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Short Communication

Modifications in the bradykinin main chain are not necessary for antagonistic activity in rat blood pressure assay

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Abstract: Previous studies demonstrated that the presence of a bulky acyl substituent at the *N*-terminus of B_2 antagonists significantly influenced the interaction of the peptide with B_2 receptors, thus increasing potency in the blood pressure test. Reported results also suggested that even minor changes in the structure of analogues might be of importance in designing more potent B_2 antagonists. On the other hand, it was learned that the effects of acylation might vary substantially with the chemical character of the acyl group. It seemed that either the positive or the negative charge on the *N*-terminal acyl group influenced the activity of the analogues because a suppressed antagonistic potency due to these modifications was observed.

Bearing all these findings in mind, it was decided to check how *N*-terminal acylation would affect the pharmacological activity of bradykinin. Of the many acylating agents tested previously on B_2 antagonists, it was decided to use acridin-9-ylacetic acid (Ana) and anthracen-9-ylacetic acid (Ata). The potencies of the analogues were assessed by their ability to inhibit vasodepressor response of exogenous BK in conscious rats and by their ability to inhibit the contractions of isolated rat uterus evoked by BK.

Keywords: bradykinin; B_2 antagonists; rat blood pressure assay; rat uterotonic test *in vitro*

INTRODUCTION

Bradykinin (BK) is an endogenous tissue peptide hormone with the sequence Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg and produced in the body in response to many kinds of injuries and inflammatory states. BK is also an important mediator of pain, and the shock actions of BK are mediated by at least two classes of receptors, B_1 and B_2 [1]. Both these are G-protein coupled receptors. In brief, B_1 receptors are characterized by low affinity and particular sensitivity to des-(Arg^9)-BK and its selective antagonist is des-(Arg^9)-[Leu^8]-BK. Since the B_1 antagonists do not affect any important BK responses, they are of relatively low interest. The classic B_2 receptors are of high affinity and seem to require the full BK sequence for effective activation. Accumulated evidence indicates that most of the clinically relevant effects of BK are functions of B_2 receptors, this is why research on their antagonists is a topic of great interest.

The first report on the ability of BK analogues to antagonize the action of this hormone in a standard kinin assay was made by Stewart and Vavrek [2]. The critical change necessary to confer B_2 antagonistic activity on the analogues was the replacement of Pro^7 with a D-aromatic amino acid residue. This change, followed later by the replacement of the phenylalanine residues at positions 5 and 8 with β-thienylalanine (Thi), which was a modification previously implemented to design potent BK agonists, coupled with the D-Phe^7 substitution and addition of D-Arg residue on *N*-terminus of the analogue, gave the first practically useful B_2 antagonist [3]. Hundreds of analogues with single or multiple modifications were later synthesized in many laboratories. However, these compounds have a relatively low potency, which necessitates the use of high concentrations during physiological experiments. This may account for the partial agonistic effects via stimulation of the BK receptors of other tissues [4].

A major improvement in the potency of BK antagonists was achieved in 1991 when potent B_2 blockers carrying conformationally constrained amino acid residues in their *C*-terminal ends were first synthesized by scientists from Hoechst AG, and later by others [5,6]. In all cases, these were analogues substituted in position 7 with aromatic D-amino acid residues.

Previously, it was demonstrated that the presence of a bulky acyl substituent (1-adamantaneacetic acid, 4-tert-butylbenzoic acid, 1-adamantanecarboxylic acid,

Abbreviations: The symbols of the amino acids and peptides are in accordance with the 1983 'Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature' and 'Abbreviations and symbols in peptide science: a revised guide and commentary' published in *J. Pept. Sci.*, **12**, 1–12 (2006). Other abbreviations are as follows: Aca, adamant-1-ylcarboxylic acid; Aaa, adamant-1-ylacetic acid; Ana, acridin-9-ylacetic acid; Ata, anthracen-9-ylacetic acid; TIS, triisopropylsilane; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

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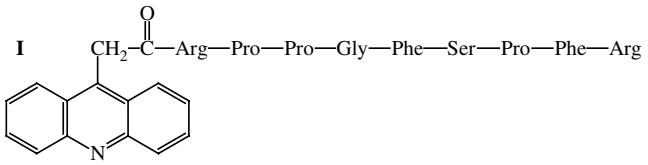
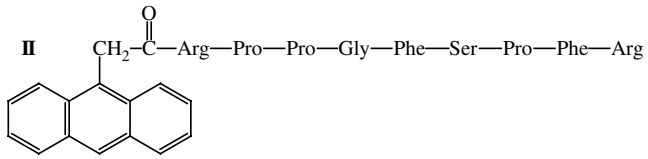
etc.) at the *N*-terminus of B_2 antagonists significantly influenced the interaction of the peptide with B_2 receptors, thus increasing potency in the blood pressure test [7]. Reported results also suggested that even minor changes in the structure of analogues might be of importance in designing more potent B_2 antagonists. On the other hand, it was learned that the effects of acylations might vary substantially with the chemical character of the acyl group [8,9]. It seemed that either the positive or the negative charge on the *N*-terminal acyl group influenced the activity of the analogues, as was observed by a suppressed antagonistic potency due to these modifications.

Bearing all these findings in mind, it was decided to verify how *N*-terminal acylation would affect the pharmacological activity of BK. Of the many acylating agents tested previously on B_2 antagonists, it was decided to use Ana and Ata. The structures of the new peptides **I** and **II** are given below.





EXPERIMENTAL

Thin-layer chromatography (TLC) was carried out on a silica plates (Merck). Spots were visualized with iodine or ninhydrin and a solvent system of butan-1-ol : acetic acid : water : ethyl acetate (1 : 1 : 1 : 1, v/v) [a] was used throughout.

Analytical high-performance liquid chromatography (HPLC) was carried out on a Waters Breeze System equipped with Waters 1525 binary pump, Waters 2487 dual λ absorbance detector, and Waters 717 plus autosampler. The purity of the peptides was determined on a Waters C_{18} column (5 μm, 100 Å; 150 × 4.6 mm). The following solvents were used: [A] 0.1% aqueous trifluoroacetic acid (TFA); [B] 80% acetonitrile in aqueous 0.1% TFA (v/v). A linear gradient from 0 to 70% of [B] for 40 min was applied for peptides at a flow rate of 1 ml/min. Preparative HPLC was carried out on a Waters Millennium System equipped with Waters 600 pump with 600 controller and Waters 2487 dual λ absorbance detector using a Kromasil C_8 column (5 μm, 100 Å, 25 × 250 mm; in a gradient running from 10 to 50% of [B] for 100 min at a flow rate of 10 ml/min, detection at λ = 226 nm). FAB/MS of 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 (±eV): 337 nm nitrogen laser).

Peptide Synthesis

The peptides were synthesized by the solid-phase method on a Symphony/Multiplex Multiple Peptide Synthesizer (Protein Technologies Inc., USA) using the Fmoc-strategy and starting from Fmoc-Arg(Pbf)-Wang resin [10] (loading 0.33 mmol/g, 50 μmol). Fmoc was removed by 20% piperidine in DMF. A five-fold excess of the respective Fmoc-amino acids was activated *in situ* using TBTU (1 equiv.)/HOBt (1 equiv.) in DMF and coupling reactions were base-catalysed with DIEA (4 equiv.). The amino acid side chain protecting groups were tBu for Hyp and Pbf for Arg and D-Arg. All the Fmoc-protected amino acids and Fmoc-Arg(Pbf)-Wang resin were purchased from NovaBiochem, Bad Soden, Germany. Ana and Ata purchased from Sigma, USA) were coupled in the final coupling step using the same procedure as that for 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 v/v/v) for 4 h. The total volume of the TFA filtrate was reduced to about 1 ml and the peptides were precipitated with cold diethyl ether. The solvents were evaporated under reduced pressure and the resulting materials dissolved in water and lyophilized. The crude products were desalted on a Sephadex G-15 column, eluted with aqueous acetic acid (30%) at a flow rate of 4.0 ml/h, and detected at λ = 254 nm. The eluates were fractionated and the fractions containing the major peak were pooled and lyophilized. All the peptides were purified by preparative HPLC. Physicochemical properties of the new analogues **I–II** are presented in Table 1.

Biological Evaluation

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, intact Wistar albino rats ($N = 58$; 325–392 g) were maintained on a regular chow diet and tap water at ambient temperature (22 ± 1 °C).

Two days before the experiment, two arterial lines were led out under pentobarbital (pentobarbital sodium −5–40–50 mg/kg i.p.) anesthesia for either injection of BK or infusion of the BK analogues. A Micro-Renathane

Table 1 Some physicochemical properties of BK analogues **I–II**

Analogue	Formula	HPLC T_R	$[M + H^+]$	
			Calculated	Found
(I) Ana-BK	$C_{65}H_{82}N_{16}O_{12}$	24.3	1279.4	1280.2
(II) Ata-BK	$C_{66}H_{83}N_{15}O_{12}$	30.6	1278.4	1278.9

High-performance liquid chromatography (HPLC) was carried out on a Waters (analytical) chromatograph equipped with a UV detector (λ = 226 nm). The purity of the peptides was determined on a Waters C_{18} column (5 μm, 100 A; 150 × 4.6 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA); [B] acetonitrile (0.1% aqueous TFA (80 : 20 v/v)). A linear gradient from 0 to 70% of [B] for 40 min was applied at a flow rate of 1 ml/min.

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polyurethane-based elastomer tubing (MRE-040, Braintree Scientific, USA) was inserted into the right carotid artery, while RenaPulse High Fidelity Pressure Tubing (RPT 040, Braintree Scientific, USA) with a tip made of Micro-Renathane MRE-040 tubing was indwelled into the right iliac artery. Both these tubings were particularly recommended for blood pressure measurement in experimental animals by the American Heart Association and reported to provide high fidelity of pulse waveform transmission with low susceptibility to intraluminal thrombosis. A Y-type connector was mounted to the tubing from the carotid artery. All catheters were exteriorized subcutaneously at the back of the neck. We allowed 40–44 h for recovery from the surgical catheterization to reduce the effect of neurohumoral activation related to trauma. In particular, we tried to minimize incoherent reactions related to an increase in endogenous catecholamines and BK, which might have interfered with our assay.

On the day of the experiment, the rats were placed in plastic cages. 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. The pressure signal was amplified, digitized at 1.5 kHz, visualized using dedicated software (UNI-BIO, Kared, Poland), and saved on a computer hard disc. MAP and HR were assessed from the continuous sequence of the obtained blood pressure values using dedicated software (Analiza-2, Kared, Poland).

The ACE inhibitor, enalapril (Merck Sharp and Dohme Research Lab., Rahway, NJ, USA; 1 mg/kg), was injected into the iliac catheter. Forty-five minutes were provided for stabilization of blood pressure. The rats displaying MAP equal to or lower than 85 mmHg, or HR higher than 350 beats/min were excluded from the experiments ($N = 5$). Two doses of BK (BK acetate salt, Sigma, USA, dissolved in 5% D-glucose solution at a concentration of 1.25 μg/ml) 125 ng or 250 ng per animal were randomly injected two to four times, every 4–5 min, into one branch of the carotid catheter until the evoked vasodepressor responses were stable. To provide homogeneity of the assay, only the rats that exhibited vasodepressor response to 125 ng BK (ΔMAP) within the range of 20 to 45 mmHg were enrolled into this study. Furthermore, the rats that displayed a vasodepressor response to 125 ng BK at a rate higher than 82% of that evoked by 250 ng BK were also excluded from the BKA assay ($N = 4$). The BK dose of 250 ng was then employed as a standard for the BKA activity assay.

The BK analogues dissolved in a 5% D-glucose solution were infused to the other branch of the carotid catheter. A constant rate of infusion (125 μl/ min) was maintained using an infusion pump (F5z Dialyse 15, Dascon BV, Uden, Netherlands). The testing of the BK analogue potency was initiated with an 8-min infusion of the concentration of 1 μg/ml (this gave a dose of 125 ng/min). During this infusion, a single 250 ng dose of BK was injected twice: at 280 s, and 450 s of the infusion. The concentration of the infused BK antagonist was then increased to 1.6, 6 or 12, and 32 or 64 μg/ml (if necessary it was increased up to 1024 μg/ml) to give the doses of 0.2, 0.75 or 1.5, 4 or 8 (128) μg/min/rat, respectively. As soon as the vasodepressor response to BK fell below 10% of the standard response, the procedure was completed. In the actual BK antagonist dose–response pattern required, additional concentrations of BK antagonists were also employed: 3, 96, 256, and 360 μg/ml (doses 0.375, 12, 32, 45 μg/min/rat, respectively).

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

The in vitro rat uterotonic test. All analogues were evaluated in the *in vitro* rat uterotonic assay using a modified Holton method [11] in Munsick solution [12] 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. standard 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. the negative decadic 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 AND DISCUSSION

The two new BK analogues (**I** and **II**) were synthesized by stepwise coupling of Fmoc-amino acids to the growing peptide chain starting from a Fmoc-Arg(Pbf)-Wang resin. Cleavage of the analogues from the resin with side chain deprotection was performed by treatment with a TFA/H_2O/TIS solution. The crude products were purified on Sephadex G-15 and by RP-HPLC.

The antagonistic potencies of analogues **I** and **II**, compared to that of the antagonist D-Arg-[Hyp^3,$Thi^{5,8}$, D-Phe^7]-BK synthesized by Stewart's group [3], which may be considered as their parent compound and its acylated form Aaa-D-Arg-[Hyp^3, $Thi^{5,8}$, D-Phe^7]-BK obtained previously, are summarized in Table 2. The potencies of the analogues were assessed by their ability to inhibit vasodepressor response of exogenous BK in conscious rats and by their ability to inhibit the contractions of isolated rat uterus evoked by BK. In the first assay, both compounds exhibited weak antagonistic potency. Analogue **I** in lower doses is an almost 40 times weaker antagonist than the Stewart's peptide and 80 times weaker than its acylated

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counterpart. Analogue **II** showed a lower potency than both model peptides (8 and 16 times, respectively). When inhibiting the effect of 90% BK, both peptides are inactive. In the rat uterus test, the analogues did not display antagonistic activity. Nevertheless, their agonistic potency (related to the activity of BK) is reduced to 5 and 14%, respectively.

The present study, which was a continuation of previous investigations to find structural requirements, resulted in high B_2 antagonistic activity in the case of BK analogues. As mentioned in the introduction, it was previously reported that the presence of a bulky acyl group (1-adamantaneacetate, 4-tert-butylbenzoate, 1-adamantanecarboxylate, etc.) at the *N*-terminus of B_2 antagonists significantly influenced the interaction of the peptide with B_2 receptors, thus increasing potency [7]. It was also demonstrated that either the positive or the negative change on the *N*-terminal acyl group might be responsible for the transformation of activity in the uterotonic test [9]. This was noticed for analogues acylated on their *N*-terminus with Ana and phthalic acid. Both were weak antagonists in the blood pressure test and weak agonists in the uterotonic test. Replacement of acridine rings with impassive anthracene rings resulted in an analogue with weak antagonistic activities in both tests.

In the present work, two of the previously used groups (acridin-9-ylacetylate and anthracen-9-ylacetylate) have been placed in the *N*-terminus of the BK molecule. As seen in Table 2, the modification transformed the activity of the analogues from agonistic to weak antagonistic in the rat blood pressure test and depressed its agonistic activity in the interaction with rat uterine receptors. It should be emphasized that these are the first reported B_2 blockers without any changes in the main peptide chain. Since 1985, it had been established that a critical change necessary to confer B_2 antagonistic activity upon BK analogues was the replacement of Pro^7 with a D-aromatic amino acid residue [2]. The first report stating that BK antagonists did not require a D-aromatic amino acid residue at position 7 was also made by Stewart and Vavrek [13]. In 1996, Reissman and coworkers described a new type of antagonist for the B_2 receptors containing MePhe in position 2 without any changes in the other positions (especially at position 7) [14]. It was also discovered that introduction of a conformationally constrained dipeptide fragment (ethylene-bridged dipeptide Phe-Phe) in positions 7 and 8 of the BK antagonist molecule resulted in preservation of antagonistic activity in the blood pressure test [15]. Recently, it was demonstrated that L-amino acids [16] or achiral amino acids [17] are also accepted in position 7. Analogues that contain either an MePhe residue or 1-aminocyclohexane-1-carboxylic acid in this position exhibited antagonistic activity in the rat blood pressure test. Nevertheless, all these active analogues were substituted in the main chain of the molecule. In this work, it is presented that the first two analogues, which do not contain any changes in the main chain, were able to antagonize the BK activity in the rat blood pressure test.

Moreover, it is very interesting that there is also a lack of antagonistic properties for both analogues in the uterotonic test. This is a very promising finding as it may be crucial for designing selective B_2 antagonists. Obviously, further studies are needed to confirm these interesting findings.

In summary, the study presented here provided new information on the structure–activity relationship of BK analogues and may have an impact on designing selectively acting antagonists of BK.

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Table 2 Pharmacological Properties of the New Analogues of BK

Analogue	Uterotonic potency % of activity of BK	Vasodepressor potency		
		ED_{20} (mg/min)	ED_{50} (mg/min)	ED_{90} (mg/min)
D-Arg-[Hyp^3,Thi^5,D-Phe^7,Thi^8]-BK	6.88 ± 0.08[a]	1.73 ± 0.43[b]	—	124.17 ± 27.04[b]
Aaa-D-Arg-[Hyp^3,Thi^5,D-Phe^7,Thi^8]-BK	7.43 ± 0.11[a]	0.84 ± 0.09[b]	—	13.94 ± 1.69[b]
(I) Ana-BK	5.0%	65.05 ± 23.41	838.2 ± 414.6	Inactive
(II) Ata-BK	14%	14.67 ± 4.30	959.0 ± 337.9	Inactive

Agonistic activity calculated as percentage of BK activity (considered as 100%), and antagonistic activity calculated as pA_2 (negative decadic logarithm of analogue concentration shifting the log dose-response curve for BK by a factor of 0.3 to the right – calculation was made from the linear portions of the curves). ED_{20}, ED_{50}, and ED_{90}, represent doses of BK antagonist (μg/kg/min) that inhibit the vasodepressor response to 250 ng of BK by 20, 50, and 90%, respectively.

[a] Value taken from Ref. 7.

[b] Value taken from Ref. 9.

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