



# Citrullyl-Hydroxyprolyl-Proline (ChPP): An Artificially Synthesized Tripeptide as Potent ACE Inhibitor

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## Abstract

Currently bioactive peptides are the main focus in attempts to identify novel angiotensin-converting enzyme inhibitors (ACEIs) for the treatment of hypertension due to their fewer side effects. In the present study we aimed to investigate the antihypertensive activity of 4 synthetic tripeptides Ornithyl-Hydroxyprolyl-Proline (Orn-Hyp-Pro OhPP) Ornithyl-Prolyl-Hydroxyproline (Orn-Pro-Hyp OPhP) Citrullyl-Hydroxyprolyl-Proline (Cit-Hyp-Pro ChPP) and Citrullyl-Prolyl-Hydroxyproline (Cit-Pro-Hyp CPhP) in vitro and in vivo. The ACE inhibitory activity and mode were analyzed with a modified spectrophotometric method and Lineweaver-Burk plots respectively. It showed that peptide Citn-Hyp-Pro (ChPP) exhibited the highest inhibition potency with an  $IC_{50}$  value of 40.48  $\mu$ M and displayed a competitive inhibition of ACE. Molecular docking simulations suggested that ChPP could form several critical hydrogen bonds with the major residues His353 and His513 located in the S2 active site of ACE. The systolic blood pressure of spontaneously hypertensive rats monitored by the tail-cuff method was significantly reduced by 15.54% at 4 h after oral administration of 20 mg/kg BW ChPP. Also ChPP remarkably downregulated the angiotensin II receptor type 1 (*agtr1*) and miR-132/-212 levels in SHR which was similar to that observed in SHR treated with captopril. It showed us that the tripeptide ChPP could be explored as a promising ACE inhibitor (ACEI) for treatment of hypertension.

**Keywords** Peptide design · Tripeptide ChPP · Molecular docking · Antihypertensive activity · Transcriptional regulation · *agtr1* · miR-132-212

## Introduction

Hypertension (HT), characterized by elevated systemic blood pressure (BP), is one of the most common chronic diseases that affects over one billion people worldwide (Mills et al. 2016). It is a major risk factor for cardiovascular disease (CVD). Angiotensin-I converting enzyme (ACE EC3.4.15.1), a zinc-containing dipeptidase, plays an important role in the regulation of BP by catalyzing the

cleavage of angiotensin-I (Ang I) to the potent vasopressor angiotensin-II (Ang II) (Hayes et al. 2016) and the degradation of the vasodilator bradykinin (Regoli and Gobeil 2015). Consequently, ACE is considered as a therapeutic target for the treatment of HT.

Apart from the direct vasoconstriction, Ang II is also an elicitor for expression of some genes and miRNAs associated with HT development. Ang II induced mRNA expression of its receptor AT1R, which is a G-coupled receptor mediating many cardiac functions (Vamos et al. 2014), and expression of miR-132/212 was up-regulated in aortas from Ang II-infused mice and in Ang II-induced HT patients (Jin et al. 2012; Eskildsen et al. 2013), suggested they might play an important role in Ang II-induced hypertension.

Many potent synthetic ACE inhibitors (ACEIs) are currently used for the clinical management of HT, such as captopril, enalapril, and perindopril (Ling et al. 2018). Despite their established effectiveness, therapies with these drugs are associated with some clinically undesirable side effects, such as dry cough, skin rash, angioedema, and renal impairment

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(Yu et al. 2018), necessitating the need to develop new ACEIs with higher efficacy and fewer side effects.

Accordingly, bioactive peptides with ACE-inhibitory activities have attracted more and more attention due to their low toxicity. An increasing number of ACE-inhibitory peptides, either naturally derived (Lee and Hur 2017; Miralles et al. 2018) or artificially synthetic (Panyayai et al. 2018), have been identified. Among them, proline (Pro) residue is preferred at C-terminal of a potent ACE inhibitors and confers resistance to degradation by digestive enzymes (Miralles et al. 2018; Jao et al. 2012), and hydroxyproline (Hyp) in peptides, especially at the penultimate position, played an important role in interacting effectively with the active pocket of ACE (Saiga et al. 2003; Taga et al. 2018). In addition, the activity of ACE-inhibitory peptides is also related to their molecular mass, and small peptides with 2–12 amino acids long and molecular weights less than 3000 Da are considered to better fit the active sites of ACE (Sun et al. 2019; Toopcham et al. 2017). Several studies also reported that peptides having a hydrophobic amino acid at the N-terminus possess potent ACE inhibitory activity, and a basic amino acid at the N-terminus also can enhance the affinity of the peptide for ACE further increasing antihypertensive activity (Lee and Hur 2017; Sun et al. 2019; Toopcham et al. 2017).

Besides 20 standard amino acids, there are many non-standard amino acids, among which citrulline (L-Cit) has no net charge and increases the hydrophobicity of protein with its content increase, and ornithine (L-Orn) is a basic amino acid. They all possess antioxidant activity and vascular protective properties (Allerton et al. 2018; Coles 2007; Butterworth and Canbay 2019), which might be beneficial in reducing side effects (Sun et al. 2017). Therefore, in this study, based on these ideas above, four tripeptides containing an N-terminal L-Cit or L-Orn, with Pro-Hyp or Hyp-Pro dipeptide motifs at the C-terminus were designed. Then, their ACE inhibitory activity, inhibition mode, antihypertensive effects, and interaction with ACE were analyzed. The results suggested that ChPP is a potent candidate for hypertension prevention.

## Materials and Methods

### Materials

The tripeptides Ornithyl-Hydroxypropyl-Proline (Orn-Hyp-Pro, OhPP), Ornithyl-Prolyl-Hydroxyproline (Orn-Pro-Hyp, OPhP), Citrullyl-Hydroxypropyl-Proline (Cit-Hyp-Pro, ChPP), and Citrullyl-Prolyl-Hydroxyproline (Cit-Pro-Hyp, CPhP) were synthesized and provided by GL Biochem (Shanghai) Ltd (Shanghai, China) with purity > 95%. Hippuryl-L-histidyl-L-leucine (HHL) and Hippuric acid (HA) were purchased from Sigma Chemical Co. (Louis, USA). All

chemicals used were chemically pure. Spontaneously hypertensive rats (SHRs) were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

### Ethics Statement

All animal studies were performed in conformance with the Guide for the Care and Use of Laboratory Animals of the Jiangsu Vocational College of Medicine and approved by the Committee on the Ethics of Animal Experiments of the Jiangsu Vocational College of Medicine (Permit Number: 20,178,102).

### ACE inhibition Assay

Prior to ACE inhibition assay, the ACE extracts from rabbit lung were prepared and its activity was determined according to our previous study (Qian et al. 2019). Subsequently, the tripeptides OhPP, OphP, ChPP, and CPhP were dissolved in 100 mmol/l borate buffered saline (BBS) containing 10% Triton X-100 and 0.1% DMSO as cosolvents at pH of 8.3. ACE inhibitory activity was assayed according to the procedure (listed in Table 1) described by Gao et al. (2011). After reaction stopped by HCl, 1.5 ml of ethyl acetate was added and mixed with a vigorous shaking for 1 min followed by a centrifugation at 3000 rpm for 5 min. Point five milliliter of the upper lipid phase was transferred into a new tube, and 2.5 ml of 0.5% diaminobenzidine (DAB) was added in and mixed well by vortex. Afterwards the tubes were heated to 40 °C and placed for 30 min in dark for chromogenic reaction between HA and DAB. Finally optical density value of the reacts were detected at 456 nm using a spectrophotometer (UV-160A, Shimadzu Corporation, Kyoto, Japan). Mixture of 0.5 ml of ethyl acetate and 2.5 ml of 0.5% DAB was used as a blank to eliminate background noise. Each assay was finished with triplicates.

ACE inhibition rate (%) was calculated according to Eq. 1. The IC<sub>50</sub> value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

**Table 1** Procedures of ACE inhibition assay

	Control (μl)	Sample (μl)	Blank (μl)
HHL(5 mmol/l)	100	100	100
Inhibitor	0	100	100
HCl (1 mol/l)	0	0	250
BBS	25	25	25
ACE	25	25	25
Incubation at 37°C for 1 h			
HCl	250	250	0
Inhibitor	100	0	0

$$\text{ACE inhibition rate(\%)} = \frac{A - S}{A - C} \times 100 \quad (1)$$

where A was the absorbance of the reaction with the buffer instead of tested samples, S was the absorbance of the reaction with tested sample, and C was the absorbance of the reaction in which HCl was added prior to HHL.

### Kinetics of ACE Inhibition

The substrate HHL (0.5, 1, 2 and 4 mmol/l) was mixed with ACE (80 U/l) and a series of synthetic tripeptides, respectively, and incubated at 37 °C for 10 min. Lineweaver–Burk plots were constructed for determining the mode of inhibition. The  $K_m$  and  $K_i$  were calculated based on the amount of HA released.

### Molecular Docking

The 3D structures of the four peptides were generated and energy-minimized with ChemOffice 2004 (CambridgeSoft Co., MA, USA). The crystal structure of human ACE was obtained from the RCSB Protein Data Bank (1O86.pdb). Before docking, the ACE protein was first extracted along with its cofactors chloride and zinc, and the polar hydrogens were then added. Afterwards, Molecular docking simulations were then performed using the AutoDock Vina software (v1.1.2) (Morris et al. 2009) in order to predict the binding affinities and conformations of each compound with ACE. The ranked docking pose of the peptide tested in the active site of ACE was determined according to the binding affinity (kcal/mol).

### Cells Viability Assay

Human umbilical vein endothelial cells (HUVECs) (BNCC347734) were obtained from BeNa Culture Collection (Jiangsu, China) and cultured according to the description of Villars et al. (2002). The procedure is to determine the cell viability in 96 well plates using a Cell Counting Kit-8 (VICMED, Jiangsu, China) after 24 h stimulation with the candidate tripeptides, consulting the method described by Qian et al. (2017). Briefly, the HUVEC cells frozen at – 80 °C were removed and quickly melted in a water bath at 37 °C. Then, the cells were centrifuged at 1000 rpm for 3 min. After removing the supernatant, the cells were resuspended with ECM medium and seeded into a 9 cm culture dish containing the prepared medium (ECM medium supplemented with 1% ECGS, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) and cultured in the incubator with 5% CO<sub>2</sub> and 95% humidity at 37 °C. To investigate cytotoxicity, 100 l cell suspension, about  $1 \times 10^5$  was seeded in a 96-well plate and cultured in an incubator for 24 h so

that they stucked to the bottom. Subsequently, 10 µl of ChPP, CPhP, OhPP or OPhP solutions with different concentrations (1.25, 2.5 and 5.0 µg/ml) were added to each well of the 96-well plates, and then incubated in the incubator for 12 h, followed by adding 10 µl ccK-8 solution to each well. After incubation in the incubator for 4 h, the absorbance at 450 nm was determined by a microplate reader. The viability were evaluated with the following equation (Eq. 2):

$$\text{Viability(\%)} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (2)$$

### Antihypertensive Tests In Vivo

Twelve-week-old male SHR (about 320 g body weight BW SPF) with tail systolic blood pressure (SBP) of over 180 mmHg were employed. They were housed in cages and cared according to standards for experimental rat care (Qian et al. 2019). SHR were acclimatized for 7 days prior to experiment and randomly divided into three groups: ChPP group (n = 6), captopril group (n = 6), and control group (normal saline n = 6). A standard laboratory rat diet (SLA-COM, Shanghai, China) and tap water were freely available. After administration with 10 or 20 mg tripeptide/kg BW, or 2 mg/kg BW captopril, or normal saline with equal volume by gavage, the SBP of each rat was measured by the tail-cuff method with a programmable electrophygmomanometer (model Softron BP-98A; Softron Beijing Incorporated, Beijing, China) at 0.5 h interval. For an accurate SBP reading, rats were placed in a chamber maintained at 37 °C to adapt to the SBP measurement device environment for at least 10 min prior to measurement. Data were expressed as means ± SD (n = 6).

### Effects of Candidate Peptides on Transcriptional Levels of miR-132 miR-212 and Agtr1

To further determine the ACE inhibitory activity of these designed tripeptides, transcriptional levels of agtr1 and miR-132/-212 regulated by Ang II were assayed, respectively. Following administration by oral gavage, the microRNA and large fragment RNA were isolated from the hearts of SHR at timepoint of the lowest blood pressure using E.Z.N.A™ miRNA kit (OMEGA Biotek, Norcross, GA, USA). Reverse transcription of mRNA and miRNA was completed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following methods reported by Qian et al. (2019), and the transcriptional levels were evaluated by qRT-PCR with AceQ™ qPCR SYBR Green Master Mix (Vazyme Biotech, Nanjing, China). Primers for reverse transcription and qRT-PCR were listed in supplemental Table S1. GAPDH gene and

small nuclear RNA (SnRNA) U6 were used as endogenous control to determine relative mRNA and miRNA expression, respectively. All reactions were run in triplicate and presented as means  $\pm$  SD ( $n=6$ ). The relative quantification analysis of expression levels of mRNA and miRNA was done by  $2^{-\Delta\Delta CT}$  method.

## Statistical Analysis

Values are presented as the mean  $\pm$  SD or SE. Statistical significance of differences was evaluated by Student's *t* test ( $p < 0.05$ ) using the software SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

## Results

### ACE Inhibitory Activities of the Tripeptides

ACE was prepared as the crude extracts from rabbit lung and its activity was determined as  $0.747 \pm 0.043$  U/ml. For exploring novel ACE-inhibitors, 4 L-proline based derivatives were designed and chemically synthesized (Table 2). It suggested that the hydrophobicity of the peptides with N-terminal Citr residue were less than those with N-terminal Orn residue based on the ClogP and TPSA values. As shown in Table 2, the activity of ChPP ( $IC_{50} = 40.48 \pm 0.47$   $\mu\text{mol/l}$ ) was significantly higher than that of CPhP ( $IC_{50} = 139.15 \pm 2.69$   $\mu\text{mol/l}$ ). Meanwhile, OhPP ( $IC_{50} = 212.35 \pm 4.90$   $\mu\text{mol/l}$ ) exhibited

a significantly higher activity than OPhP ( $IC_{50} = 1272.35 \pm 187.69$   $\mu\text{mol/l}$ ).

### ACE Inhibitory Kinetics

The ACE inhibition mode of the synthetic tripeptides was evaluated using the Lineweaver–Burk plots. As it can be seen in Fig. 1, for the designed peptides, all the lines crossed at the same y-intercept, but at different slopes and x-intercepts, suggesting a competitive inhibition pattern towards ACE.

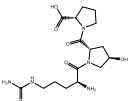
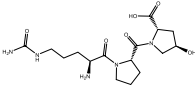
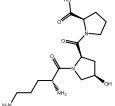
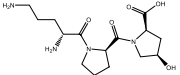
Besides, the inhibition constant ( $K_i$ ), an indicator of binding affinity to ACE enzyme, was calculated for these competitive inhibitor. As listed in Table 2, the  $K_i$  value followed an order: ChPP (0.59  $\mu\text{mol/l}$ ) < CPhP (0.81  $\mu\text{mol/l}$ ) < OhPP (1.85  $\mu\text{mol/l}$ ) < OPhP (12.23  $\mu\text{mol/l}$ ), which was consistent with their ACE inhibitory activity.

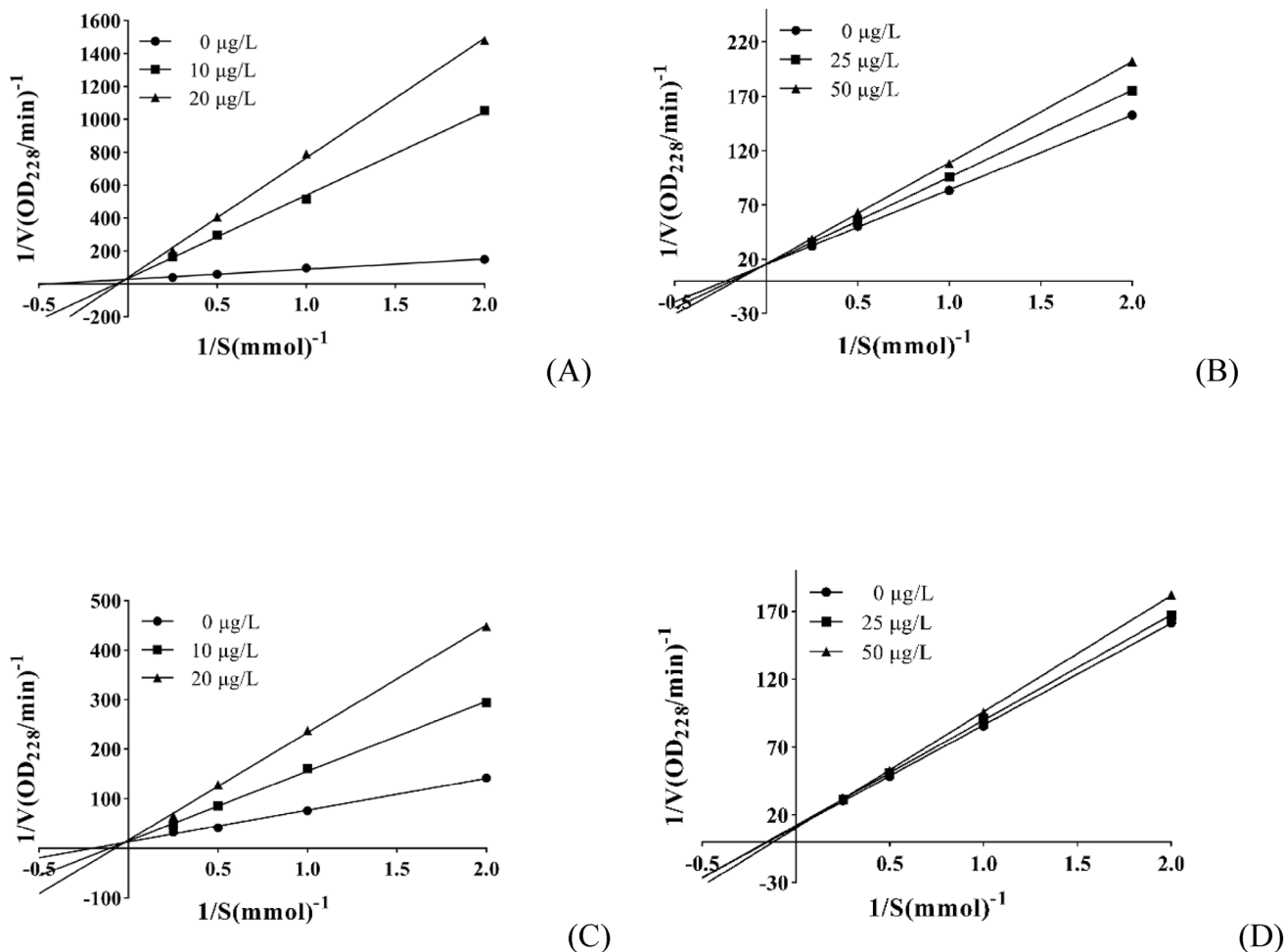
### ACE Molecular Docking

To further understand the inhibition mechanism of the tripeptides, molecular docking studies were performed by the AutoDock Vina software (v1.1.2) (Trott and Olson 2010). The binding affinities of ChPP, CPhP, OhPP, and OPhP were shown in Table 2. It is noted that ChPP exhibits the highest binding activity with a binding affinity value of  $-7.6$  kcal/mol, followed by CPhP ( $-7.5$  kcal/mol), OhPP ( $-6.4$  kcal/mol), and OPhP ( $-6.4$  kcal/mol), respectively.

The active site of ACE is composed of three active pockets: S1 (Ala354 Glu384 and Tyr523), S2 (Gln281 His353 Lys511 His513 and Tyr520), and S1' (Glu162 residue) (Rohit et al. 2012). Additionally, zinc ion ( $Zn^{2+}$ ) in the active site plays an essential role in ACE catalytic activity and is

**Table 2** Structure and characteristics of four tripeptides designed

No.	Structure	Abbr. name	Molecular weight (Da)	CLogP	TPSA	ACE $IC_{50}$ ( $\mu\text{mol/l}$ )	Inhibitory mode	$K_i$ (mmol/l)	Affinity (kcal/mol)
1		Citr-Hyp-Pro ChPP	385.42	-2.20	179.29	$40.48 \pm 0.47$	Competitive	0.59	-7.6
2		Citr-Pro-Hyp CPhP	385.42	-2.20	179.29	$139.15 \pm 2.69$	Competitive	0.81	-7.5
3		Orn-Hyp-Pro OhPP	342.40	-3.88	150.19	$212.35 \pm 4.90$	Competitive	1.85	-6.4
4		Orn-Pro-Hyp OPhP	342.40	-3.88	150.19	$1272.35 \pm 187.69$	Competitive	12.23	-6.4



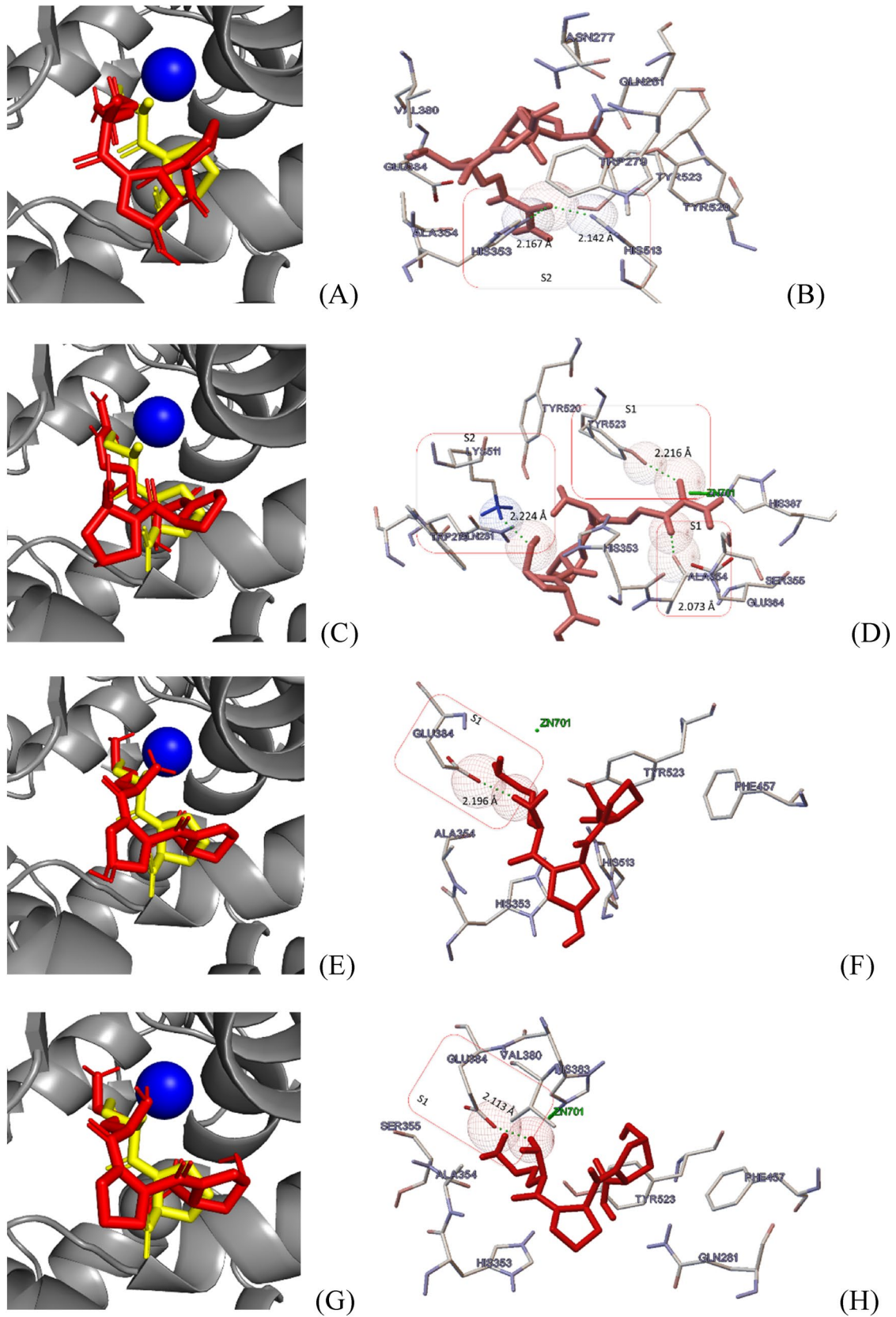
**Fig. 1** Lineweaver–Burk plots of ACE inhibition of ChPP (a), CPhP (b), OhPP (c), OPhP (d) and by different peptide concentrations at different substrate concentrations

tetrahedrally coordinated by His383, His387, and Glu411 residues in ACE (Spyroulias et al. 2004). The optimal docking pose of the tripeptides in relation to ACE is displayed in Fig. 2. The carbonyl group at N-terminal of ChPP could coordinate with His353 and His513 in S2 active pocket by hydrogen bonds with distance of 2.167 and 2.142 Å, respectively (Fig. 2a, b). The interaction between CPhP and ACE was mediated through hydrogen bonds between carboxyl group at C-terminal and carbonyl group at N-terminal of CPhP and Tyr523 and Ala354 in S1 active pocket of ACE with distance of 2.216 and 2.073 Å, respectively, and hydrogen bonds between amine group at N-terminal of CPhP and Lys511 in S2 active pocket of ACE with distance of 2.224 Å (Fig. 2c, d). The 2-amine group of Ornithine residue of OhPP formed hydrogen bond interaction with Glu384 in S1 active pocket of ACE with distance of 2.196 Å (Fig. 2e, f). Interaction of OPhP with ACE was same as that exhibited between OhPP and ACE with distance of 2.113 Å (Fig. 2g, h).

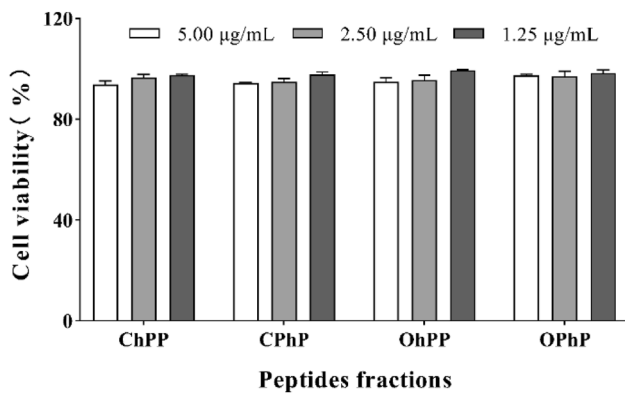
### Effect of ACE Inhibitory Peptides on HUVECs Viability

The effect of candidate tripeptides on the viability of HUVECs was estimated using the CCK-8 assay. The results showed that all candidate tripeptides slightly reduced the viability of HUVECs by 1.80–8.12% compared with control group (Fig. 3), suggesting that the concentration of candidate tripeptides (5 µg/ml) showed no cellular toxicity.

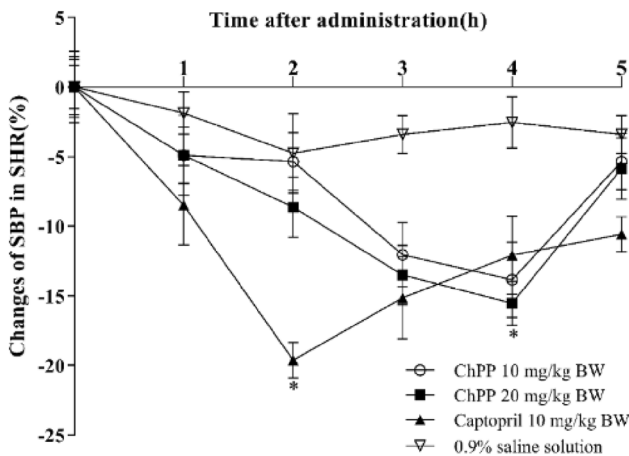
Docking confirmation of candidate tripeptides (red) and ligand compound captopril (yellow) with tACE. Zinc ion is shown as blue sphere (a, c, e, g). Interactions between peptides (sticks) and the residues of tACE (lines) (b, d, f, h). Green dotted lines indicates the formation of hydrogen bonds. Image obtained with AutoDock Tools software.



**Fig. 2** Molecular docking simulation of peptides ChPP CPhP OhPP and OPhP against tACE (Color figure online)

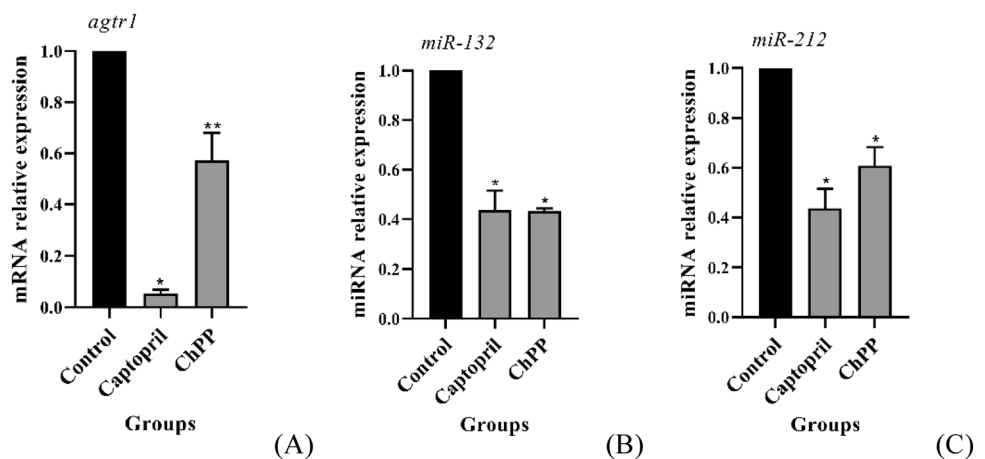


**Fig. 3** Cell viability of HUVECs stimulated by the peptides fractions with varied concentrations. Value expressed as means ±SD of three determinations



**Fig. 4** Changes of systolic blood pressure (SBP) of SHR after oral administration of tripeptide ChPP with different doses. The significance of differences [ $p < 0.05$  was calculated by *t* test ( $n = 6$ )]

**Fig. 5** Quantitative PCR analysis of the level of *agtr1* mRNA and miR-132/212 expression. **a** *agtr1*; **b** miR-132; **c** miR-212 (\*significant difference  $p < 0.05$ ; \*\*significant difference  $p < 0.01$ )



### Effect of ACE Inhibitory Peptides on the SBP of SHRs

Based on the above results, the antihypertensive activity of the tripeptide ChPP was further investigated in acute anti-hypertensive tests using SHRs. Male SHRs were administered intragastrically with the ChPP. Captopril was used as a positive control. ChPP (20 mg/kg BW) induced a significant reduction of SBP, with the largest decline of SBP by about 15.54% occurring at 4 h ( $p < 0.05$ ), which was slightly less than that of captopril (10 mg/kg BW) (Fig. 4). SBP in the ChPP group then began to recover and almost returned to the basal level at 5 h. Increasing doses of ChPP to 20 mg/kg BW reduced SBP to a greater extent. Nevertheless, the changes did not reach statistical significance (Fig. 4).

### Effects of Tripeptides on Transcriptional Levels of *Agtr1* and miR-132/-212

To further illuminate the ACE inhibitory effect of the tripeptides, the effects of tripeptides on expression levels of *Agtr1* mRNA and miR-132/212 were assayed by qRT-PCR in the aorta of SHRs (Fig. 5). As depicted in Fig. 5, ChPP significantly downregulated the transcriptional levels of *Agtr1* and miR-132/-212 ( $p < 0.05$ ). It is noted that *Agtr1* level was significantly lower in captopril group than that of ChPP group ( $p < 0.05$ ), while the levels of miR-132/-212 were comparable between the two groups.

### Discussion

Acting as a vital player in the renin-angiotensin system (RAS) by activating vasoconstrictor Ang II and inactivating vasodilator bradykinin, the overactivity of ACE results in high blood pressure, which makes it become an important target for clinical drugs, such as captopril, lisinopril, and enalapril, belonging to ACEIs structurally containing C-terminal proline residue. Recently, natural peptides composed

of amino acids are preferred because of the side effects of synthetic peptidomimetic drugs.

In this study, we designed four tripeptides and evaluated their ACE inhibitory activity *in vitro* and antihypertensive effect *in vivo*. Our results are consistent with previous studies reporting that the presence of hydrophobic amino acids at the C-terminus could potentiate the ACE inhibitory activity of the peptide (Miralles et al. 2018; Jao et al. 2012; Taga et al. 2018; Ruiz et al. 2004). Ruiz et al. (2004) revealed that ACE only binds weakly with peptide inhibitors that have penultimate Pro residues while the presence of Pro at the C-terminal has demonstrated to enhance binding to the enzyme (Ruiz et al. 2004). In addition, it is also demonstrated that substitution of Hyp with Pro in “X-Hyp-Gly” type tripeptides dramatically decreased the ACE-inhibitory activity of tripeptides (Taga et al. 2018). In contrast, the ACE inhibitory activity was dramatically decreased by prolyl hydroxylation in the case of C-terminal Pro-containing “Gly-X-Pro” type tripeptide (Taga et al. 2018). Our results exhibited the same pattern that the ACE-inhibitory activity of “X-Hyp-Pro” type tripeptide was stronger than that of “X-Pro-Hyp” type ones. Table 2 also shows that the ACE-inhibitory activity of ChPP is significantly higher than that of OhPP. It might be due to that Orn is more hydrophilic than Citn, leading to the greater hydrophilicity of the peptide OhPP than that of ChPP (as reflected by the Clog/TPSA values in Table 2), which increased the solubility of OhPP in the aqueous phase, but on the other hand, restricts the entry of the peptide to the ACE active site as reported by prior studies (Li et al. 2017; Ashok and Aparna 2017), thus reducing the ACE inhibitory activity.

The result of ACE inhibition mode analysis revealed that all the designed peptides here can compete with the substrates for the catalytic site of ACE or can alter the ACE conformation thereby reducing the enzymatic activity (Jao et al. 2012). It also indicated that the peptides can bind to the ACE at positions including both the active and other regulatory sites, consequently preventing catalysis.

The  $K_i$  value of ChPP (0.59  $\mu\text{mol/l}$ ) was lower than that of other three peptides indicating higher binding ability of ChPP to ACE which is in line with their respective  $IC_{50}$  values. Many Peptides are found to be competitive inhibitors for ACE, such as commercial tripeptides IPP and VPP (FitzGerald et al. 2004). However, there were also some mixed inhibition peptides, such as Phe-Glu-Asp-Tyr-Val-Pro-Leu-Ser-Cys-Phe (FEDYVPLSCF) and Phe-Asn-Val-Pro-Leu-Tyr-Glu (FNVPLYE) with  $IC_{50}$  of 10.77 and 7.72  $\mu\text{mol/l}$ , respectively (Ahn et al. 2012). Forghani et al. (2016) also obtained three peptides (EVSQGRP, VSRH-FASYAN, and SAAVGSP) from *Stichopus horrens*, exhibiting mixed inhibition patterns with  $IC_{50}$  of 50, 80, and 210  $\mu\text{mol/l}$ , respectively (Forghani et al. 2016).

The binding affinity of all four tripeptides are in line with their *in vitro* ACE inhibitory activity ( $IC_{50}$ ) in this study. It is known that His353 and His513 were conserved in various ACE homologues (Sturrock et al. 2004) and played a vital role in ACE activity. The stability of the ACE-captopril complex was mainly attributed to the hydrogen bonds between the carbonyl group of captopril and His353 and His513 residues of ACE, respectively (Bhuyan and Mugesh 2011). The best ACE inhibitory activity of ChPP might be due to its interaction with His353 and His513 in ACE S2 pocket, even though the interaction between C=O groups of ChPP and  $Zn^{2+}$  at optimal state (with distance  $> 3 \text{ \AA}$ ) was not a potential active site, compared with the interaction between the S-H group of captopril and  $Zn^{2+}$  (with distance about 2.5  $\text{ \AA}$ ) (data not shown).

The *in vivo* antihypertensive activity of ChPP in SHR mice in our study was comparable or even better than antihypertensive peptides under similar conditions reported in previous studies (Jung et al. 2006; Ko 2012). Jung et al. (2006) found that administration of 10 mg/kg BW peptides MIFPGAG-GPEL caused SBP reduction of 22 mmHg (about reduction of 11%) in SHR mice at 3 h after administration, and the effect was maintained for 9 h. Ko (2012) described that administration of the peptide VEGY (dose: 10 mg/kg BW) led to a reduction of SBP by about 11.2% and activities were maintained for 6 h. Thus, our results clearly indicated that ChPP can be explored as a peptide candidate for the hypertension management.

In RAS, Ang II is the major executor downstream of ACE. Ang II not only elevates blood pressure but also stimulates expression of some genes and miRNAs involved in cardiovascular system (Vamos et al. 2014; Mitra et al. 2010; Jin et al. 2012; Eskildsen et al. 2013). Mitra et al. (2010) described that expression of Ang II type 1 receptors (agtr1) in cardiovascular regions of the brain was increased as increasing level of Ang II or prolonging exposure to Ang II (Mitra et al. 2010). Besides, chronic infusion of Ang II could up-regulate level of miRNA-132/-212 in the heart and aorta of in hypertensive rats (Jin et al. 2012; Eskildsen et al. 2013). From the data, we could find that the mRNA expression of agtr1 significantly was suppressed by captopril more than by Peptide, but the expression of miRNA-132/-212 were same. It might be attributed to the direct interaction between Ang II and agtr1, but the intermediation between Ang II and miRNA-132/-212. And, there were other factors involved in the expression regulation of miRNA-132/-212 (Kumarswamy et al. 2014). Totally, we could conclude that ChPP could serve as a potent bioactive agent, inhibiting ACE to produce Ang II to lower blood pressure, although endogenous level of Ang II was not detected. Furthermore more studies should be executed to evaluate its safety compared to current ACEIs.



## Conclusions

This study demonstrated that ChPP, one of 4 synthetic tripeptidic derivatives of proline, exhibited the most potent *in vitro* ACE inhibitory activity with a competitive inhibition mode. What's more, molecular docking simulation confirmed its interaction with ACE S2 active pocket, and it reduced the SBP almost to a similar extent to that of captopril. The down-regulation of Agtr1 mRNA and miR-132/-212 further suggested that ChPP might be explored as a potential antihypertensive candidate for further utilization.

**Author Contributions** Bingjun Qian designed the experiments, analyzed the data, and wrote the paper; Lili Yu and Oscar D. Villarreal finished Molecule docking; Siyi Huang and Jianghua Huo finished analysis of ACE inhibitory activity and blood pressure monitoring; Chongchong Tian finished RT-PCR analysis and revised the paper.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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