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New bradykinin B_2 receptor antagonists — influence of C-terminal segment modifications on their pharmacological properties★

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In the present study we describe the synthesis and some pharmacological properties of eight new analogues of bradykinin (BK). Two peptides were designed by substitution of position 7 or 8 of the known [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK antagonist (Stewart's antagonist) with L-pipecolic acid (L-Pip). The next two analogues were obtained by replacement of the D-Phe residue in position 7 of the Stewart's peptide with L-β^2-isoproline (L-β^2-iPro) or L-β^3-homoproline (L-β^3-hPro). The four analogues mentioned above were also prepared in N-acylated form with 1-adamantaneacetic acid (Aaa). Biological activity of the compounds was assessed by isolated rat uterus and rat blood pressure tests. Our results showed that L-Pip in position 7 slightly increased antagonistic potency in the blood pressure test, but it turned the analogue into an agonist in the rat uterus test. Replacement of Thi by L-Pip in position 8 also enhanced antagonism in the rat pressure test but preserved the antagonism in the rat uterus test. L-β^2-iPro or L-β^3-hPro in position 7 decreased the potencies in both tests. We also demonstrated that acylation of the N-terminus did not increase, as was claimed previously, the antagonistic potencies of the resulting peptides. The results thus support the hypothesis about the existence of different types of BK receptors in the rat uterus and blood vessels. Our studies provide new information about the structure–activity relationship of BK antagonists which may help in designing more potent BK receptor blockers.

Keywords: bradykinin, B_2 antagonists, rat blood pressure test, *in vitro* rat uterotonic test, sterically restricted residue

INTRODUCTION

Over the past two decades it has become clear that various kinins and their receptors are involved in numerous physiological functions. From the standpoint of therapeutic intervention, the well known pro-inflammatory effects of bradykinin (BK) and its metabolites mediated through B_1 and B_2 receptors (Fein *et al.*, 1997; Ahluwalia & Perretti, 1999; McLean *et al.*, 2000; Couture *et al.*, 2001; Marceau & Regoli, 2004) are prominent among those processes. Some severe disease conditions, such as chronic in-

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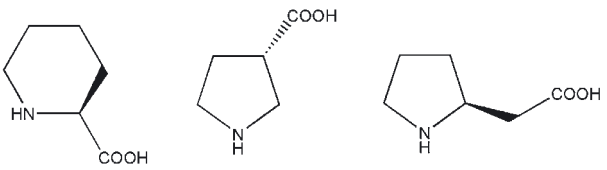
Abbreviations: Aaa, 1-adamantaneacetic acid; Acc, 1-aminocyclohexane-1-carboxylic acid; Apc, 1-aminocyclopentane-1-carboxylic acid; D-1-Nal, 1-naphthyl-D-alanine; D-2-Nal, 2-naphthyl-D-alanine; DMF, *N,N,*-dimethylformamide; D-Tic, D-1,2,3,4,-tetrahydroiso-quinoline-3-carboxylic acid; HOBt, 1-hydroxybenzotriazole; HR, heart rate; Hyp, L-hydroxyproline; L-Pip, L-pipecolic acid; L-β^2-iPro, L-β^2-isoproline; L-β^3-hPro, L-β^3-homoproline; MAP, mean arterial pressure; NMM, *N*-methylmorpholine; NMP, 1-methyl-2-pyrrolidone; Oic, octahydroindole-2-acetic acid; Pbf, 2,2,4,6,7-pentamethyldihydrobenzo-furan-5-sulfonyl; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; Thi, L-thienylalanine; TIS, triisopropylsilane.

flammatory pain, sepsis, edema, head trauma and asthma, have their basis in the inflammatory response. In this situation, development of the B_1 and B_2 receptor antagonists could provide novel therapeutics. The therapeutic targets have been the subject of intense research for some time now (Fujie *et al.*, 1993; Perkins *et al.*, 1993; Sharma, 1993; Fink, 1998; Stewart *et al.*, 1999; Heitsch, 2003; Howl & Payne, 2003; Zausinger, 2003) and a limited number of published human clinical studies have been conducted concerning pain, asthma, rhinitis and septic shock (Whalley *et al.*, 1987; Proud *et al.*, 1995; Akbary *et al.*, 1996; Fein *et al.*, 1997; Turner *et al.*, 2001) showing some level of efficacy with BK antagonists. A key question that awaits appropriate clinical trials is to what extent the results from animal studies translate to humans. The therapeutic areas currently of most interest are driven by medical needs and include pain, cardiovascular function, asthma and cancer.

The B_2 receptor had not been clearly defined until 1985 when the first generation of antagonists based on [D-Phe^7]BK was produced (Vavrek & Stewart, 1985). In those early compounds (e.g. [Thi^{5,8},D-Phe^7]BK), the transition to partial agonist or an antagonist was caused by a structural change introduced by a non-natural amino-acid residue (D-Phe). The added rigidity at this position in subsequent peptide antagonists such as icatibant (Hoe 140, [D-Arg^0,Hyp^3,Thi^5,D-Tic^7,Oic^8]BK) (Hock *et al.*, 1991) and NPC17731 ([D-Arg^0,Hyp^3,D-HypE(transpropyl)^7, Oic^8]BK) (Trifilieff *et al.*, 1993) showed that the spatial orientation of the C-terminal region of the peptide molecule was critical for antagonism. Icatibant exhibits a high affinity for the B_2 receptor, no residual agonist activity in most mammalian species and an impressive resistance to peptidases. The combination of these properties contributes to its prolonged action (several hours) in animal models.

In our laboratory we have explored several structural factors that might affect the antagonistic properties of BK analogues (Lammek, 1994; Prahl *et al.*, 1997a; Labudda-Dawidowska *et al.*, 2005; Labudda *et al.*, 2006). We confirmed that the antagonistic activity at the B_2 receptor might be attributed to peptides having at position 7 a suitable achiral, non-aromatic, conformationally constrained amino acid, e.g. 1-aminocyclohexane-1-carboxylic acid (Acc). We described BK analogues modified by introduction of an ethylene-bridged dipeptide unit, -&D-Phe-(&)D-Phe-, in positions 6–7 or 7–8 and Acc or 1-aminocyclo-pentane-1-carboxylic acid (Apc) residues in positions 6, 7 or 8 of the known [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK antagonist (Stewart's antagonist). We demonstrated the importance of appropriate localization in the molecule of the modification used for ensuring the antagonistic activity. Our



L-pipecolic acid (L-Pip) L-β^2-isoproline (L-β^2-iPro) L-β^3-homoproline (L-β^3-hPro)

Figure 1. Non-coded amino acids used in the study.

results, obtained for analogues modified at position 7 of the previously synthesized antagonist with 1-naphthyl-D-alanine (D-1-Nal) or 2-naphthyl-D-alanine (D-2-Nal) residues (the first one exhibited low potency, the latter was distinctly effective) highlighted the importance of conformation of the C-terminal part of the peptide for its antagonistic properties. It should be emphasized that the great difference observed in the activities is due to a minor change in the structure of the analogues; they differ only in the connection of the naphthalene ring to the backbone of the molecule — through either position 1 or 2 (Prahl *et al.*, 1997b). We reported that acylation of the N-terminus of B_2 antagonists with various bulky acyl groups (e.g. 1-adamantaneacetyl, 1-adamantanecarbonyl, 4-tert-butylbenzoyl, palmitoyl, etc.) improved the antagonistic potency in rat blood pressure assay (up to 33-fold) (Lammek, 1994).

Bearing all this in mind, we decided to check how the replacement of amino-acid residues at positions 7 or 8 of the [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK antagonist synthesized by Stewart's group (Schachter *et al.*, 1987) with L-pipecolic acid (L-Pip) residue (which may be considered as homoproline, see Fig. 1) would influence pharmacological properties of the resulting analogues (**I** and **III**). L-Pip should reduce the flexibility of the peptide backbone similarly to the Acc modification. Like the Acc residue, the L-Pip ring also consists of six atoms, but it has a nitrogen atom in its structure. The next two peptides (**V** and **VII**) were designed by substitution of position 7 of the Stewart's antagonist with other proline derivatives, either its structural isomer, L-β^2-isoproline (L-β^2-iPro), or its homologue, L-β^3-homoproline (L-β^3-hPro), see Fig. 1, the structures differing only slightly. It is worth emphasizing that this position in the BK molecule is occupied by a Pro residue. The aforementioned four analogues (**I, III, V** and **VII**) were also synthesized in the N-acylated form using 1-adamantaneacetic acid (Aaa) (**II, IV, VI** and **VIII**).

MATERIALS AND METHODS

Experimental. High-performance liquid chromatography (HPLC) was carried out on a Waters

(analytical and semi-preparative) chromatograph equipped with a UV detector (λ = 226 nm). The purity of the peptides was determined on a Hypersil C_{18} column (5 μm, 100 Å; 250 × 4.6 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] 80% acetonitrile in aqueous 0.1% TFA (v/v). A linear gradient from 20 to 80% of [B] in 30 min was applied for peptides at a flow rate of 1 mL/min. Semi-preparative HPLC was carried out using a Waters C_{18} column (15 μm, 100 Å; 7.8 × 300 mm) in a linear gradient from 15 to 45% of [B] in 60 min at a flow rate of 2.5 mL/min. The FAB/MS of the peptides were recorded on a TRIO-3 mass spectrometer at 7 keV with argon as the bombarding gas and on a Bruker BIFLEX III MALDI TOF mass spectrometer (ionization: 337 nm nitrogen laser).

Peptide synthesis. The peptides were synthesized by the solid-phase method using the Fmoc strategy and starting from Fmoc-Arg(Pbf) Wang resin (Wang, 1973) (GL Biochem Shanghai Ltd., 1% DVB, 100–200 mesh, 0.4 mmol/g). Fmoc was removed by 20% piperidine in DMF. A 3-fold excess of respective Fmoc-amino acids was activated *in situ* using TBTU (1 eq) / HOBt (1 eq) in a mixture of DMF/NMP (1:1, v/v) containing 1% Triton, and coupling reactions were base-catalyzed with NMM (2 eq). The amino acid side-chain protecting groups were Bu^t for Hyp and Ser and Pbf for Arg and D-Arg. All the Fmoc-protected amino acids were commercially available (NovaBiochem, Bad Soden, Germany). L-β^2-iPro was prepared from 4-hydroxyproline using a known procedure (Klein *et al.*, 1997; Cardillo *et al.*, 2000). For the synthesis of L-β^3-hPro we used modified version of the procedure described by Cardillo *et al.* (2002). Aaa was coupled in the final coupling step (peptide **II, IV, VI, VIII**) using the same procedure as that for the Fmoc-amino acids. Cleavage of the peptides from the resin with side-chain deprotection was performed by treatment with TFA/H_2O/TIS (95.5:2.5:2.5, by vol.) for 4 h. The total volume of the TFA filtrate was evaporated *in vacuo* to about 1 mL. The peptides were precipitated with cold diethyl ether and filtered through a Schott funnel. All the peptides were purified by semi-preparative HPLC.

The physicochemical properties of peptides **I-VIII** are presented in Table 1.

Effect of BK analogues on rat blood pressure. The antagonistic potency of the BK analogues was assessed by their ability to inhibit the vasodepressor response to exogenous BK in conscious rats. Male Wistar rats (n = 89; 318–458 g) were maintained on a regular chow diet and tap water at ambient temperature (22±1°C). The assay of the antagonistic potency of the BK analogues was the same as described in detail in references (Labudda-Dawidowska *et al.*, 2005; Labudda *et al.*, 2006; 2007) with minor modifications.

Table 1. Some physicochemical properties of analogues I–VIII

Analogue	Formula	HPLC T_R [min]	$[M+H]^+$	
			Calculated	Found
I	$C_{53}H_{83}N_{19}S_2O_{13}$	13.75	1258.9	1258.9
II	$C_{65}H_{99}N_{19}S_2O_{14}$	19.35	1433.9	1434.0
III	$C_{55}H_{85}N_{19}S_2O_{13}$	15.68	1253.1	1252.9
IV	$C_{67}H_{101}N_{19}S_2O_{14}$	19.93	1428.1	1428.4
V	$C_{52}H_{81}N_{19}S_2O_{13}$	11.50	1245.2	1245.4
VI	$C_{64}H_{97}N_{19}S_2O_{14}$	17.58	1420.2	1420.7
VII	$C_{53}H_{83}N_{19}S_2O_{13}$	12.57	1259.2	1258.5
VIII	$C_{65}H_{99}N_{19}S_2O_{14}$	18.41	1434.2	1434.1

The rats had two arteries cannulated and all catheters were exteriorized subcutaneously at the back of the neck. A Y-type connector was mounted to the carotid artery line. Following a 40–44 h recovery, mean arterial pressure (MAP) and heart rate (HR) were monitored through a Gould-Statham P23-ID pressure transducer (Gould, Cleveland, OH, USA) connected to the iliac catheter. Angiotensin converting enzyme (kininase II) inhibitor, enalapril (Merck Sharp and Dohme Research Lab., Rahway, NJ, USA; 1 mg/kg), was injected into the iliac artery to stabilize the blood pressure. To provide homogeneity of further assay, rats with MAP within the range of 85–135 mmHg and HR 270–350 beats/min were arbitrarily enrolled to the study. Two doses of BK (bradykinin acetate salt; Sigma, USA), 125 ng or 250 ng per animal, dissolved in 5% D-glucose at a concentration of 1.25 μg/mL, were randomly injected four or five times, every 4 to 5 min, into one branch of the carotid catheter. The rats which exhibited vasodepressor response to 250 ng BK (ΔMAP) within the range of 20 to 45 mm Hg were enrolled to the study. In accordance with the previous study (Labudda *et al.*, 2007), those rats which displayed the vasodepressor response to 125 ng BK higher than 82% of that evoked by 250 ng BK were not included in the blood pressure test. The BK dose of 250 ng was used as a standard in further assay.

The BK analogues dissolved in 5% D-glucose were infused (constant infusion rate 80 μL/min) to the other branch of the carotid catheter. The testing was initiated with the 10-min infusion at a concentration of 1 μg/mL (this gave a dose of 0.08 μg/min per rat). During this infusion, the 250 ng dose of BK was injected twice: at 270 s and at 570 s. The concentration of the infused BK antagonist was then increased to 4, 16, 64, 256, 1024 μg/mL and, if necessary, up to 2048 or 3580 μg/mL, to give the doses

of 0.32, 1.28, 5.12, 20.48, 81.92, 163.84 and 286.40 μg/min per rat, respectively. The BK analogue infusion at concentrations of 1024 μg/mL and higher was limited to 5 min and BK was injected only once by the end of the infusions. As soon as the vasodepressor response to BK was lower than 10% of the standard response, the procedure was completed.

The percent inhibition of the vasodepressor response to 250 ng BK by the tested BK antagonist was plotted against the logarithm of the dose. From these dose response curves, effective doses ED_{20}, ED_{50} and ED_{90}, were determined representing doses of the BK antagonist (μg/min) that inhibit vasodepressor response to the agonist (250 ng of BK) by 20, 50 and 90%, respectively. The results are reported as mean values ± S.E.M.

The *in vitro* rat uterotonic test. All the analogues were evaluated in the *in vitro* rat uterotonic assay using a modified Holton's method (Holton, 1948) in Munsick solution (Munsick, 1960) on a strip of rat uterus. Wistar rats in estrus induced by injection of estrogen 48 h before the experiments were used. BK was used as a standard over a concentration range of 10 pM to 10 nM. Cumulative dose-response curves were constructed both in the absence and presence of various doses of the analogues, i.e. doses of BK were added successively to the organ bath in doubled concentrations and at 1-min intervals, without the fluid being changed, until the maximum contraction was obtained. The height of a single isometric contraction in response to different doses of BK was measured. The antagonist was applied to the organ bath 1 min prior to the cumulative dosing of BK. The antagonistic activity was expressed as pA_2, i.e. negative common logarithm of the analogue concentration which shifted the dose-response curve of BK by a factor of two (comparison of the linear portions of the dose-response curves). Each analogue was tested on uteri taken from 3–5 rats. As far as the agonistic activity of the analogues is concerned, it was calculated by comparing the threshold doses of cumulative dose–response curves of the analogue and that of standard BK. The activity of BK was taken as 100%.

RESULTS

The eight new BK analogues (**I–VIII**) were synthesized in excellent purity (higher than 98% according to analytical HPLC) and satisfactory yield for biological testing. Pharmacological properties of the new compounds and Stewart's antagonist (Schachter *et al.*, 1987) used as a positive control are summarized in Table 2. The potency was assayed in the rat uterus test and blood pressure test using conscious rats as described in the Materials and Methods section.

Basal hemodynamic characteristics of the tested rats are typical of unrestrained rats at daytime. The whole population appeared homogeneous, since no significant differences among selected rat subsets were found. Prior to the administration of BK, MAP averaged 113.68 ± 1.59 mm Hg (ranging from 94 to 135 mm Hg) and HR was 311.30 ± 2.70 beats/min (ranging from 278 to 345 beats/min). BK evoked a significant vasodepressor response (19.45 ± 1.10 and 26.42 ± 1.36 mm Hg, for doses 125 and 250 ng, respectively). The relatively low HR recorded during experiments confirmed good recovery of the rats from the surgical procedures and also good acclimatization to the experimental conditions. No signs of discomfort were noticed during the test.

Four out of eight tested BK analogues: **III, V, VI** and **VII** elicited a direct vasodepressor response at low doses. Although endogenous BK is not the major vasodilatory contributor that takes part in the regulation of arterial pressure, the evoked decrease in MAP may suggest some BK agonistic potency. On the other hand, all of the aforementioned BK analogues inhibited the vasodepressor responses of exogenous BK at high doses.

It is of interest that some of the tested BK analogues (**V** and **VI**) evoked tachyphylaxis to BK: when BK was administered at intervals shorter than 5 min, the second vasodepressor response was regularly lower. From a practical point of view, the development of tachyphylaxis may disturb the antagonistic potency assay. Unlike in our previous tests (Labudda-Dawidowska *et al.*, 2005; Labudda *et al.*, 2007), we decided to take the response to the initial dose of BK during infusion of the BK analogues for the data analysis. The possibility that a BK analogue may induce tachyphylaxis to BK indicates that one should evaluate the rough data with caution. Eventually, in bioassays consisting of repetitive BK dosage during BK analogue infusions, higher antagonistic potency of BK analogues may be estimated than that calculated from an assay based on a less extensive dosage pattern.

The potencies of our analogues in the rat blood pressure test are summarized in Table 2. Compound **I** possessed high antagonistic potency, comparable to that of the Stewart's peptide. Its acylated form was, however, highly potent only at low doses (20% inhibition of the effect of BK by 1.3 μg/min). Data for analogue **III**, which was designed by replacement of Thi at position 8 of the model peptide by the L-Pip residue, showed that this modification resulted in a potent antagonist of BK in the test. At low doses (ED_{20}), its activity was approximately 4 times higher than that of the Stewart's peptide and it was about 5 times more potent when inhibiting

Table 2. Pharmacological properties of analogues I–VIII

Analogue		Uterotonic potency (% of activity of BK or pA_2)	Vasodepressor potency		
			ED_{20} [μg/min]	ED_{50} [μg/min]	ED_{90} [μg/min]
[D-Arg^0–Hyp^3,Thi^{5,8},D-Phe^7]-BK Stewart's antagonist		pA_2 = 7.70 ± 0.13	0.43 ± 0.03	3.19 ± 0.33	52.60 ± 10.59
[D-Arg^0–Hyp^3,Thi^{5,8},L-Pip^7]-BK	**(I)**[a]	18.3%	0.23 ± 0.04*	2.05 ± 0.26	44.98 ± 10.64
Aaa[D-Arg^0–Hyp^3,Thi^{5,8},L-Pip^7]-BK	**(II)**	0.5%	1.30 ± 0.46	40.67 ± 14.38	4456 ± 1575*
[D-Arg^0–Hyp^3,Thi^5,D-Phe^7,L-Pip^8]-BK	**(III)**	pA_2 = 7.43 ± 0.26	0.10 ± 0.016*	0.73 ± 0.09	11.35 ± 1.76
Aaa[D-Arg^0–Hyp^3,Thi^5,D-Phe^7,L-Pip^8]-BK	**(IV)**	pA_2 = 7.99 ± 0.25	0.16 ± 0.03*	1.43 ± 0.22	28.99 ± 6.15
[D-Arg^0–Hyp^3,Thi^{5,8}, L-β^2–iPro^7]-BK	**(V)**	<0.01%	agonist	weak antagonist	weak antagonist
Aaa[D-Arg^0–Hyp^3,Thi^{5,8}, L-β^2–iPro^7]-BK	**(VI)**[b]	<0.01%	agonist	weak antagonist	weak antagonist
[D-Arg^0–Hyp^3,Thi^{5,8}, L-β^3–hPro^7]-BK	**(VII)**	1.9%	15.94 ± 2.37*	127.02 ± 34.11	1441 ± 413*
Aaa[D-Arg^0–Hyp^3,Thi^{5,8}, L-β^3–hPro^7]-BK	**(VIII)**	0.1%	3.58 ± 0.80	76.05 ± 23.29	6141 ± 2483*

Notes: In uterotonic test, agonistic activity was calculated as percentage of BK activity (set to 100%); antagonistic activity was calculated as pA_2 (negative common logarithm of analogue concentration shifting the log dose-response curve for BK by a factor of 0.3 to the right: calculations were made from linear portions of curves); ED_{20}, ED_{50} and ED_{90} represent doses of BK antagonist (μg/min) that inhibit vasodepressor response to 250 ng of BK by 20, 50 and 90%, respectively. *values ED_{20} or ED_{90} extrapolated from dose-response curve; [a]partial agonist properties: exhibited tendency towards decrease of MAP; [b]produced substantial tachyphylaxis to BK (when BK was administrated repeatedly at intervals shorter than 5 min the second vasodepressor response to BK was regularly lower).

the effect of BK by 90%. Acylation of compound **III** with Aaa decreased the antagonistic activity about 2-fold. Notwithstanding, peptide **IV** was still more potent than the reference one. Analogues **V** and **VI** were weak B_2 agonists at low doses. They showed, however, a weak antagonism of BK at high doses. Compound **VIII** possessed weak antagonistic properties as compared to those of the model peptide. Unlike the other tested pairs of analogues, the non-acylated counterpart **VII** showed only negligible antagonistic activity.

In the *in vitro* uterus assay compounds **I, II, V, VI, VII** and **VIII** displayed agonistic potency much lower than that of BK. The dose-response curves for the analogues were parallel to that for bradykinin. The analogues showed no antagonistic activity. Peptide **III** and its acylated counterpart **IV** exhibited rather potent antagonism, comparable or even higher, respectively, than that of the Stewart's peptide.

DISCUSSION

In the present study we continue our previous investigations of BK analogues to find structural requirements which result in high B_2 antagonistic activity. Recently we reported that the Acc residue could be accepted in positions 7 and 8 of the model B_2 antagonist molecule — the Stewart's antagonist. We found that the Acc^8 modification resulted in an increase of B_2 antagonistic potency in blood pressure test and preservation of the antagonistic properties in the rat uterus test. As a result of this substitution in position 7, we obtained analogues inactive in the rat uterus test but showing BK antagonistic potency in the blood pressure test. Nevertheless, their activity was lower than that of the model peptide. In the present work we substituted positions 7 or 8 of the same model peptide with the L-Pip residue. Compared to Acc, the ring of L-pipecolic acid also consists of six atoms, but has a nitrogen atom in its structure. Previous theoretical and experimental studies have shown that 1-aminocycloalkane-1-carboxylic acids impart considerable stereochemical rigidity to peptide backbones, which are constrained to adopt conformations in the $3_{10}/\alpha$-helical regions of the ϕ and ψ spaces (Paul *et al.*, 1986; Toniolo *et al.*, 1989; Toniolo & Benedetti, 1994; Paradisi *et al.*, 1995; Sikorska & Rodziewicz-Motowidło, 2008). Similar to Acc modifications, L-Pip changed the character of the relevant fragment of the molecule from aromatic to aliphatic and reduced its conformational freedom. From the presented results it is clear that replacement of the residue at position 8 by L-Pip, similar to Acc^8 modification, is advantageous for B_2 antagonistic potency. The resulting analogue retains the antagonistic properties also in the utero-

tonic test. A similar though less distinct effect was noticed following L-Pip^7 substitution. Analogue **I** is another example of B_2 antagonist with suitable non-aromatic conformationally constrained amino-acid residue at this position that supports our previous results (Prahl *et al.*, 1997a; 1997b; Derdowska *et al.*, 2001; Labudda-Dawidowska *et al.*, 2005; Labudda *et al.*, 2006).

When designing compounds **V** and **VII**, we focused once more on position 7, which is occupied by a Pro residue in the BK molecule. We decided to substitute D-Phe^7 in Stewart's peptide with a structural isomer of proline, L-β^2-iPro or its homologue, L-β^3-hPro. As mentioned above, in the blood pressure test, analogue **V** was a weak B_2 agonist at low doses, exhibiting weak antagonism at higher doses, while compound **VII** possessed antagonistic properties much lower than that of the model compound. Peptides **V** and **VII** differ from one another by the presence of the proline derivatives with only slightly different structures (see Fig. 1). In our opinion, this minor structural difference may have a significant impact on bioactive conformations of the molecules and consequently may influence their interaction with B_2 receptors. These findings seem to support our previous results obtained for modification of position 7 of the BK analogues with the D-1-Nal and D-2-Nal residues (Prahl *et al.*, 1997a).

Our hypothesis that acylation of the N-terminus of BK antagonists with bulky groups regularly improves the antagonistic potency in the blood pressure test, substantiated by our earlier results (Lammek, 1994; Lammek *et al.*, 1990; 1991), does not appear to be valid for each pair of the analogues. In this study, the acylated compounds, with one exception, were less potent than the non-acylated ones (analogues **I** and **II**, **III** and **IV**, **V** and **VI**). In the case of the rat uterus test, acylation resulted in either retention (peptides **V** and **VI**) or suppression of the agonistic activity (peptides **I** and **II, VII** and **VIII**), or enhancement of antagonistic potency (peptides **III** and **IV)**.

Two of the new analogues (**III** and **IV**) are potent B_2 antagonists in both assays. However, other tested peptides which showed a strong (**I** and **II**) or moderate (**VIII**) antagonistic potency in the blood pressure test turned out to be agonists in the rat uterus test. This would support the idea of the presence of different types of BK receptor in the rat uterus and blood vessels.

The question of the existence of more than two BK receptor subtypes within a single species remains a great issue in the BK receptor field. The possibility of interspecies differences has clearly been shown because predicted amino-acid sequences of cloned receptors in different species vary. The most conclusive experimental evidence for species-related differences in BK receptors relates to studies with B_2 receptor antagonists. For peptide antagonists, affinities are generally greater in preparations taken from the rat and rabbit than in that taken from the guinea pig; contrary is the case for the non-peptide antagonist WIN64338 (Hall & Morton, 1997). Indeed, an analysis of these and other data has led one group to propose the existence of B_{2A} (rabbit, dog, and possibly man) and B_{2B} (guinea pig, hamster, rat) receptors (Regoli *et al.*, 1994). The strongest evidence for a B_3 receptor comes from the work of Farmer and colleagues who reported that various B_1 and B_2 receptor antagonists had very weak affinities and did not displace specific BK binding in the guinea-pig trachea (Farmer *et al.*, 1989). However, subsequent studies by this group and other ones gave conflicting results and no clear-cut conclusions can be made regarding the existence of B_3 receptors (Hall & Morton, 1997). It should be noted, however, that up to the present there is no evidence for more than one gene for B_2 receptor within a single species and B_2 receptor gene knockout mouse has lost all responses to exogenous BK (Borkowski *et al.*, 1995). Alternative splicing of a single gene transcript cannot also be ruled out. Some of the pharmacological discrepancies observed with BK and its antagonists may be explained by the interaction with orphan G-protein-coupled receptors for which ligands are not known. Recently, Boels and Schaller (2003) described the discovery and tissue-specific expression of a novel human gene encoding GPCR 100 (*GPR100*). This orphan receptor is present in locations where kinins act (such as heart, skeletal muscle, some endocrine glands and brain areas) and is claimed to be a new high-affinity receptor for BK and kallidin.

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REFERENCES

Ahluwalia A, Perretti M (1999) B_1 receptors as a new inflammatory target. Could this B the 1? *Trends Pharmacol Sci* **20**: 100–104.

Akbary A, Wirth KJ, Schölkens BA (1996) Efficacy and tolerability of icatibant (Hoe 140) in patients with moderately severe chronic bronchial asthma. *Immunopharmacology* **33**: 238–242.

Boels K, Schaller HC (2003) Identification and characterization of GPR100 as a novel human G-protein-coupled bradykinin receptor. *Br J Pharmacol* **140**: 932–938.

Borkowski JA, Ransom RW, Seabrook GR, Trumbauer M, Chen H, Hill RG, Strader CD, Hess JF (1995) Targeted disruption of a B_2 bradykinin receptor gene in mice eliminates bradykinin action in smooth muscle and neurons *J Biol Chem* **270**: 13706–13710.

Cardillo G, Gentilucci L, Melchiorre P, Spampinato S (2000) Synthesis and binding activity of endomorphin-1 analogues containing β-amino acids. *Bioorg Med Chem Lett* **10**: 2755–2758.

Cardillo G, Gentilucci L, Qasem AR, Sgarzi F, Spampinato S (2002) Endomorphin-1 analogues containing β-proline are μ-opioid receptor agonists and display enhance enzymatic hydrolysis resistance. *J Med Chem* **45**: 2571–2578.

Couture R, Harrisson M, Vianna RM, Cloutier F (2001) Kinin receptors in pain and inflammation. *Eur J Pharmacol* **429**: 161–176.

Derdowska I, Prahl A, Neubert K, Hartrodt B, Kania A, Dobrowolski D, Melhem S, Trzeciak HI, Wierzba T, Lammek B (2001) New analogues of bradykinin containing a conformationally restricted dipeptide fragment in their molecules. *J Peptide Res* **57**: 11–18.

Farmer SG, Burch RM, Meeker SA, Wilkins DE (1989) Evidence for a pulmonary B_3 bradykinin receptor. *Mol Pharmacol* **36**: 1–8.

Fein AM, Bernard GR, Criner GJ, Fletcher EC, Good JT Jr, Knaus WA, Levy H, Matuschak GM, Shanies HM, Taylor RW (1997) Treatment of severe systemic inflammatory response syndrome and sepsis with a novel bradykinin antagonist, deltibant (CP-0127). Results of a randomized, double-blind, placebo-controlled trial. CP-0127 SIRS and Sepsis Study Group. *J Am Med Assoc* **277**: 482–487.

Fink MP (1998) Therapeutic options directed against platelet activating factor, eicosanoids and bradykinin in sepsis. *J Antimicrob Chemother* **41**: 81–94.

Fujie H, Hayashi I, Oh-ishi S (1993) The kinin released from high molecular weight-kininogen is responsible for inflammatory exudation in rats: detection of kinin-free-kininogen in the exudates by immunoblot analysis. *Life Sci* **53**: 1691–1701.

Hall JM, Morton IKM (1997) Immunopharmacology of bradykinin receptors. In *The Kinin System*. Farmer SG, *Handbook of Immunopharmacology*. The Academic Press, London.

Heitsch H (2003) The therapeutic potential of bradykinin B_2 receptor agonists in the treatment of cardiovascular disease. *Expert Opin Investig Drugs* **12**: 759–770.

Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke S, Breipohl G, König W, Knolle J (1991) Hoe 140 a new potent and long acting bradykinin-antagonist: in vitro studies. *Br J Pharmacol* **102**: 769–773.

Holton P (1948) A modification of the method of Dale and Lindlow for standardization of posterior pituitary extract. *Br J Pharmacol* **3**: 328–334.

Howl J, Payne SJ (2003) Bradykinin receptors as a therapeutic target. *Expert Opin Ther Targets* **7**: 277–285.

Klein SI, Czekaj M, Molino BF, Chu V (1997) Constrained β-alanine based GpIIb/IIIa antagonists. *Bioorg Med Chem Lett* **7**: 1773–1778.

Labudda-Dawidowska O, Wierzba TH, Prahl A, Kowalczyk W, Gawiński Ł, Plackova M, Slaninová J, Lammek B (2005) New bradykinin analogues modified in the C-terminal part with sterically restricted 1-aminocyclohexane-1-carboxylic Acid. *J Med Chem* **48**: 8055–8059.

Labudda O, Wierzba T, Sobolewski D, Kowalczyk W, Śleszyńska M, Gawiński Ł, Plackova M, Slaninová J, Prahl A (2006) New bradykinin analogues substituted in positions 7 and 8 with sterically restricted 1-aminocyclopentane-1-carboxylic acid. *J Peptide Sci* **12**: 775–779.

Labudda O, Wierzba T, Sobolewski D, Śleszyńska M, Gawiński Ł, Plackova M, Slaninová J, Prahl A (2007) New bradykinin analogues acylated on the N-terminus: effect on rat uterus and blood pressure. *Acta Biochim Polon* **54**: 193–197.

Lammek B (1994) Design and synthesis of B_2-antagonists of bradykinin. *Pol J Chem* **68**: 913–920.

Lammek B, Wang YX, Gavras I, Gavras H (1990) A new highly potent antagonist of bradykinin. *Peptides* **11**: 1041–1043.

Lammek B, Wang YX, Gavras I, Gavras H (1991) A novel bradykinin antagonist with improved properties. *J Pharm Pharmacol* **43**: 887–888.

Marceau F, Regoli D (2004) Bradykinin receptor ligands: therapeutic perspectives. *Nat Rev Drug Discov* **3**: 845–852.

McLean PG, Perretti M, Ahluwalia A (2000) Kinin B_1 receptors as novel anti-inflammatory targets. *Emerg Ther Targets* **4**: 127–141.

Munsick R (1960) Effect of magnesium ion on the response of the rat uterus to neurohypophysial hormones and analogues. *Endocrinology* **66**: 451–457.

Odya C, Goodfriend T, Pena C (1980) Bradykinin receptor-like binding studied with iodinated analogues. *Biochem Pharmacol* **29**: 175–185.

Perkins MN, Campbell E, Dray A (1993) Antinociceptive activity of the bradykinin B_1 and B_2 receptor antagonists, des-Arg^9[Leu^8]-BK and Hoe 140, in two models of persistent hyperalgesia in the rat. *Pain* **53**: 191–197.

Plevin R, Owen P (1988) Multiple B_2 kinin receptors in mammalian tissues. *Trends Pharmacol Sci* **9**: 387–389.

Prahl A, Derdowska I, Dawidowska O, Neubert K, Hartrodt B, Wierzba T, Juzwa W, Lammek B (2003) New bradykinin analogues substituted in positions 6 and 7 with naphthylalanine. *Polish J Chem* **77**: 881–887.

Prahl A, Wierzba T, Winklewski P, Musiał P, Juzwa W, Lammek B (1997a) Antagonists of bradykinin modified with conformationally restricted dipeptide fragment. *Polish J Chem* **71**: 929–935.

Prahl A, Wierzba T, Winklewski P, Wszędybył M, Cherek M, Juzwa W, Lammek B (1997b) Influence of C-terminal modifications of bradykinin antagonists on their activity. *Collect Czech Chem Commun* **62**: 1940–1946.

Proud D, Bathon JM, Togias AG, Naclerio RM (1995) Inhibition of the response to nasal provocation with bradykinin by HOE-140: efficacy and duration of action. *Can J Physiol Pharmacol* **73**: 820–826.

Regoli D, Gobeil F, Nguyen QT, Jukic D, Seoane PR, Salvino JM, Sawutz DG (1994) Bradykinin receptor types and B_2 subtypes. *Life Sci* **55**: 735– 749.

Schachter LR, Uchida Y, Londridge DJ, Łabędź T, Whalley ET, Vavrek RJ, Stewart JM (1987) New synthetic antagonists of bradykinin. *Br J Pharmacol* **92**: 851–855.

Sharma JN (1993) Therapeutic prospects of bradykinin receptor antagonists. *Gen Pharmacol* **24**: 267–274.

Sikorska E, Rodziewicz-Motowidło S (2008) Solution conformations of bradykinin antagonists modified with Cα–Cα cyclized nonaromatic residues. *J Peptide Sci* **14**: 819–829.

Steranka L, Manning D, Dehas C, Ferkany J, Borosky S, Connor J, Vavrek R, Stewart J, Shyder J (1988) Bradykinin as a pain mediator: Receptors are localized to sen-

sory neurons and antagonists have analgesic actions. *Proc Natl Acad Sci USA* **85**: 3245–3249.

Stewart JM, Gera L, York EJ, Chan DC, Bunn PA Jr (1999) Bradykinin antagonists: present progress and future prospects. *Immunopharmacology* **43**: 155–161.

Toniolo C, Crisma M, Valle G, Bonora GM, Polinelli S, Becker EL, Freer RJ, Sudhanand R, Rao RB, Balaram B, Sukumar M (1989) Conformationally restricted formyl methionyl tripeptide chemoattractants: a three-dimensional structure-activity study of analogs incorporating a C alpha, alpha-dialkylated glycine at position 2. *Pept Res* **2**: 275–281.

Toniolo C, Benedetti E (1994) Structures of polypeptides from α-amino acids disubstituted at the α-carbon. *Macromolecules* **24**: 4004–4009.

Paul PKC, Sukumar M, Bardi R, Piazzesi AM, Valle G, Toniolo C, Balaram P (1986) Stereochemically constrained peptides. Theoretical and experimental studies on the conformations of peptides containing 1-aminocyclohexanecarboxylic acid. *J Am Chem Soc* **108**: 6363–6370.

Paradisi M, Torrini I, Zecchini GP, Luente G, Gauvuzzo E, Mazza F, Pochetti G (1995) γ-Turn conformation induced by α,α-disubstituted amino acids with a cyclic six-membered side chain. *Tetrahedron* **51**: 2379–2386.

Trifilieff A, Amrani Y, Landry Y, Gies JP (1993) Comparative action of new highly potent bradykinin receptor antagonists in the guinea-pig trachea. *Eur J Pharmacol* **239**: 227–229.

Turner P, Dear J, Scadding G, Foreman JC (2001) Role of kinins in seasonal allergic rhinitis: icatibant, a bradykinin B_2 receptor antagonist, abolishes the hyperresponsiveness and nasal eosinophilia induced by antigen. *J Allergy Clin Immunol* **107**: 105–113.

Vavrek RJ, Stewart JM (1985) Competitive antagonists of bradykinin. *Peptides* **6**: 161–164.

Wang SS (1973) p-Alkoxybenzyl alcohol resin and p-alkoxybenzyloxycarbonyl hydrazide resin for solid phase synthesis of protected peptide fragments. *J Am Chem Soc* **95**: 1328–1333.

Whalley ET, Clegg S, Stewart JM, Vavrek RJ (1987) The effect of kinin agonists and antagonists on the pain response of human blister base. *Naunyn-Schmiedeberg's Arch Pharmacol* **336**: 652–655.

Zausinger S (2003) Bradykinin receptor antagonists in cerebral ischemia and trauma. *IDrugs* **6**: 970–975.