Structure-based design and optimization of antihypertensive peptides to obtain high inhibitory potency against both renin and angiotensin I-converting enzyme

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The human renin–angiotensin system (RAS) plays an essential role in regulating blood pressure and systemic vascular resistance. Renin and angiotensin I-converting enzyme (ACE) are two key enzymes in RAS and have long been recognized as attractive antihypertensive targets. Here, a synthetic strategy was proposed integrating quantitative structure–activity relationship (QSAR), molecular dynamics (MD) simulation and binding free energy analysis to discover novel dual renin and ACE peptidic inhibitors. With the strategy a number of candidates were generated virtually, from which eight promising peptides were selected and synthesized for biological assay. Consequently, three peptides (RYLP, YTAWVP and YRAWVL) were successfully identified to have satisfactory inhibitory profile against both renin and ACE with IC50 values of <1 mM and <10 μM, respectively. Structural analysis and energetic dissection revealed different binding modes of peptide to renin and ACE; a peptide only inserts its C-terminus into the active site of ACE, whereas the whole peptide packs tightly against renin. In addition, when limited to structural diversity it is hard to reconcile the renin and ACE inhibitory activities of short peptides such as dipeptides. These findings can be used to guide peptide optimization with improved biological activity.

Keywords: hypertension; cardiovascular disease; quantitative structure–activity relationship; peptide; renin; angiotensin I-converting enzyme

1. Introduction

Hypertension is one of the major risk factors for cardiovascular disease, with an impact on global health. In 2010, 27.6% of adults worldwide had hypertension. This proportion is projected to increase to ~30% by 2025, which will result in 1.56 billion people with hypertension globally [1]. Multiple studies have suggested that various dietary factors are associated with blood pressure and hypertension. Elevated blood pressure, particularly systolic blood pressure, has been linked to multiple adverse clinical outcomes including stroke, heart failure, myocardial infarction, renal insufficiency, peripheral vascular disease, retinopathy, dementia and premature mortality [2]. Due to the complex nature of hypertension, it is not surprising that single antihypertensive agents normalize blood pressure for less than a
majority of hypertensive patients. Combination antihypertensive therapy, consisting of agents from two or more different antihypertensive drug classes, not only increases the likelihood of achieving the target blood pressure goal, but also offers the potential for greater protection against target organ damage by targeting separate pathologic mechanisms [3]. Without thorough monitoring, however, various problems can arise from the combination therapy. In some cases, patients must try several different combinations of drugs before finding the best therapy to successfully treat a medical condition. The problem is complicated as both treatment and confounder can change over time and the confounder acts as an important mediating variable through which the treatment exerts its beneficial effect [4].

Instead of combination therapy, multi-target peptidic drugs have recently attracted considerable interest as potential alternative therapies for the treatment of hypertension. Small peptides derived from food sources or synthesized artificially have shown an effect on blood pressure in clinical trials. This biological mechanism is primarily due to inhibition of renin and angiotensin I-converting enzyme (ACE), thereby regulating blood pressure through the renin–angiotensin system (RAS) [5]. Renin stimulates the production of angiotensin I from angiotensinogen, a α2-globulin that is produced constitutively and released into the circulation mainly by the liver. Angiotensin I by itself is inactive. However, when acted upon by ACE it gets converted to angiotensin II, which is active and responsible for most of the presser effects [6]. Since the renin and ACE form rate-limiting steps in this cascade and angiotensinogen is their only known substrate, simultaneous inhibition of these two steps would be a promising antihypertensive strategy.

Here, we performed rational design and optimization of novel multi-target peptides to inhibit both renin and ACE by combining computational design [7] and experimental assay. In the protocol, a QSAR model was developed based on a variety of known ACE-inhibitory peptides, which were then used to monitor and design new peptide structures with promising properties. Potent peptides were further optimized to suppress renin using molecular dynamics (MD) simulations and binding free energy calculations. The optimization was monitored to ensure restoration of the ACE inhibitory capability of these peptides. Furthermore, the biological activities of newly designed peptides were also evaluated to solidify the results arising from theoretical investigations.

2. Materials and methods
2.1 Complex crystal structures
The co-crystallized complex structure of renin with angiotensinogen was retrieved from the Protein DataBank (PDB) database [8] (PDB: 2X0B) (Figure 1a). Here, only the minimal hexapeptide sequence (FHLVIH, referring to P3P2P1P1P2P3′) containing the N-terminal hydrolysed site of angiotensinogen was considered. This consists of a central split site LeuP1-ValP1′ capped by two residue diads PheP3-HisP2 and IleP2′-HisP3′ at each side of the site.

The complex structure of ACE with a snake venom peptide inhibitor BPPb (GLPPRPKIPP, referring to P10P9P8P7P6P5P4P3P2P1) was recently solved by Masuyer and colleagues using X-ray crystallography (PDB: 4APH) (Figure 1b) [9]. The inhibitor is a member of the large family of natural bradykinin-potentiating peptides; the family was first found to have antihypertensive properties.

The crystal structure data were utilized as templates to generate other peptide complexes with renin or ACE using a previously described strategy [10]. Briefly, the additional residues of co-crystallized peptide relative to target peptide were removed manually, and then the
remaining residues were mutated computationally to those in target peptide with the SCWRL method [11]. The virtual mutagenesis method can only change (mutate) the side chains of peptide, during which the backbone is fixed. Thus, the resulting protein–peptide complexes were refined by molecular mechanism (MM) optimization and equilibrated with molecular dynamics (MD) simulations. In addition, a number of pieces of experimental and theoretical evidence support the suggestion that peptide ligands prefer to adopt a consensus fashion binding to their common receptor. However, different peptides may have a significant difference in atomic details due to the variations in their side chains and local packing. The SCWRL method has previously been shown to be a powerful tool to accurately reproduce the position of the side-chain atoms from the backbone trace of proteins and peptides. For example, Knapp and colleagues have systematically compared the performance of five widely used methods in reproducing the side-chain atoms of peptide ligands determined by X-ray crystallography and found that only the SCWRL method can obtain a sub-angstrom level error for the predicted side-chain atoms [12].

2.2 Quantitative structure–activity relationship (QSAR)

A total of 289 ACE-inhibitory peptides (see Tables S1 in the supplementary material which is available via the multimedia link on the online article webpage) with known biological activity were retrieved from the ACEpepDB database (http://www.cfrti.com/pepdb/). The lengths of these peptides range from two to six amino acids; no peptides with seven or more amino acids were included in the set since previous studies had suggested that peptides up to six amino acids are sufficient to bind ACE tightly. These peptides are diverse in terms of the compositional and structural features they represent, with physicochemical properties of charged, polar, hydrophobic and aromatic moieties [13].

Here, the amino acid descriptor $z$-scale [14] was employed to characterize the sequence pattern of peptides. The $z$-scale consists of three components $z_1$, $z_2$ and $z_3$ representing electrostatic, steric and hydrophobic features, respectively (Table 1), which were derived from 29 physicochemical properties of the amino acids using principal component analysis (PCA). $z$-Scale has been widely used in the QSAR community to study diverse peptide properties such as activity, affinity, bitterness and stability [15]. When we employ $z$-scale to parameterize, for
example, a tripeptide LKS, each amino acid residue in the tripeptide can in turn be characterized by three $z$-scale components $z_1$, $z_2$, and $z_3$. In this way, the tripeptide is converted, as shown below, to a vector of $3 \times 3 = 9$ descriptors:

$$\begin{array}{c}
\text{L} \\
\downarrow \quad \downarrow \quad \downarrow \\
-4.19, & -1.03, & -0.98, & 2.84, & 1.41, & -3.14, & 1.96, & -1.63, & 0.57 \\
\text{K} \\
\downarrow \\
-1.39 \\
\text{S} \\
\downarrow \\
-1.40 \\
\end{array}$$

Since ACE inhibitory peptides adapt their C-terminus to dock into the ACE active site and the N-terminal residues of these peptides are just an elongation from the C-terminus, the investigated peptides with different lengths (2–6 amino acids) were aligned by C-terminus. Before employing $z$-scale to parameterize a peptide less than six amino acids long, the peptide was extended with several pseudo residues X at its N-terminus to make it as a pseudo hexapeptide (for example, tripeptide LKS was converted to a pseudo hexapeptide XXXLKS). When employing $z$-scale ($z_1$, $z_3$, and $z_3$) to parameterize the hexapeptide, a total of $6 \times 3 = 18$ descriptors is obtained, of which 1–3 represent the first residue in the peptide, 2–6 represent the second one, and so on. Instead of $z$-scale descriptors, each pseudo residue was characterized by three zero components in the parameterization.

The correlations between the structural variables and bioactivities of a panel of peptides can be modelled by partial least squares (PLS) regression [16]. PLS is a latent variable regression technique which has been widely used in peptide QSAR studies. Instead of finding hyperplanes of minimum variance between the response $Y$ (biological activities) and independent variables $X$ (structural variables), this method finds a linear regression model by projecting the predicted variables and the observable variables to a new space. The obtained models were validated by internal five-fold cross-validation and external blind validation.

Table 1. The amino acid descriptor $z$-scale [14].

| Amino acid | $z_1$ | $z_2$ | $z_3$ |
|------------|-------|-------|-------|
| Ala (A)    | 0.07  | -1.73 | 0.09  |
| Val (V)    | -2.69 | -2.53 | -1.29 |
| Leu (L)    | -4.19 | -1.03 | -0.98 |
| Ile (I)    | -4.44 | -1.68 | -1.03 |
| Pro (P)    | -1.22 | 0.88  | 2.23  |
| Phe (F)    | -4.92 | 1.30  | 0.45  |
| Trp (W)    | -4.75 | 3.65  | 0.85  |
| Met (M)    | -2.49 | -0.27 | -0.41 |
| Lys (K)    | 2.84  | 1.41  | -3.14 |
| Arg (R)    | 2.88  | 2.52  | -3.44 |
| His (H)    | 2.41  | 1.74  | 1.11  |
| Gly (G)    | 2.23  | -5.36 | 0.30  |
| Ser (S)    | 1.96  | -1.63 | 0.57  |
| Cys (C)    | 0.71  | -0.97 | 4.13  |
| Asn (N)    | 3.22  | 1.45  | 0.84  |
| Asp (D)    | 3.64  | 1.13  | 2.36  |
| Thr (T)    | 0.92  | -2.09 | -1.40 |
| Tyr (Y)    | -1.39 | 2.32  | 0.01  |
| Gln (Q)    | 2.18  | 0.53  | -1.14 |
| Glu (E)    | 3.08  | 0.39  | -0.07 |
2.3 MD simulation and MM/PBSA analysis

The complex system of a protein receptor (renin or ACE) with its peptide ligand was minimized and equilibrated by MD simulations [17]. The complex was solvated in a rectangular box full of TIP3P water molecules [18] and the boundary of the box is at least 10 Å away from the complex. Counter ions were placed based on the Columbic potential to keep the whole system neutral. The AMBER03 force field [19] was used to perform the MD simulations, during which the SHAKE algorithm [20] was employed to constrain hydrogen atoms.

After simulations, the molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) method [21] was utilized to estimate peptide binding free energy to renin or ACE. MM/PBSA analysed hundreds of snapshots extracted from the MD trajectories, and then calculated the nonbonded interaction energy, $\Delta E_{\text{int}}$, between protein and peptide using the MM approach and the solvent effect, $\Delta G_{\text{slv}}$, associated with the interaction by the PBSA model.

Consequently, the total binding energy of peptide to protein can be expressed as $\Delta G = \Delta E_{\text{int}} + \Delta G_{\text{slv}}$. Here, the sander and mm-pbsa modules in the AMBER package [22] were used to carry out MD simulations and MM/PBSA calculations, respectively. Although the entropy penalty may contribute unfavourably to protein–peptide binding, the thermodynamic effects associated with the penalty are still elusive and hard to characterize reliably. In addition, given that many of the peptide samples studied here share a similar binding mode towards their common protein receptor, the entropy change upon peptide binding could be regarded as a constant. Therefore, the contribution of entropy penalty to peptide binding free energy was not considered in this study.

2.4 Enzymatic inhibition assay

Renin inhibition assay was conducted by fluorescence spectrometry using the renin inhibitor screening assay kit as described previously [23]. Briefly, the assay mixture contained 10 μM of renin substrate, human recombinant renin and the peptide sample in 50 mM Tris-HCl buffer containing 100 mM NaCl. The renin substrate and peptide samples were mixed and pre-warmed to 37°C for 10 minutes prior to the assay before addition of renin to the mixture to initiate the reaction. The increase in fluorescence intensity was measured for 10 minutes at 37°C. The spectrofluorometer was set at an excitation wavelength of 340 nm, an emission wavelength of 490 nm, an excitation bandwidth of 5 nm and an emission bandwidth of 10 nm. The half maximal inhibitory concentration (IC50) value was expressed as the peptide concentration at which renin’s catalytic activity was reduced by 50%.

The ACE inhibitory potency of peptides was assayed using a method reported by Hernández-Ledesma and colleagues [24]. Briefly, 50 μL of each sample was added to 0.1 mL of 0.1 M sodium borate buffer containing 0.3 M NaCl and 5 mM substrate hippuryl-l-histidyl-l-leucine (HHL). ACE was then added and the reaction mixture was incubated at 37°C for 60 minutes. After centrifugation, 1 mL of the upper layer was transferred into a glass tube and evaporated at room temperature for 3 hours under vacuum. The hippuric acid was dissolved in 3.0 mL of distilled water. The absorbance was measured at 228 nm using spectrophotometer. The activity of each sample was tested for triplicate. The IC50 value was determined as the peptide concentration that is required to inhibit 50% of ACE’s catalytic activity.
3. Results and discussion

3.1 Comparison of peptide binding behaviours to renin and ACE

The complex crystal structures of renin and ACE with their cognate peptide ligands FHLVIH and GLPPRPKIPP, respectively, are shown in Figure 1. As can be seen, the hexapeptide fragment FHLVIH is deeply embedded within the catalytic pocket of renin, forming a tightly packed interface between the enzyme and peptide, while the BPPb inhibitor GLPPRPKIPP locates in a long cleft on ACE surface and adapts its C-terminus to interact with the enzymatic active site.

Subsequently, nonbonded interactions across the complex interfaces of renin and ACE with their peptide ligands were detected and plotted using the LIGPLOT program [25]. It is evident from Figure 2 that intensive nonbonded networks of hydrogen bonds, hydrophobic forces and van der Waals contacts can be found at the interfaces [26], conferring substantial stability and specificity for complex architectures. The central residues His\textsuperscript{P2}, Leu\textsuperscript{P1} and Ile\textsuperscript{P1′} of FHLVIH define three hydrogen bonds with renin, and strong hydrophobic forces and van der Waals contacts can be observed at the N-terminus of the peptide (Figure 2a). However, a number of hydrogen bonds and salt bridges are formed between the C-terminus of the BPPb peptide and the active site residues Ser\textsuperscript{516}, Ser\textsuperscript{517}, Lys\textsuperscript{511}, His\textsuperscript{513}, Tyr\textsuperscript{520} and Tyr\textsuperscript{523} of ACE. In addition, efficient hydrophobic interactions are also found around these regions (Figure 2b).

Next, the relative contribution of different residues to peptide recognition by renin and ACE was examined using computational alanine scanning [27]. In the scanning, peptide residues were in turn mutated to alanine and then the binding free energy changes (\(\Delta\Delta G\)) upon the mutation were calculated using MD simulation and MM/PBSA analysis. The \(\Delta\Delta G\) profiles of two peptides FHLVIH and GLPPRPKIPP are shown in Figure 3. All the six residues in FHLVIH have considerable or moderate effects on peptide affinity (\(\Delta\Delta G > 0.5\) kcal/M), suggesting a tight interaction between the whole peptide ligand and renin receptor. In contrast,
only the C-terminal residues (e.g. P1, P2, P3 and P4) of GLPPRPKIPP play an important role in peptide binding to ACE ($\Delta\Delta G > 1$ kcal/M), whereas most N-terminal residues (e.g. P7, P8 and P9) appear to contribute modestly to the binding ($\Delta\Delta G < 0.5$ kcal/M). This is expected given that ACE is a dipeptidyl carboxypeptidase that recognizes and binds the C-terminus of substrate angiotensin I. Previous studies found that the C-terminal six residues of peptide inhibitors are sufficient to determine ACE–peptide recognition and interaction [12]. Thus, the significant difference of peptide binding modes to renin and ACE can be used to guide the structure optimization of peptide inhibitors with improved inhibitory activity against both the enzymes.

The conformational variation may play an important role in the binding of highly flexible peptides to renin and ACE receptors; this, is induced by diverse intermolecular forces such as electrostatic interaction, van der Waals contact, hydrophobic force, hydrogen bonding, entropy penalty and conformational strain energy. Here, electrostatic interaction, van der Waals contact, hydrophobic force and hydrogen bond have been investigated explicitly, while entropy penalty and conformational strain energy were ignored in this study. The entropy penalty is discussed above. For the strain energy: (i) there are no available methods that can be used to efficiently, reliably compute conformational straining upon protein–peptide binding, (ii) a series of peptide ligands are usually homologous, possessing similar sequence pattern and binding mode, and thus their strain energy upon the binding could be regarded as a constant, and (iii) peptides are highly flexible and thus only a limited conformational effect can be imposed with their binding to protein receptors.

3.2 QSAR modelling of ACE-inhibitory peptides

Amino acid descriptors characterize the relative importance of different amino acids at the different positions of a peptide. However, this, cannot be used to estimate the absolute value of peptide affinity directly given that the amino acids at different positions in a peptide sequence may not contribute equally to peptide binding and some additional factors that were not included in the descriptors, such as interactive effect and flexibility, would also play an important role in the binding. Therefore, QSAR modelling was used to establish the statistical correlation between the descriptors and peptide activity.

Figure 3. Binding free energy change profiles of peptides FHLVIH (a) and GLPPRPKIPP (b) upon the mutation of peptide residues to alanine.
An n-mer peptide of ACE binder was characterized in terms of its sequence pattern using the information encoded in z-scale descriptors; if \( n < 6 \), then the \( 6 - n \) missing N-terminal residues were characterized by zero vectors. Subsequently, we employed PLS regression [16] to correlate the obtained descriptors with experimentally measured activities of 289 ACE inhibitory peptides. It is worth noting that all the 289 samples are active peptides and that no random controls are used in the development of QSAR predictor. Previously, a number of studies suggested that, if a regression model is trained only by active samples, this model, from a statistical point of view, would predict the inactive ones to have low activity but not inactivity [28,29]. This is acceptable given that our goal is to discover high-activity peptides, and thus low-activity samples suggested by the predictor would no longer be considered in a subsequent inhibition assay.

Model validation is essential for a predictive QSAR model; Golbraikh and Tropsha pointed out that the high cross-validation \( q^2 \) appears to be the necessary but not the sufficient condition for the model to have a high predictive power, and that external validation is the only way to establish a reliable QSAR model [30]. Thus, in addition to traditional cross-validation, external test set validation was also carried out in this study. The 289 peptide samples were split randomly into a calibration set consisting of 200 peptides and a test set containing the other 89 peptides; the calibration set was utilized to build the QSAR model, while the test set was used to blindly validate the built model.

The best number of latent variables of the regression was determined by five-fold cross-validation, that is, the latent variables were added step-by-step to the PLS regression model and cross-validation was performed for each step to obtain coefficient of determination \( q^2 \). The number of latent variables at which the \( q^2 \) achieved the maximum was considered the best and used to develop the predictive model. With this strategy, three latent variables were determined in the final model, where regression fitting \( r^2 \) and cross-validation \( q^2 \) are 0.767 and 0.694, respectively (Figure 4). Furthermore, the model was employed to perform

Figure 4. Plot of regression fitting \( r^2 \) and cross-validation \( q^2 \) against the number of latent variables used in the PLS model. The model with three latent variables achieves a maximal \( q^2 = 0.694 \).
prediction on the 89 test peptides and a good generalization ability can also be found with predictive \( p^2 = 0.652 \).

The scatter plots of experimental ACE inhibitory activities and calculated values for calibration set and test set are shown in Figure 5. It is evident that all sample points are distributed evenly around the slope line fitted through them and no obvious outliers can be found in the plots. In addition, a very similar profile of sample points distributed over the calibration and test spaces is observed, indicating that the QSAR modelling performance is consistent in both internal fitting and external prediction. Previously, Ren and colleagues systematically characterized the single position mutation energy profile (SPMEP) of peptide binding to ACE and developed two structure-based QSAR predictors by using linear PLS and nonlinear SVM separately [13].

Here, the statistics obtained from different QSAR modelling are tabulated in Table 2. As can be seen, although the two SPMEP predictors were obtained by exploiting the information derived from ACE–peptide complex structures and one of them was built using the nonlinear SVM method, they have only a moderate performance in internal fitting, cross-validation and external prediction compared with the sequence-based linear model established in the current work. Thus, it is suggested that our model would be a good choice to carry out extrapolation on the peptides that the model has never been seen; it could be used in the next study to help guide the structural optimization of renin inhibitory peptides with maintaining potent ACE-inhibitory potency.

The PLS method employed a weighting strategy to measure the relative importance of different residues in a peptide, which can be converted to variable importance in the projection (VIP) [16]. As can be seen in Figure 6, the C-terminal residues such as P1, P2 and P3 seem to contribute significantly to peptide binding, with their VIP values ranked at the top of the 18 variables in the PLS model. This is in line with the important role played by the C-terminus in ACE inhibitory peptides as revealed above by the structural and energetic analysis of the

![Figure 5. Plot of calculated ACE inhibitory activities (pIC\textsubscript{50}) against experimental values for the 200 calibration peptides and 89 test peptides.](image-url)
ACE–peptide complex. In particular, the hydrophobic variables in P1, P2 and P3 residue positions exhibit a very significant effect on peptide binding and the steric variable in P1 position also contributes considerably to the binding, suggesting that the presence of bulky, nonpolar amino acids in the C-terminus would improve peptide affinity to ACE.

3.3 Optimization of dual ACE and renin peptidic inhibitors

In order to obtain peptides with potent inhibition against both ACE and renin, we employed a structure-based strategy to optimize the renin inhibitory peptides and, during the procedure, these peptides were monitored in a real-time manner using the QSAR model built above to maintain their ACE inhibitory capability. Considering the symmetry of renin-hydrolysed substrate, only dipeptides (referring to P1P1'), tetrapeptides (referring to P2P1P1'P2') and hexapeptides (referring to P3P2P1P1'P2'P3') were considered here as renin inhibitors. The optimization started from the renin hexapeptide substrate FHLVIH, which can be further reduced to dipeptide LV and tetrapeptide HLVI. Subsequently, systematic single point mutation profiles were generated separately for the dipeptide, tetrapeptide and hexapeptide based

| Method             | Class          | Regression | $r^2$  | $q^2$  | $p^2$  |
|--------------------|----------------|------------|--------|--------|--------|
| SPMFEP [13]        | Structure-based| PLS        | 0.667  | 0.534  | 0.510  |
| SPMFEP [13]        | Structure-based| SVM        | 0.725  | 0.653  | 0.632  |
| z-scale (the current work) | Sequence-based| PLS        | 0.767  | 0.694  | 0.652  |

Table 2. Comparison of statistics obtained from different QSAR modelling.

![Figure 6. Variable importance in the projection (VIP) in the PLS model. A variable in the model is expressed as the $n$-X form, where $n$ represents the residue position $P_n$ in the hexapeptide that the variable belongs to and the X is the variable type, including H – hydrophobic, S – steric and E – electrostatic.](image-url)
on their complex structures with renin, where each residue of the three peptides was in turn mutated to other 19 types of amino acid using the SCWRL method [11], followed by an MD simulation procedure to relax the mutated complex systems. Finally, a total of 38 \((2 \times 19)\), 76 \((4 \times 19)\) and 152 \((6 \times 19)\) mutants as well as three unmutated peptides were obtained for the dipeptide, tetrapeptide and hexapeptide systems, respectively. Based on MM/PBSA analysis of renin complex structures with the three unmutated peptides and their corresponding mutants, we defined three matrices of \(2 \times 19\), \(4 \times 19\) and \(6 \times 19\) sizes to describe the single position mutation free energy profiles of peptide interaction with renin; element \(m(i,j)\) of a matrix represents the renin–peptide binding free energy change \(\Delta \Delta G\) upon mutation of native residue to amino acid type \(j\) at the \(i\) position of peptide \((i = P1\) and \(P1’\) for dipeptide, \(P2\), \(P1\), \(P1’\) and \(P2’\) for tetrapeptide, and \(P3\), \(P2\), \(P1\), \(P1’\), \(P2’\) and \(P3’\) for hexapeptide; \(j = 1, 2, 3 \ldots 19\)).

From the three matrices we can obtain the favourable amino acid types \((\Delta \Delta G < -1 \text{ kcal/M})\) for each position of the three peptides. As can be seen in Figure 7, the dipeptide, tetrapeptide and hexapeptide exhibit a similar favourable amino acid profile, that is, most positions \((P1, P1’, P2’\) and \(P3’\)) prefer to hydrophobic and/or bulky amino acids such as L, A, P, V and W, while the N-terminus \((P3\) and \(P2\)) of the peptide tends to hold polar or charged residues such as Q, R and Y. The combination of these favourable amino acids would result in four dipeptides, 12 tetrapeptides and 48 hexapeptides, considered promising renin inhibitors; their potencies \((\text{pIC}_{50}\) values) against ACE were predicted using the QSAR model and plotted in Figure 8.

It is evident that variations of the activity distributions of hexapeptides and tetrapeptides are significantly larger than that of dipeptides, although their mean values are basically consistent. This is expected because longer peptides should possess higher structure diversity and thus exhibit more variable biological activity. Therefore, we selected one dipeptide, two hexapeptides and five hexapeptides from the 64 promising renin inhibitor candidates to perform the biological assay. The one dipeptide and two hexapeptides were predicted to have \(\text{pIC}_{50} > 5\) \((\text{IC}_{50} < 10 \mu\text{M})\), and the five hexapeptides were predicted to have \(\text{pIC}_{50} > 5.5\) \((\text{IC}_{50} < 3.2 \mu\text{M})\), against ACE.

Next, the selected one dipeptide, two hexapeptides and five hexapeptides were synthesized and purified, and their inhibitory potencies against both renin and ACE were tested using the protocol described in the Methods section. The results obtained are tabulated in Table 3. The difference between QSAR predicted and experimentally measured activities is significant; this could be that some additional factors such as flexibility and entropy were not included in the

![Figure 7](image_url)  
Figure 7. Favourable amino acids at each position of renin inhibitory peptides; their combinations would result in four dipeptides, 12 tetrapeptides and 48 hexapeptides.
predictor which may lead to a systematic bias for the predicted results. However, there is a moderate correlation between the predicted and experimental activities with a Pearson correlation coefficient $R_p = 0.558$. Therefore, we think the predictor could be used, at least for qualitative purposes, to rank the peptide candidates. In addition, the inhibitory potencies of these tested peptides on renin and ACE are not consistent; the former and latter are at millimolar and micromolar levels, respectively, suggesting that peptides may serve as good inhibitors of ACE but moderate binders to renin. This is in line with a previous report that natural peptides can only exhibit moderate inhibition on renin but possess higher activity for ACE [31].

In addition, we also found that longer peptides generally possess a higher inhibition potency to renin, but the peptide length seems to have no significant correlation with ACE

| Peptide     | Renin inhibition | ACE inhibition |
|-------------|------------------|---------------|
|             | MM/PBSA-derived binding free energy $\Delta G$ (kcal/mol) | Experimental $IC_{50}$ (mM) | QSAR-predicted $IC_{50}$ (μM) | Experimental $IC_{50}$ (μM) |
| IF          | -8.6             | 8.46 ± 0.74   | 8.6 | 27.8 ± 4.2 |
| RAWP        | -14.7            | 0.38 ± 0.02   | 7.4 | 28.3 ± 4.8 |
| RYLP        | -13.2            | 0.54 ± 0.04   | 2.3 | 9.4 ± 1.7  |
| YTAWVP      | -18.3            | 0.098 ± 0.007 | 0.65 | 8.7 ± 1.2 |
| YTWMAP      | -15.0            | 0.13 ± 0.009  | 2.7 | 54.2 ± 8.5 |
| YRAWVL      | -19.6            | 0.056 ± 0.004 | 1.2 | 2.6 ± 0.4 |
| YNAWAP      | -17.4            | 0.072 ± 0.005 | 1.8 | 38.0 ± 5.5 |
| WRAWAP      | -14.9            | 0.21 ± 0.01   | 2.4 | n.d.$^a$  |

Table 3 Characteristics of the one dipeptide, two hexapeptides and five hexapeptides selected to perform the *in vitro* assay.

Figure 8. Distribution of the predicted pIC$_{50}$ values of 4, 12 and 48 renin inhibitory dipeptides, tetrapeptides and hexapeptides, respectively.

$^a$n.d. not determined.
inhibition. This is expected given that longer peptides can bind to renin more efficiently due to the tightly packed interface between renin’s active pocket and the whole peptide ligand, whereas peptides only adapt their C-terminus to interact directly with ACE and addition of residues in the peptide N-terminus would thus make a limited contribution to activity improvement. Furthermore, there is a significant difference between the absolute values of predicted and experimental ACE-inhibitory activities (Table 3). This is acceptable given that some additional factors such as peptide flexibility and entropy effect were not included in the QSAR model, possibly leading to a systematic bias for predicted results. However, a moderate correlation between the predicted and experimental values can be observed for the designed peptides with Pearson’s correlation coefficient $R_p = 0.558$ (Figure 9), suggesting that the model can be used at least for qualitative purposes to rank the relative activities of peptide candidates.

Here, we selected three peptides, i.e. RYLP, YTAWVP and YRAWVL, as potent dual inhibitors of renin and ACE as they exhibit a satisfactory inhibition profile against both enzymes (i.e. IC$_{50}$ <1 mM for renin and <10 μM for ACE). It is seen that the three peptides have common structure features, that is, the peptides are mainly composed of hydrophobic and/or bulky amino acids, while only a few polar or charged residues such as R, T and N are located at the peptide N-terminus. Such a physicochemical profile can be explained by the fact that hydrophobic forces and van der Waals contacts are primarily responsible for peptide binding to renin and ACE, but some N-terminal polar residues may constitute additional polar interactions such electrostatic attractions and hydrogen bonds between renin and peptide; the additional polar residues at the peptide N-terminus do not have a substantial influence on ACE inhibition due to the primary interactions that occur at the peptide C-terminus with ACE.

![Figure 9. Plot of predicted ACE-inhibitory activities (pIC$_{50}$) against experimental values for the designed peptides.](image-url)
4. Conclusions
A computational protocol that combined QSAR modelling, MD simulation and MM/PBSA analysis is described to design antihypertensive peptides with high inhibitory activity against both renin and ACE. With the protocol we were able to generate a number of promising candidates, from which several peptides were confirmed in vitro as good dual renin and ACE inhibitors. We conclude with the following remarks.

(i) The peptides RYP, YTAWVP and YRAWVL were successfully designed to have a satisfactory inhibitory profile against both renin and ACE with IC$_{50}$ at micromolar and nanomolar levels, respectively.

(ii) Peptides bind to renin and ACE in different modes, that is, a peptide only inserts its C-terminus into the active site of ACE, whereas the whole peptide packs tightly against renin.

(iii) When limited to structural diversity, it is hard to reconcile the renin and ACE inhibitory activities of short peptides such as dipeptides; longer peptides such as tetrapeptides and pentapeptides could serve as good candidates to develop antihypertensive agents with high potency on both renin and ACE.

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