industrial HTS. Virtual screening also provides academic institutions lacking HTS capacities applicable methods of hit identification [20]. Normally, either ligand-based or structure-based screening is chosen for a certain target bioactivity. Tan et al. [21] proposed that the integration of both techniques could give complementary and improved results for screening processes. We used this integrated approach in the present study. After docking by ligand-based and structure-based techniques (all 529 peptide structures were screened), we kept the structures with both a SimilarityScore and a RerankScore lower than those of IPP for further investigation. A total of 387 structures (see Supplementary Table S1) had scores lower than those of IPP.

The results are shown in Table 1; there are five groups in which all members (23 members in each group) have both a SimilarityScore and a RerankScore lower than those of IPP and another six groups that contain more than 20 members with these lower scores (for more details about docking scores, see Supplementary Table S1). Among these 11 groups, there are five groups whose members contain an amino acid with an electrically charged side chain at the penultimate position (*i.e.*, Xaa-Arg-Pro, Xaa-His-Pro, Xaa-Lys-Pro, Xaa-Glu-Pro and Xaa-Asp-Pro), three groups that have phosphorylated amino acids (pTyr, pSer and pThr) at this position, and three groups in which an aromatic ring-containing amino acid (Xaa-Phe-Pro, Xaa-Trp-Pro and Xaa-Tyr-Pro) occupies this position.

In their study of 308 food-derived small peptides, Wu et al. [22] proposed that for ACE inhibitor tripeptides, the most frequent residues for the C-terminus were ring-containing amino acids, whereas amino acids with a positively charged side chain were common in the middle position, and hydrophobic amino acids were

Table 1
Number of structures with both SimilarityScore and RerankScore lower than those of IPP.

No.	Tripeptide groups[a]	Common structures[b]	No.	Tripeptide groups	Common structures
1	Xaa-Ala-Pro	5	13	Xaa-Met-Pro	17
2	Xaa-Arg-Pro	21	14	Xaa-Phe-Pro	21
3	Xaa-Asn-Pro	14	15	Xaa-Pro-Pro	12
4	Xaa-Asp-Pro	20	16	Xaa-pSer-Pro	22
5	Xaa-Cys-Pro	6	17	Xaa-pThr-Pro	21
6	Xaa-Gln-Pro	17	18	Xaa-pTyr-Pro	23
7	Xaa-Glu-Pro	23	19	Xaa-Ser-Pro	9
8	Xaa-Gly-Pro	9	20	Xaa-Thr-Pro	15
9	Xaa-His-Pro	23	21	Xaa-Trp-Pro	22
10	Xaa-Ile-Pro	9	22	Xaa-Tyr-Pro	23
11	Xaa-Leu-Pro	18	23	Xaa-Val-Pro	14
12	Xaa-Lys-Pro	23	Total		387

[a] Xaa is any amino acid among 23 investigated ones.
[b] Structures with both SimilarityScore and RerankScore lower than those of IPP.

Table 2
ADME properties (calculated by Percepta) and ACE inhibition of selected peptides.

No.	Peptides	RerankScore	IC_{50} (μM)	Log *P*	Lipinski's[a]	OralBio[b]
1	Trp-Met-Pro	−166.92	16.6 ± 0.1	Optimal	Good	17.5551
2	Trp-Tyr-Pro	−160.04	39.4 ± 0.2	Optimal	Moderate	8.1285
3	Trp-Val-Pro	−157.15	30.3 ± 1.9	Optimal	Good	15.6372
4	Trp-Gln-Pro	−155.88	5.6 ± 0.1	Hydrophilic	Moderate	0.8309
5	Tyr-Lys-Pro	−154.97	178.3 ± 3.8	Hydrophilic	Moderate	2.2081
6	Trp-His-Pro	−154.86	71.1 ± 0.6	Hydrophilic	Moderate	2.5754
7	Trp-Lys-Pro	−153.19	55.3 ± 0.9	Optimal	Moderate	1.8899
8	Trp-Phe-Pro	−152.45	95.7 ± 2.5	Optimal	Good	36.0245
9	Trp-Thr-Pro	−151.90	12.8 ± 0.5	Hydrophilic	Moderate	2.3846
10	Tyr-Met-Pro	−151.63	142.0 ± 5.0	Hydrophilic	Good	6.9838
11	Tyr-Leu-Pro	−151.53	274.4 ± 8.2	Hydrophilic	Good	9.186
12	Tyr-Tyr-Pro	−149.95	231.7 ± 13.9	Hydrophilic	Moderate	3.947
13	Ile-Tyr-Pro	−148.98	15.8 ± 0.4	Hydrophilic	Good	11.0819
14	Tyr-Trp-Pro	−148.16	83.3 ± 1.6	Optimal	Moderate	10.6072
15	Phe-Met-Pro	−147.97	176.0 ± 2.6	Hydrophilic	Good	28.9321
16	Trp-Trp-Pro	−147.15	44.0 ± 0.4	Optimal	Moderate	13.5831
17	Ile-Pro-Pro	−123.93	5.0[c]	Hydrophilic	Good	25.1891

[a] In agreement with the Lipinski's rule-of-five.
[b] Oral bioavailability – remain amount after dosing 50 mg.
[c] from reference [25].

common for the N-terminus. In the present study, groups of tripeptides with electrically charged (both positive and negative) amino acids showed low docking scores for all members, which seems to be in agreement with the proposal by Wu et al. Our finding that phosphorylated amino acids at the penultimate position of peptides gave a low docking score (see Supplementary Table S1) is also consistent with a previous report about docking of the peptides [23].

3.3. ADME properties evaluation and ACE inhibition

To be more focused on the most potent candidates, we then ranked the structures obtained from the virtual screening step again by RerankScore, and 100 structures with lowest RerankScore were extracted (see Supplementary Table S2) and subjected to Percepta for an ADME properties evaluation. Only structures evaluated as in "moderate" and "good" agreement with Lipinski's rule-of-five [24] and no significant first-pass metabolism were retained and subjected to ACE inhibiton assays. Of the 100 selected peptides, only 16 structures satisfied these criteria and were used for *in vitro* experiments. These peptides were synthesized and tested for ACE inhibitor activity. The results are shown in Table 2.

Due to the differences in assay conditions used for evaluation of inhibitor activity, it is difficult to compare results between studies; however, assuming that IC_{50} values reflect the inhibitory strength, we can somewhat use the values for comparing the inhibitory capacity of samples across different studies. In this context, compared with many previous reports on ACE inhibitor peptides, these 16 peptides showed quite high inhibitory activity with IC_{50} values ranging from 5.6 to 274.4 μM. The highest inhibition was recorded for Trp-Gln-Pro and the lowest was for Tyr-Leu-Pro. We could not find any published work concerning ACE inhibitor activity on 14 of the peptides, with WHP and IYP being reported elsewhere [26,27]. Surprisingly, tripeptides with phosphorylated amino acids at the penultimate position were reported with low docking scores in previous step, but none were contained in the final 16 candidates. This may be because peptides containing phosphorylated amino acids have too many oxygen atoms in their structures and consequently, they violated Lipinski's rule-of-five and did not meet the criteria used for oral drugs. In addition to their high inhibition activity, all 16 peptides were predicted to be stable compounds with oral bioavailability ranging from 1.66% to 72% (or 0.83 to 36.0 mg after a virtual dose of 50 mg). These are quite high values compared to those of a report describing that nearly all orally active peptides have oral bioavailability lower than 5% [28]. Of course, these are the predicted values and the experimental results may be lower than predicted data, but the values still show the peptides to be potential candidates as viable ACE inhibitors.

From the results presented in Table 2, it can also be inferred that the correlation between RerankScores (average scores from two tautomers of each peptide) and IC_{50} values was approximately 35% (correlation coefficient *R* value = 0.35). This is an acceptable value in the drug discovery field, where HTS is still the main "player" but the hit rate is only 0.01–0.1% [29].

3.4. Stereochemical effects

As is the case for other protein enzymes composed of chiral amino acids residues, it is logical that ACE inhibition depends on the steric configuration of its inhibitor [30]. In the present study we investigated the effect of the form of amino acids on the inhibition activity of inhibitory peptides by changing the amino- and carboxyl-termini residues from L-form to D-form. The modified peptides were also subjected to an *in vitro* ACE inhibition assay and docking experiments.

As shown in Table 3, the change to D-form amino acids at both the amino and carboxyl termini resulted in a decrease of inhibition activities. The change at the N-terminus led to less of a decrease in activity compared to the change at the C-terminus. Changing the N-terminal residues to the D-form resulted in a decrease in inhibition activities by approx. 20–50 fold, whereas changing the proline residue of the C-terminus to the D-form resulted in a decrease of inhibition activities by approx. 70–200 fold. These results not

Table 3
ACE inhibition activity of modified peptides.

No.	Tripeptides	IC_{50} values (μM)
1	Trp-Gln-Pro	5.6 ± 0.1
2	Trp-Thr-Pro	12.8 ± 0.5
3	Ile-Tyr-Pro	15.8 ± 0.4
4	Trp-Met-Pro	16.6 ± 0.1
5	D-Trp-Gln-Pro	91.5 ± 1.8
6	D-Trp-Thr-Pro	169.9 ± 1.2
7	D-Ile-Tyr-Pro	723.0 ± 37.9
8	D-Trp-Met-Pro	110.0 ± 6.3
9	Trp-Gln-D-Pro	>1165
10	Trp-Thr-D-Pro	>1243
11	Ile-Tyr-D-Pro	>1278
12	Trp-Met-D-Pro	>1157

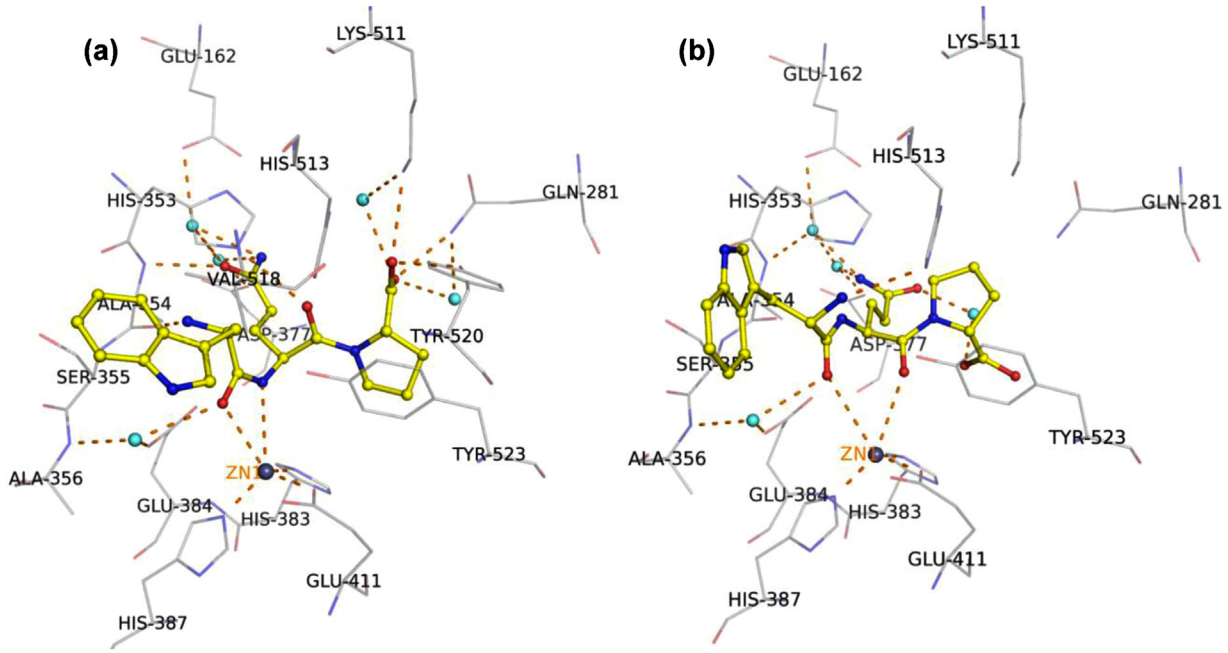

Fig. 2. Close view of interaction between tripeptides WQP with L-proline (a) and D-proline (b) at C-terminus and ACE; yellow skeletons are tripeptides, cyan spheres are water molecules, gray spheres are zinc ion; PyMOL 1.3 (The PyMOL molecular graphics system, Schrödinger, LLC) was used for creating the image while HBPLUS [31] was used to calculate the interaction.

only confirmed the critical role of proline at the C-terminus in ACE inhibitory peptides but also emphasized the significance of the L-form amino acid at this position.

Our docking studies of tripeptides with the D-form at the C-terminus also showed an increase in the RerankScore or a decrease in the binding strength (see Supplementary Table S3). Analysis of the docking poses using HBPLUS [31] revealed that a configurational change at this position resulted in a change of the peptide-ACE interaction, especially in the interactions of the carboxyl group. In the L-form, both of the oxygen atoms of proline's carboxyl group interact with residues of the protein, usually Lys511, Tyr520, His513 and His353; however, in the D-form, only one of these atoms had an interaction with the residues, and the number of interactions at this position was also decreased. Peptides with L-proline at the C-terminus also showed the same spatial distribution as that of lisinopril's L-proline moiety (see Supplementary Fig. S1; the image was rebuilt from PDB 1O86). Fig. 2 provides an example of tripeptides (with an L-form and D-form proline at the C-terminus) positioning inside the active site of ACE. Images of other peptides inside the active site are provided in Supplementary Fig. S2.

Surprisingly, the total number of interactions in the tripeptides with a D-form amino acid at the C-terminus was more than that of the tripeptides with an L-form (see Supplementary Tables S4 and S5), but the inhibition activity was much lower. This result lets us assume that the number of interactions is less important than the position of the interaction and also helps us to reconfirm the important role of configuration in the inhibitory activity of ACE inhibitor peptides.

4. Conclusion

In this study, we used three-step virtual screening as a guiding tool for wet chemistry in designing angiotensin-converting enzyme inhibitors. Sixteen peptides with "acceptable" Lipinski's rule-of-five violations were obtained, 14 of which have not been reported elsewhere, and all of them showed quite high inhibition activity against ACE compared with previously reported inhibitory peptides. Predicted to have no significant first-pass metabolism and high bioavailability, the peptides should be recognized as potent ACE inhibitors and investigated further *in vivo*. Our investigation of configurational changes also showed that the L-form of proline is essential for the inhibitory activity of tripeptides that ended with this amino acid. One question remains to be answered: why does only one small difference at the position next to P_1 (—CO—NH— in tripeptides and —(COOH)C—NH— in dicarboxylate-containing drugs) result in a major difference (approx. thousands of times) of inhibition strength? The detailed reason for this difference should be identified to determine the structure-activity relationship of potent ACE inhibitors.

Conflicts of interest

The authors declare no conflicts of interest and no financial conflicts in this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmgm.2014.08.002.

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