https://doi.org/10.31925/farmacia.2021.2.16 **ORIGINAL ARTICLE**

IN VITRO AND *EX VIVO* STUDIES ON ANGIOTENSIN-I CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY OF SHORT SYNTHETIC PEPTIDES

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Abstract

In our days the interest in the studies of new angiotensin-converting-enzyme (ACE) inhibitors as modulators of the renin-angiotensin-system (RAS) is growing, not only because of their importance as drugs for arterial hypertension treatment, but also for their therapeutic potential during COVID-19 infection. This study presents data for the design and synthesis of short peptides by SPPS strategy and investigates *in vitro* and *ex vivo* their potential as ACE inhibitors in the light of structural changes. The obtained results give insight into the structure-activity relationship of peptide sequences and show differences regarding the effects of peptides in two experimental procedures (inhibitory potency on purified ACE activity and AT-I induced rat ileum contractions). Three of the newly synthesized peptides with terminal proline, LAP, LKP and VAP, showed relatively high inhibitory activities.

Rezumat

Interesul pentru studiul noilor inhibitori ai enzimei de conversie a angiotensinei (ECA), modulatori ai sistemului renină-angiotensină (RAS), este în creștere, nu numai datorită importanței lor ca medicamente pentru tratamentul hipertensiunii arteriale, ci și pentru potențialul lor terapeutic în infecția COVID-19. Acest studiu prezintă date pentru proiectarea și sinteza peptidelor scurte prin strategia SPPS și investighează *in vitro* și *ex vivo* potențialul lor ca inhibitori ai ECA. Rezultatele obținute oferă o perspectivă asupra relației structură-activitate a secvențelor peptidice și evidențiază diferențe în ceea ce privește efectele peptidelor în două proceduri experimentale (potența inhibitoare a activității ECA purificate și contracțiile induse de AT-I asupra ileonului de șobolan). Trei dintre peptidele nou sintetizate cu prolină terminală, LAP, LKP și VAP, prezintă activități inhibitoare relativ ridicate.

Keywords: ACE, peptides, synthesis, inhibitors, ileum contractions

Introduction

Recently the inhibitors of angiotensin-converting enzyme (ACE) and angiotensin receptor 1 (AT1R) apprehended the attention of worldwide scientists due to their potential benefit/harms for patients infected by Coronavirus disease (COVID-19) [4].

Angiotensin I-converting enzyme (ACE) is one of the important vasopressor mechanisms. ACE impact is not only on converting angiotensin I to angiotensin II, but it has a vasopressor action on the renin-angiotensin system (RAS), and also inactivates bradykinin that possesses vasodilating properties [7, 14, 22]. It is known that in humans, an ACE polymorphism has been associated with determinants of renal and cardiovascular functions [28]. The homologue of ACE, termed ACE2, has been identified and predominantly expressed in the vascular endothelial cells of the kidney and heart [5, 25]. It contains a single zinc-binding catalytic domain, which is 42% identical to the human ACE active domain. ACE2 functions as a carboxy-peptidase, cleaving a single residue from AT-I, generating Ang 1-9 [5, 25], and a single residue from AT-II to generate Ang 1-7 [25]. These *in vitro* biochemical data suggest that ACE2 may modulate the RAS and thus affect blood pressure regulation. In addition, it has been shown that ACE2 can cleave other peptide substrates [5, 25]. It is well known, that there are two isoforms of ACE in human tissues - somatic and testicular ACE. Somatic sACE has two structurally homologous domains (N-and C-), while testicular tACE has a single domain, identical to the C-domain of somatic sACE [3, 24]. There are studies that show differences in the inhibitory and substrate selectivity of the N- and C-domain of human, rat and mouse ACE [26]. The inhibition of RAS by a cascade of proteins plays a key role in the treatment of hypertension and cardiovascular diseases.

Different classes of synthetic inhibitors of Angiotensin converting enzyme (ACE) are used as drugs in the treatment of hypertension and associated diseases [16,

19]. Even though the synthetic drugs inhibitors as captopril, lisinopril, fosinopril, enalapril etc. are often used due to their well-known effects, they have undesirable side effects as dry cough, skin rashes, impaired renal function, hyperkaliaemia etc. [1]. Globally, there is an increasing interest in alternative sources of bioactive compounds with antihypertensive action as food of different origins - milk protein, egg protein, different cheeses, white and red wine, vegetable proteins and marine sources [2, 10, 11, 27]. It is expected that ACE inhibitors isolated from different natural sources can effectively control blood pressure and in the same time they have minimal side effects. It is worth to notice that often poor correlations between the *in vitro* ACE inhibitor activity of bioactive peptides and the *in vivo* antihypertensive activity have been observed. This can be partly due to digestion by means of gastrointestinal proteinases in the stomach and/or due to their low bioavailability [20].

One of the most investigated milk derived low molecular weight bioactive peptides inhibitors of ACE such as Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), show they resisted the *in vivo* degradation by gastrointestinal enzymes and were able to exert antihypertensive activity through the inhibition of ACE in aorta [17]. Up to day a lot of studies focused on the ACE inhibitor peptides with hydrophobic residue, usually L-proline, at the C-terminus. Very often the content of bioactive peptides in natural sources is quite low and this necessitates the search for alternative strategies for designing new peptides by chemical means.

In this paper it is presented the synthesis of some short chain peptides by innovated chemical synthesis pathway and the comparison of their chemical structure with their action on ACE activity from two sources: purified enzyme and rat ileum segments.

Materials and Methods

Materials

Fmoc-Ala Angiotensin-I (AT-I), Angiotensin-I converting enzyme (from rabbit lung) (ACE) and N-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG) were purchased from Sigma Aldrich. Lisinopril (LIS) (N^2-[-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline) was obtained from SuanPharma (Spain). AT-I, lisinopril and the tested compounds were dissolved in distilled water and prepared right before the experiments.

The Fmoc protected amino acids Fmoc-Ala, Fmoc-Val, Fmoc-Leu, Fmoc-Lys(Boc), Fmoc-Ile, Fmoc-Trp and H-Pro-2-ClTrt resin (L-proline 2-chlorotrityl ester polymer-bound), Fmoc-Lys(Boc)-Wang resin were purchased from Iris Biotech (Germany). All other reagents and solvents used were of analytical or HPLC grade purity and were obtained from Sigma Aldrich.

Synthesis of the new peptides

The conventional solid-phase peptide synthesis based on Fmoc (9-fluorenylmethoxycarbonyl) chemistry was employed to synthesize short peptides with expected ACE inhibitory activity. H-Pro-2-ClTrt resin, Fmoc-Lys(Boc)-Wang resin, HBTU (3-[Bis(dimethylamino)-methyliumyl]-3H-benzotriazol- 1-oxide hexafluorophosphate) and DIPEA (N,N-diisopropylethylamine) were used as solid-phase carrier, coupling reagents and base respectively.

The conventional manual solid-phase peptide synthesis (SPPS) based on Fmoc strategy was used for the obtained short peptide sequences as Val-Ala-Pro (VAP), Val-Ala-Trp (VAW) [29], Leu-Ala-Pro (LAP), Leu-Lys-Pro (LKP), Ile-Ala- Lys (IAK) as potential inhibitors of ACE.

The target peptides were synthesized by the general procedure: the synthesis begins with the coupling of 0.30 mmol of the first Fmoc-amino acid to 4 equiv. the pre-activated resin. 4 eq. coupling agent TBTU (0.9563 g, 0.30 mmol), 8 eq. base DIPEA (0.7621 g, 0.60 mmol), dissolved in 2 mL DMF. The reaction mixture was allowed to mix for 1 hour and 30 min using a Vortex mixer.

Completion of coupling reactions was monitored by the Kaiser test and the Fmoc groups were removed by adding 20% piperidine in DMF. After completion of the reaction acetaldehyde/chloranil test was carried and in the case of absence of a free -NH_2 group, the reaction continued with the next step. Deblocking was performed with 20% piperidine solution. The second Fmoc amino acid was added in the following way: to 2 mL DMF were added 4 equiv. Fmoc-AA, 4 eq. coupling agent TBTU (0.9563 g, 0.30 mmol) and 8 eq. base DIPEA (0.102 ml, 0.60 mmol). The mixture thus obtained was added to the resin in the reaction vessel. The reaction system was Vortex mixed for 1.5 h. Fmoc protection was accomplished by deblocking with 20% solution of piperidine. The final step of the synthesis of the tripeptide was its removal from the resin. The most commonly used reagent for removal of the synthesized peptide from the resin is trifluoroacetic acid (TFA). During the reaction highly reactive carbocations are formed. This requires the use of a quencher: triisopropyl silane (TIS) that prevents from adverse reactions. A solution consisting of 95% TFA: 2.5% H_2O: 2.5% TIS, was added to the resin and reaction mixture was mixed for 3 h under magnetic stirring at room temperature. After completion of the reaction the flask contents were filtered and washed with TFA, evaporated under nitrogen and precipitate by diethyl ether under ice. The organic solvents were removed and the peptides were freeze dried.

The representative reaction scheme for SPPS of target peptide H-Leu-Lys-Pro-OH is shown on Figure 1.

All target peptides was purified by High performance liquid chromatography (HPLC), VWR Hitachi "LaChrom Ellite", pump L-2130, UV-detector L-2400, column RP C-18 Vydac 238 TP, 250 x 4.6 mm, mobile phase CH_3CN/0.1 % TFA:H_2O/0.1 % TFA (30:70 v/v) and analyses by MS analysis Figure 2.

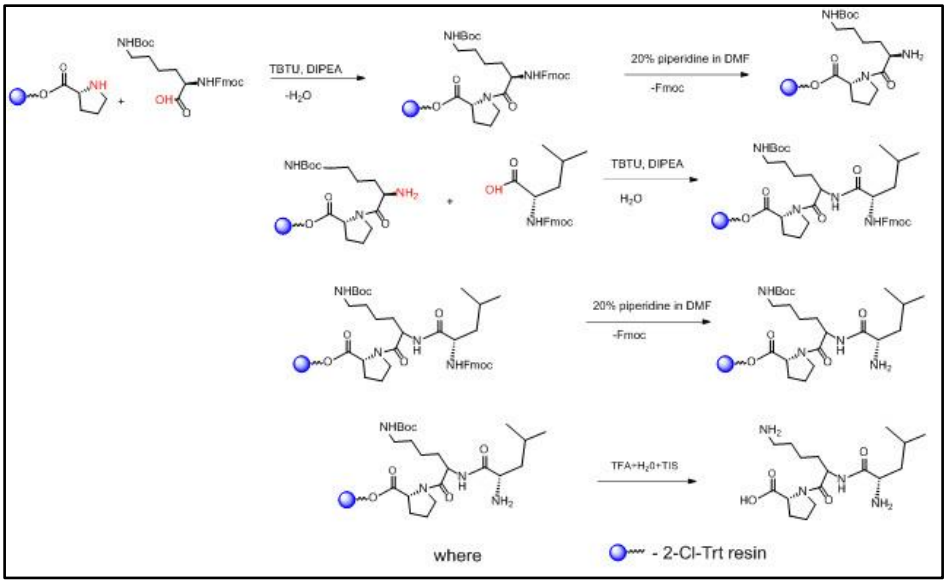

Figure 1.

Reaction scheme of the synthesis of H-Leu-Lys-Pro-OH

Reagents and conditions: H-Pro-2-ClTrt resin, Fmoc-Lys(Boc), Fmoc-Leu, HOBT, TBTU, DIPEA, TFA/triisopropylsilane/H_2O (95:2.5:2.5), r.t., 1.5 h

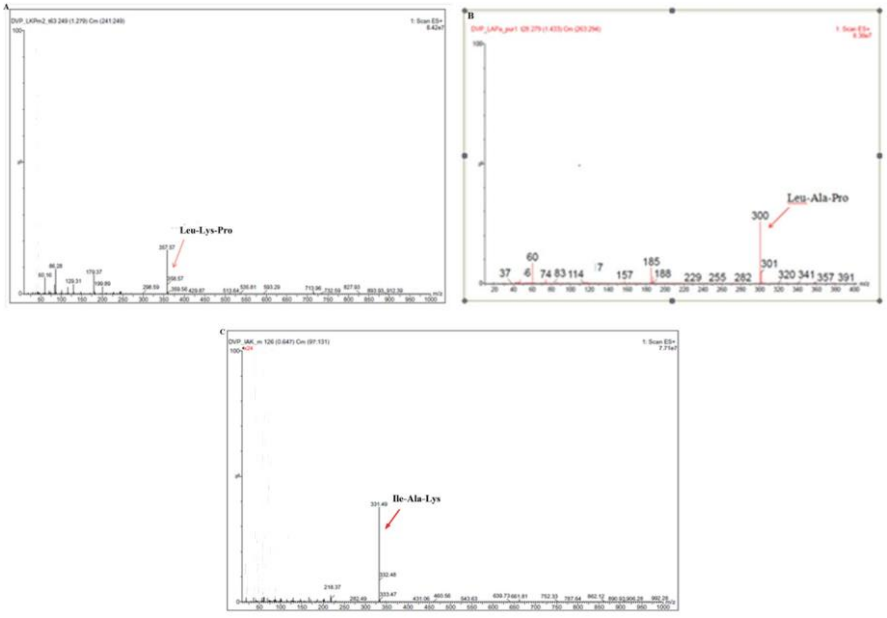

Figure 2.

Mass spectra (MS) of pure newly synthesis peptides (A,(Leu-Lys-Pro); B,(Leu-Ala-Pro); C,(Ile-Ala-Lys))

The MC spectra were recorded on an ACQUITY^{TM} UPLC system (Waters) coupled with a Micromass® Q-Tof MICRO^{TM} mass spectrometer (Waters) equipped with single quadrupole mass detector

The solution peptide synthesis protocol was used for the obtained the peptides His-Phe-Tyr (HFY) and Ser-Tyr-Gly-Leu (STGL) as substrates for proteinases (unpublished results).

Testing of the newly synthesized short peptides for ACE inhibition activity

The FAPGG degradation method of Holmquist [9] with substantial modifications was used. ACE (250 mU) was dissolved in 1 mL TRIS buffer (0.075M without NaCl, pH 8.3) and kept at -20ºC. The working solution (1 mM) of FAPGG (10 mg/25 mL) in assay buffer was prepared and kept in a brown glass bottle, stable for 2 months at 4ºC. Subsequent dilutions of tested compounds were done in the assay buffer - TRIS buffer (0.075M with 0.3 M NaCl, pH 8.3).

Briefly, the experimental procedure was as follows: 10 mL ACE (2.5 mU) plus 10 µL buffer or 10 µL of inhibitor solution were mixed with 230 µL assay buffer in plastic tubes, and the mixture was incubated at 37ºC for 5 min. The reaction was started with

0.75 mL of preheated at 37ºC solution of substrate FAPGG (0.75 mM in assay buffer final concentration). After gently mixing the tubes, the degradation of FAPGG was followed at 340 nm, for 30 min using a Carry double beam spectrophotometer, against buffer as reference.

ACE activity was expressed as rate of disappearance of FAPGG ($\Delta A\ min^{-1}$). Tris–HCl buffer was used instead of peptides solutions in the control sample.

Dose-dependent ACE inhibition was investigated using at least four different concentrations of peptides. The concentration of peptide that inhibited ACE activity by 50% (IC_{50}) was calculated using a non-linear regression from a plot of percentage of ACE activity *versus* sample concentrations using GraphPad Prism 8.01. All experiments were performed in duplicate.

Testing of the newly synthesized short peptides for rat ileum AT-I induced contractions

The *in vitro* experiments were performed on male Wistar albino rats, weighing 180 - 200 g, housed at 22 - 25°C with free access to food and water and a natural day/night light cycle.

After sacrificing the animals, the terminal ileum was carefully separated from surrounding connective tissues and cleaned in ice-cold Tyrode solution. The terminal ileum segments (about 10 - 12 mm long) were fixed in organ baths containing 4 mL of Tyrode's solution saturated with 95% O_2 and 5% CO_2, and connected to electronic transducers. The ileum preparations were preloaded with 1 g and left for 60 minutes adaptation at 37°C. The smooth muscle tone of the preparations was isometrically registered [12].

For each ileum segment 2 dose-response curves have been created. The first (a control one) was build up with cumulatively applied AT-I (10^{-9} to 10^{-6} M). The segment was then washed several times and after an interval of 60 min the examined compound was added to the organ bath. The effects of the newly synthesized compounds on basal tone and their influence on angiotensin-induced rat ileum contractions were tested. The newly synthesized inhibitors were added 5 min prior to AT-I in two different concentrations (10^{-7} M, 10^{-6} M for VAW, LKP, LAP, IAK, and 10^{-6} M, 10^{-5} M for VAP). Lisinopril (10^{-7} M, 10^{-8} M), as a well-known ACE inhibitor, was used as a reference inhibitor. The concentrations of the tested peptides and LIS were selected on the basis of preliminary studies.

Using GraphPad Prism 8.01, the ileum contraction responds (%) (without or in the presence of ACE inhibitors) were plotted *versus* different concentrations of AT-I. By non-linear regression analysis EC_{50} values were calculated. The ratio of EC_{50} values between controls and after different concentrations of inhibitors served as assessment of their ACE inhibitory power.

Animals were handled following the European Communities Council Directive 2010/63/EU and the protocol was approved by the Institutional Ethics Committee.

Results and Discussion

Inhibitory effect on the activity of purified ACE

The methodology used in our study in order to determine the activity of purified ACE implies compliance with several conditions for the achievement of relevant and repeatable results. First of all that refers to the pre-incubation of the enzyme in an environment containing NaCl/0.3 M, 5 min this is a well-known condition for higher enzyme activity. Because of the very close changes in the absorption peaks of substrate /FAPGG/ and its metabolite, relatively low Vmax values needing long incubation sample time, we preferred to build an experimental schedule by following the difference of absorption at 340 nm after 30 min incubation at 37ºC against buffer solution in the absence or presence of inhibitors in different concentrations [21].

The results of the *in vitro* experiments on activity of purified ACE by different peptides are presented in Figure 3(A - B) and the calculated IC_{50} values were presented in Table I.

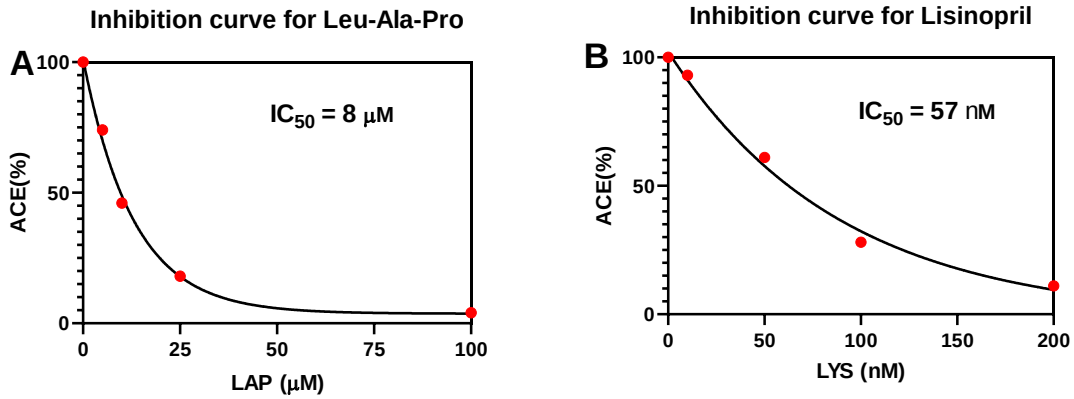

Figure 3.
Inhibition curves on ACE (2.5 mU) activity after incubation with LAP (A) and LIS (B) (substrate FAPGG 0.75 mM)

Table I

Investigated short peptides and IC_{50} values for inhibition of ACE

	Compound	IC_{50}
1	Val-Ala-Pro (VAP)	66 µM
2	Val-Ala-Trp (VAW)	148 µM
3	Leu-Lys-Pro (LKP)	19 µM
4	Leu-Ala-Pro (LAP)	8 µM
5	Ile-Ala-Lys (IAK)	188 µM
6	His-Phe-Tyr (HFY)	> 200 µM
7	Ser-Tyr-Gly-Leu (SYGL)	95 µM
8	Lisinopril (LIS)	57 nM

The inhibition of the enzyme activity depends on the concentration of the inhibitor following the logical non-linear regression curve. The higher inhibitory activity of the various peptides follows the well-known dependence of the presence of proline as C-terminus [8] which sharply increases the binding of inhibitor to the ACE active site [13, 18]. The three new tri-peptides with terminal proline with relatively high inhibitory activity are LAP, followed by LKP and VAP. The replacement in the 2 position of positively charged lysine amino acid with hydrophobic alanine lead to the increase approximately 2 times of the IC_{50} value for the inhibition of ACE. The replacement of hydrophobic proline on the 3 position with the positively charged lysine amino acid extremely changed the IC_{50} value for the inhibition of ACE.

The other synthesized peptides have relatively low inhibitory activities, HFY practically having no effect.

Inhibitory effect on AT-I rat ileum contractions

It is well known that in many cases the effectiveness of chemical compounds in *in vitro* experiments does not match the effectiveness when tested in models close to clinical conditions. Such a pattern is the follow-up of the contractile response of rat ileum under the influence of AT-I in different conditions. In this simple system the contraction of the ileum depends mainly on the ACE activity, converting AT-I to AT-II at the tissue level. In such a system, we assessed the rat ileum's response to cumulatively applied AT-I concentrations in the presence or absence of different concentrations of newly synthesized most active ACE inhibitors. In such an experiment, we could get a direct answer as to whether the structural-concentration dependence found on inhibition of the enzyme activity *in vitro* fits the potentially possible application to inhibit the enzyme *ex vivo*.

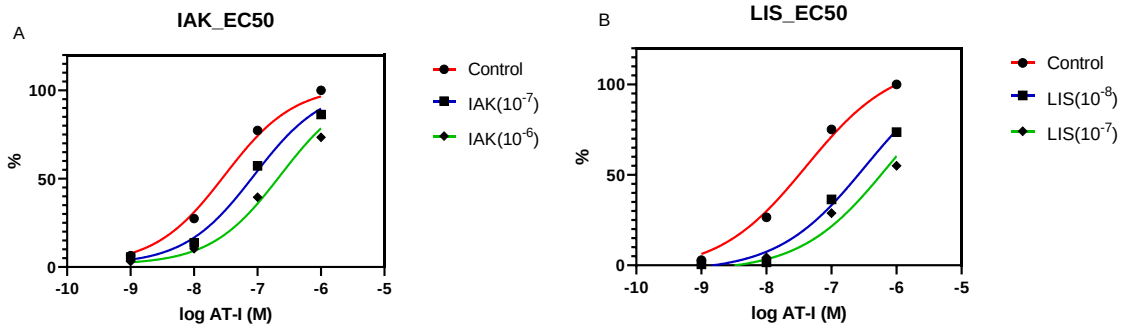

Figure 4.

Rat ileum. Cumulative dose-response curves of AT-I without or in the presence of peptides IAK (A) and LIS (B)

Figure 4 clearly shows that the cumulative dose-response curve of AT-I shifts to the right by different concentrations of inhibitors, in this case IAK and LIS. This naturally confirms the fact that ACE inhibitors are competitive inhibitors.

The ratio of this displacement, expressed most logically by comparing changes in the EC_{50} after different concentrations of inhibitors compared to that of control responses, called EC_{50} ratio, reflect more logically the power of inhibition of ACE by the different compounds in the conditions of the *ex vivo* experiment (Table II).

Table II

Calculated EC_{50} values for rat ileum contractions after different concentrations of AT-I. EC_{50} ratio is the ratio of EC_{50} in the presence of inhibitor divided by EC_{50} of agonist (AT-I) alone

	Control (AT-I) (EC_{50})	EC_{50} ratio			
Concentration		0.01 µM	0.1 µM	1 µM	10 µM
LAP	11.0^{-8}		3.562	4.55	
VAP	1.5^{-8}			1.777	5.947
LKP	5.5^{-8}		3.075	3.506	
IAK	3.04^{-8}		2.914	7.573	
VAW	1.98^{-8}		3.494	3.995	
LIS	4.09^{-8}	8.11	18.14		
Average ± SD	6.2^{-8} ± 4.3				

LAP = Leu-Ala-Pro; VAP = Val-Ala-Pro; LKP = Leu-Lys-Pro; IAK = Ile-Ala-Lys; VAW = Val-Ala-Trp; LIS = Lisinopril

From the five new synthesized tripeptides four of them showed almost equal inhibition effect on ileum ACE (EC_{50} ratio around 3.0 at 0.1 μM). VAP is almost 100 times less effective. LIS is with the most effective EC_{50} ratio - 8.11 at 0.01 μM. Comparing the effect of compounds *in vitro* and *ex vivo* on ACE activity shows significant differences. In the first case, we directly investigated the changes in the amount of substrate used, while in the second for the activity we assessed it indirectly from the contractile response of the ileum to the metabolite formed in the reaction (AT-II). It is well known that in addition to mucosal localisation, ACE in ileum is in close proximity with AT1R receptor in the muscle [6]. This suggests that differences in the effects of compounds in the two experimental procedures could be due to the achievement of different concentrations for inhibition of tissue ACE from the compounds in ileum trial depending on their physico-chemical characteristics.

Surprisingly there are very few publications discussing the effect of ACE inhibition on the *ex vivo* synthesis of AT-II and the corresponding physiological response, concerning rat jejunum [15] and rat vas deferens [23]. The results of our experiments on the inhibition of ACE show the usefulness of comparative *in vitro/ex vivo* studies to obtain important information on the QSAR of new compounds, especially in the context of their future *in vivo* use.

Conclusions

Herein we reported the design and synthesis of short peptides analogs of natural peptides and their *in vitro* and *ex vivo* interaction with ACE activity. In order to assess the chemical structure-activity relationship of the new compounds on ACE activity, two sources of enzyme were used: purified enzyme and rat ileum segments. Our results confirm that hydrophobic C-terminal proline in position 3 is crucial for the inhibitory effect. Furthermore, the replacement in the 2 position of positively charged lysine amino acid (LKP) with hydrophobic alanine significantly increases the inhibitory activity of the new compound (LAP). The combination Ala^2-Pro^3 leads to an enhanced of the inhibitory effect. However, the amino acid in position 1 is also important, because Val^1, although hydrophobic like Leu, decreases the inhibitory properties (VAP). Differences between the peptides inhibitory actions were observed when comparing their effect on a biological target, AT-I induced contractions of rat ileum. Our results show that, in addition to direct inhibition of ACE, other physico-chemical characteristics, e.g., transport through the cell membrane, are responsible for registered differences.

The new data obtained in this study appears to turn light on the design of new ACE inhibitory molecules.

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Conflict of interest

The authors declare no conflict of interest.

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