PEPTIDE INHIBITORS OF THE ANGIOTENSIN CONVERTING ENZYME WITH NONPROTEINOGENIC AMINO ACIDS

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Dedicated to the memory of Dr Karel Bláha.

To study the structural requirements of the angiotensin converting enzyme we have synthesized and tested two series of acylated tripeptides with the common structure Acyl-AA_1-AA_2-Pro and Acyl-AA_1-Arg-Pro. The structure–activity relationship indicates that the inhibitory activities result from the structure and conformation of the whole molecule. The use of nonproteinogenic amino acids in the positions AA_1 and AA_2 stabilizes to some degree the peptides against enzymatic degradation. Some of the acylated tripeptides are able to reduce the angiotensin I-induced blood pressure enhancement of normotensive rats. The peptides are applied orally. No good correlation exists between the inhibitory activity of the isolated enzyme and the in vivo activity. The structural requirements for the inhibition of the isolated ACE and the potentiation of the bradykinin action on the guinea pig ileum are different.

The angiotensin converting enzyme (ACE, peptidyl dipeptide hydrolase EC 3.4.15.1) plays an important role in the blood pressure regulation. It forms from the inactive angiotensin I the angiotensin II which increases the blood pressure. Furthermore, this enzyme inactivates the blood pressure lowering hormone bradykinin. In the last 10 years certain highly effective inhibitors of this enzyme were developed and some of these were used as therapeutic agents for the regulation of the blood pressure[1]. With the aim to get more informations about the structural requirements of this enzyme we have estimated structure–conformation–activity relationship of analogues both of the bradykinin potentiating nonapeptide $BPP_{9\alpha}$ (Glp-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) and the pentapep-

tide $BPP_{5\alpha}$ (Glp-Lys-Trp-Ala-Pro). We have studied the effect of the replacement of each amino acid in the sequences by other natural or unnatural amino acids[2–5].

Based on the results with the bradykinin potentiating peptides, we have now studied the effect of proteinogenic and nonproteinogenic amino acids on the inhibitory activity of di- and tripeptides (Tables I and II). We are interested in comparing the effects of amino acid replacements in the C-terminal part of the nona- and pentapeptide with the effect of replacements in tripeptides.

For these investigations we have synthesized acylated tripeptides of two common structures Acyl-AA_1-AA_2-Pro and Acyl-AA_1-Arg-Pro. The first structure contains neutral and hydrophobic amino acids whereas the second structure contains hydrophilic arginine. One of the key ideas for the development of orally applicable peptide inhibitors was the use of nonproteinogenic amino acids, to stabilize the peptides against enzymatic degradation. In our earlier investigations on bradykinin we found that such amino acids as *erythro*-β-phenylserine or *erythro*-α-amino-β-phenylbutyric acid stabilize the nonapeptide against enzymatic cleavage with kininase II. Therefore we used D-, α- or β-substituted, N-methyl and hydrophobic amino acids in the place of AA_1 and AA_2.*

The combination with acyl residues enhances or reduces the inhibitory activity of the di- and tripeptides. We used bulky and hydrophobic carbonic acids or dicarbonic acids e.g. adamantane-1-carbonic-, benzoic-, *p*-hydroxyphenyl-propionic, phthalic and norbornene dicarbonic acid.

Because of the different theories about the mechanism of action of the bradykinin potentiating peptides we estimated the biological activity of some compounds both on the inhibition of the isolated enzyme and on the potentiation of the bradykinin action on smooth muscles.

EXPERIMENTAL

Peptides: Syntheses of the free and acylated di- and tripeptides will be published elsewhere[7]. The peptides are purified by the use of chromatography on Sephadex LH20, Biogel P2, Fractogel

* The nomenclature and symbols of the amino acids and peptides follow the published IUPAC IUB recommendations[6]. The proteinogenic amino acids used in this studies are of L-configuration, if not stated otherwise. Other abbreviations used: MePhe, L-N-methylphenylalanine; MeAla, L-N-methylalanine; Ach, α-aminocyclohexanoic acid; Abu(Ph), DL-*erythro*-α-amino-β--phenylbutyric acid; Ser(Ph), DL-*erythro*-β-phenylserine; Ape(OH), DL-*erythro*-β-hydroxypentanoic acid; $Phe(Me_2)$, DL-2,5-dimethylphenylalanine; PhCO, benzoyl; Pht, phthaloyl; Mal, maleoyl; HPP, *p*-hydroxyphenylpropionyl; AdCO, adamantoyl; DBP, dibenzylphosphoryl; NBDC, norbonene dicarbonic acid monoacyl; AI, angiotensin I; AII, angiotensin II.

PGM 2000, Silicagel 60 or preparative HPLC (Zorbax ODS; CH_3CN–0.1M-$AcONH_4$). All endproducts are characterized by their melting point, optical activity, amino acid analysis, electrophoresis, TLC and HPLC. The used di and tripeptides are listed in Tables I and II. The employed bradykinin potentiating penta- and nonapeptides were synthesized by some of us[2–5].

Inhibition of the isolated angiotensin converting enzyme: Inhibitory activities of the acylated and nonacylated peptides were determined with an enzyme preparation from pig lungs using PhCO-Gly-His-Leu as a substrate. The method was described elsewhere (ref.[8]).

Potentiation of the bradykinin action: The potentiating activity of some peptides was estimated on the guinea pig ileum and compared with bradykinin induced contractions ($1 \cdot 10^{-8}$ M-$BPP_{9\gamma}$ (ref.[9])). The contraction was enhanced by this concentration to 280% ($n = 39$). This value corresponds to 100% potentiating activity.

Inhibition of angiotensin I-induced pressor response in conscious normotensive rats: Male Wistar rats (350–400 g) were anesthetized with ether oxygen air mixture. The animals were surgically prepared with an aortic catheter inserted via the right femoral artery and a cavel catheter inserted via the right femoral vein. Both catheters surfaced on the neck. The rats were placed in individual cages after surgery. To start experiments, the cannulae were connected with a pressure transducer. Heart rate and blood pressure were recorded continuously from the pressure signal by a electrocardiograph.

The test procedure was as follows: To establish the base line pressure response, 300 ng/kg AI was injected i.v. via the jugular vein (the increasing of blood pressure was about 40 mmHg). After recovery of blood pressure and heart rate 120 ng kg AII was injected. This procedure was repeated

TABLE I
Inhibitory activities of nonacylated di-, tri- and pentapeptides

Compound	Sequence	Inhibition of ACE IC_{50}, µmol l^{-1}
I	Arg-Pro	60
II	D-Phe-Arg	inactive
III	DL-Ape(OH)-Pro	>200
IV	D-Phe-Ala-Pro	5
V	DL-Abu(Ph)-Ala-Pro	20
VI	DL-Ser(Ph)-Ala-Pro	200
VII	Ach-Ala-Pro	80
VIII	Trp-Ala-Pro	150
IX	Pyr-Lys-Trp-Ala-Pro	0.5
X	Pyr-Lys-Trp-D-Ala-Pro	20
XI	D-Phe-Arg-Pro	40
XII	DL-Abu(Ph)-Arg-Pro	2.5
XIII	DL-Ape(OH)-Arg-Pro	100
XIV	Pro-Arg-Pro	30
XV	Ala-Arg-Pro	30
XVI	Pro-Arg-Ala	>100
XVII	Ala-Arg-Ala	>100

once more. Ten min later the test-compounds were administered to the rats. The AI and AII injections were repeated 20, 40, 60, 90, 120, 180, 240, 300, and 360 min post the application as described above. The AI challenge was used to etablish the specific ACE-inhibitory effect of the tested compounds. In preliminary studies 300 ng/kg AI and 120 ng/kg AII were found to be equipotent.

The two base line pressor responses were averaged and the obtained mean used as references for the following calculations. The percentage of inhibition was determined by comparing the reference values and the pressure response after the administration of the drugs.

TABLE II
Inhibitory activities of acylated di- and tripeptides

Compound	Sequence	IC_{50}, μmol^{-1}
XVIIIa	NBDC-DL-Phe(Me_2)-Pro	> 100
XVIIIb	Mal-DL-Phe(Me_2)-Pro	> 100
XVIIIc	Pht-DL-Phe(Me_2)-Pro	70
XIXa	HPP-DL-Ape(OH)-Pro	25
XIXb	PhCO-DL-Ape(OH)-Pro	> 200
XX	AdCO-Arg-Pro	30
XXI	AdCO-D-Phe-Arg	> 200
XXII	DBP-Leu-Trp	> 100
XXIIIa	PhCO-Phe-Ala-Pro	2.8
XXIIIb	HPP-Phe-Ala-Pro	9
XXIIIc	AdCO-Phe-Ala-Pro	> 100
XXIVa	PhCO-D-Phe-Ala-Pro	50
XXIVb	AdCO-D-Phe-Ala-Pro	> 100
XXV	PhCO-DL-Abu(Ph)-Ala-Pro	12
XXVIa	PhCO-DL-Ser(Ph)-Ala-Pro	25
XXVIb	HPP-DL-Ser(Ph)-Ala-Pro	38
XXVII	PhCO-MePhe-Ala-Pro	50
XXVIII	AdCO-Ach-Ala-Pro	200
XXIXa	PhCO-Trp-Ala-Pro	5.5
XXIXb	Pht-Trp-Ala-Pro	100
XXX	PhCO-DL-Ape(OH)-Ala-Pro	7
XXXI	PhCO-Phe-MeAla-Pro	inactive
XXXII	$(PhCO\text{-}Phe\text{-}DL\text{-}Cys\text{-}Pro)_2$	inactive
XXXIII	PhCO-Phe-Leu-Trp	15
XXXIV	PhCO-Phe-Arg-Pro	20
XXXVa	AdCO-D-Phe-Arg-Pro	25
XXXVb	HPP-D-Phe-Arg-Pro	20
XXXVIa	AdCO-DL-Abu(Ph)-Arg-Pro	70
XXXVIb	HPP-DL-Abu(Ph)-Arg-Pro	3
XXXVII	AdCO-DL-Phe(Me_2)-Arg-Pro	20
XXXVIII	PhCO-Phe-Val-Arg	200

RESULTS AND DISCUSSION

For the purpose of the study of the structural requirement of the angiotensin converting enzyme we have divided the estimated compounds from different viewpoints into free peptides (Table I) and acylated peptides (Table II). The activities of the acylated and nonacylated tripeptides are in the micromolar range. Only the additional interactions of the N-terminal part with the enzyme in the nona- and pentapeptides (Table II,Table III) enhance the activities to the nanomolar range. Structures of many ACE-inhibitors with high activity (Enalapril and analogues) demonstrated the importance of the reduced peptide bond CH_2NH or other modifications of the peptide bond. We consider our studies on the acylated peptides as a necessary prerequisite for the development of highly active inhibitors containing optimized sequences and acyl-residues in combination with modified peptide bonds. The use of structure–activity relationship studies for the optimization of the structure is restricted by the influence of small amounts of impurities. Thus, we found in two cases that the contamination of the products with 1 or 2 per cent impurity enhances the inhibitory activity by 1 or 2 orders of magnitude. The isolation and characterization of the impurities will be published elsewhere.

The inhibitory activities of the free peptides in Table I are in the same range as of acylated peptides (Table II). In the series of the AA_1-Ala-Pro sequences, the analogue with D-phenylalanine (*IV*) has the highest activity. More bulky or hydrophobic amino acids reduce the activity (*XIXb, XX – XXII*). The replacement of L-alanine in position 2 by the D-configuration decreases the activity, too. This finding agrees with structure–activity relationships of the penultimate position in $BPP_{9\alpha}$ (refs[2,3,5]) and $BPP_{5\alpha}$ (ref.[4]).

In the other series with arginine, the compounds *XVI* and *XVII* in Table I indicate that the C-terminal proline is essential for the biological activity. The

TABLE III
Differences between the potentiation of the bradykinin action on the isolated guinea pig ileum and inhibition of the isolated ACE ($BPP_{9\alpha}$ 10^{-8} mol l^{-1}; bradykinin 10^{-8} mol l^{-1})

Compound	Sequence	Potentiation, %	Inhibition, IC_{50}, mol l^{-1}
	Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	100	3×10^{-9}
IX	Pyr-Lys-Trp-Ala-Pro	2.3	0.5×10^{-6}
XXXIV	PhCO-Phe-Arg-Pro	3.8	20×10^{-6}
XXIIIa	PhCO-Phe-Ala-Pro	0.55	2.8×10^{-6}
XXI	PhCO-Phe-MeAla-Pro	0.65	inactive

effects of the N-terminal amino acids are different in the two series. Thus, compound *XII* is more active than the tripeptide *XI*, whereas compound *V* is less active than *IV*.

In the series of acylated tripeptides with the structure Acyl-AA_1-AA_2-Pro (Table II), the benzoyl residue gives the highest activities (*XXIIIa*, *XXIVa*, *XXV*, *XXVIa*). Contrary to the most of other peptides, the acylation of the tripeptide Ser(Ph)-Ala-Pro enhances the inhibitory activity (*XXVIa,b*).

Acylation of the peptides of the common structure AA_1-Arg-Pro leads to compounds with sligthly reduced or enhanced activity (*XXXIV* – *XXXVII*). Therefore, in contrast to the complete loss of activity of the peptide D-Phe-Ala-Pro by acylation with the adamantoyl residue (*XXIVb*), the combination of this hydrophobic residue with the hydrophilic peptide D-Phe-Arg-Pro enhances the activity (*XXVa*).

The comparison of the activities in Table II with free peptides in Table I indicates, that no direct relations exist between free and benzoylated peptides. The complete loss of the activity in compound *XXXII* seems to be unexpected because of the described high activity of the dipeptide D-Cys-Pro (ref.[10]). On the other hand the loss of the activity by the methylation of the penultimate peptide bond is in good agreement with our findings for analogues of the nonapeptide $BPP_{9\alpha}$ (ref.[4]).

Coupling of the hydrophobic and bulky adamantoyl residue to the hydrophobic sequences of the common structure AA_1-AA_2-Pro destroys the biologically active structure (*XXIIIc*, *XXIVb*, *XXVIII*). Contrary to this finding, the combination of the hydrophobic adamantoyl residue with arginine containing peptides yields compounds with enhanced or sligthly reduced activities (*XXXVa*, *XXXVIa*). The *p*-hydroxyphenyl-propionyl-residue seems to be favourable in the same manner as the benzoyl residue (*XXIIIb*, *XXVIb*, *XXXVb*, *XXXVIb*). The most striking fact of the acylated and nonacylated dipeptides in Tables I and II is the unexpected high activity of compounds *XVIIIc*, *XIXa* and *XX*.

Summarizing this part of our studies we might conclude that inhibitory activities of acylated di- and tripeptides depend on the combination of the acyl residue with certain sequences. It seems to be impossible to calculate the biological activity as a summary value from distinct contributions of the acyl-residue and the sequence. However, combination of hydrophobic acyl residues with hydrophilic peptides is favoured.

Oral application of some of the di- and tripeptides (*XI*, *XII*, *XX*, *XXIIIa*, *XXIVa*, *XXIVb*, *XXV*, *XXIXa*, *XXIXb*, *XXXII*, *XXXVa*, *XXXVb*, *XXXVIa*) was attempted on normotensive rats. We found that only acylated tripeptides are active after oral administration. Free tripeptides and free or acylated dipeptides reflect only a very short effect on the blood pressure. Fig. 1 shows the inhibition

of the angiotensin I induced blood pressure enhancement by 2 acylated tripeptides in comparison with the action of the commercial products Captopril, Enalapril (MK 421) and Ramipril (Hoe 498). The acylated tripeptides are less active than the commercial inhibitors. The duration of the ACE inhibition differs from 20 minutes to few hours. None or no good correlation exists between the inhibition of the isolated enzyme and the in vivo activity after oral application. Surprisingly, tripeptides with the benzoylphosphoryl residue (*XXII*) are inactive after oral application[11].

Furthermore, the results with our acylated tripeptides indicate that the acyl residue and nonproteinogenic amino acids stabilize the peptides to some degree against enzymatic degradation in the gastrointestinal tract and in the

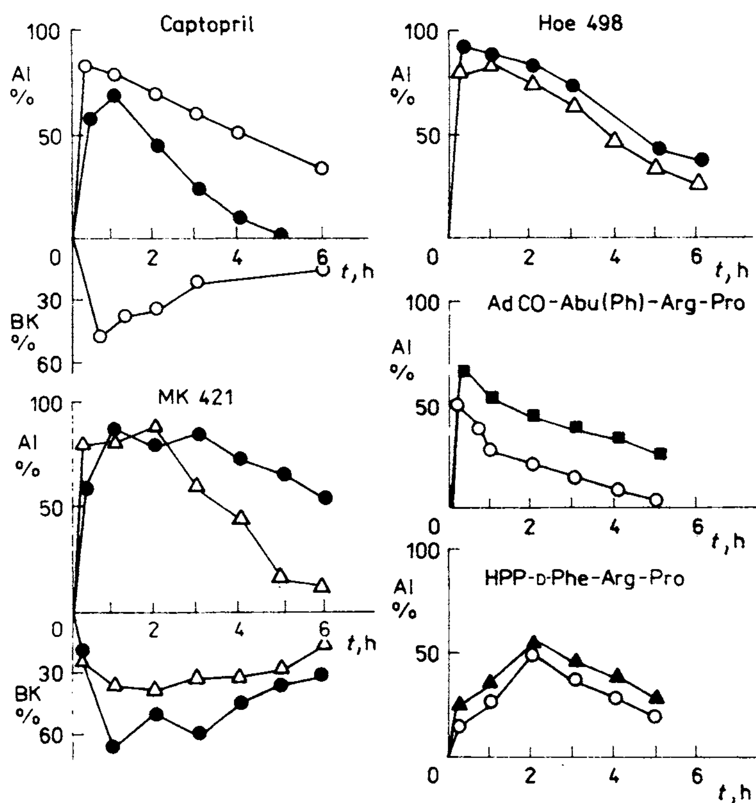

FIG. 1

Inhibition of the angiotensin I induced blood pressure enhancement (AI), and potentiation of the bradykinine action (BK) by orally given inhibitors. △ 0.3 mg kg; ● 1 mg kg; ○ 3 mg/kg; ▲ 6 mg/kg; ■ 10 mg kg. Captopril, MK 421 and Hoe 498 are commercial products

blood. For pharmaceutical use the inhibitory activity of the tripeptides must be enhanced in a second step by modifications of the peptide bond.

It seems to be commonly assumed that potentiation of the bradykinin action results from the inhibition of the bradykinin inactivation by ACE. But there exist some findings from the literature[12] and from our own studies[9,13] which contrast with this explanation. Table III lists some compounds with marked differences between the bradykinin potentiating activity and the inhibition of the isolated enzyme. Differences in these two types of activities reach in some cases 1 or 2 orders of magnitude. It seems that the inhibition of the enzyme is more sensitive to changes in the structure than the potentiating activity. These results indicate different structural requirements for the potentiating and inhibitory activity. From this finding and our results with bradykinin binding studies[13] we assume that the potentiation mechanism is based on the increase of the affinity of the bradykinin receptor. These results might be of interest also for the therapeutical use of peptide inhibitors of the ACE.

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