Discovery of a dual function peptide that combines aminopeptidase N inhibition and kinin B₁ receptor antagonism

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Abstract

Previous analyses support that aminopeptidase N is a major inactivation pathway for high affinity peptide ligands of the human and rabbit forms of the kinin B₁ receptor (agonists or antagonists). In this study, we found that the high affinity antagonist B-9958 (Lys-Lys-[Hyp³, CpG⁵, D-Tic⁷, CpG⁸]des-Arg⁹-bradykinin) is an aminopeptidase N substrate based on its capacity to compete for the hydrolysis of the chromogenic substrate L-Ala-p-nitroanilide by membranes isolated from human or rabbit arterial smooth muscle cells, its inactivation in the presence of these membranes (radioreceptor assay) and on its intense potentiation by the aminopeptidase N inhibitor amastatin in the rabbit aorta contractility assay (gain of 0.84 unit in the pA₂ scale). Analogs of B-9958 in which the N-terminal Lys residue was substituted by D-Lys or D-Arg (B-10352 and B-10356, respectively) showed reduced affinity at the human or rabbit B₁ receptors (1.2-2.8-fold), as estimated by the displacement of [³H]Lys-des-Arg⁹-BK binding, but were more potent antagonists of des-Arg⁹-BK-induced contraction of the rabbit aorta than B-9958 in the absence of amastatin; they were not potentiated by the latter inhibitor. Unexpectedly, B-10356 inhibited L-Ala-p-nitroanilide hydrolysis without being inactivated, suggesting that it is an aminopeptidase N inhibitor. This was verified as B-10356 (but not B-10352) potentiated peptides unrelated to kinins but susceptible to aminopeptidase N inactivation (angiotensin III, thrombin receptor hexapeptide agonist). B-10356 inhibits dual molecular targets (aminopeptidase N enzyme Ki 0.9-2.2 µM, kinin B₁ receptor binding Ki 0.5-1.5 nM) and this may be an advantage for specific therapeutic applications (e.g., inhibition of angiogenesis).
Introduction

The $B_1$ receptor for kinins is a G protein coupled receptor essentially unresponsive to bradykinin, unlike the related $B_2$ receptor, but rather is stimulated by fragments of bradykinin and Lys-bradykinin (also known as kallidin) devoid of the C-terminal arginine residue (des-arginine$^9$-bradykinin [des-Arg$^9$-BK], Lys-des-Arg$^9$-BK) (Leeb-Lundberg et al., 2005). At the human $B_1$ receptor and at that from specific mammalian species (the rabbit, porcine, bovine, but not the laboratory rodents), Lys-des-Arg$^9$-BK is the only natural agonist of subnanomolar potency and des-Arg$^9$-BK is comparatively much weaker (Leeb-Lundberg et al., 2005; Morissette and Marceau, 2006); therefore, the reaction that removes the N-terminal Lys residue is practically an inactivation pathway in these species. Previous analyses support that aminopeptidase N (CD13, EC 3.4.11.2) is a major inactivation pathway for high affinity ligands of the human and rabbit forms of the $B_1$ receptor in arterial tissue (Pelorosso et al., 2005; Fortin et al., 2005). This applied to the optimal agonist Lys-des-Arg$^9$-BK and to peptide antagonists that were not protected from aminopeptidase digestion in contractility assays. Other peptidases that are critical for BK inactivation, such as angiotensin converting enzyme and neutral endopeptidase, have comparatively less importance for the inactivation of Lys-des-Arg$^9$-BK in arterial tissue (Pelorosso et al., 2005; Fortin et al., 2005).

In the kallikrein-kinin system, the $B_1$ receptor plays a particular role as one of the gene products that is most strongly regulated in the vasculature under the influence of cytokines, MAP kinases and transcription factors such as nuclear factor-$\kappa$B (Marceau et al., 1998; Leeb-Lundberg et al., 2005); by contrast, the $B_2$ receptor, sensitive to native kinins bradykinin and Lys-bradykinin, is constitutively expressed. The pathological upregulation of vascular $B_1$ receptor expression has been observed in a number of experimental systems related to myocardial infarction (Tschöpe et
al., 2004), diabetes (Lawson et al., 2005), tumor irrigation (Cardoso et al., 2004), genetic hypertension (Farias et al., 2004; Hagiwara et al., 2004) and angiogenesis driven by either ischemia or chronic inflammation (Emanuelli et al., 2002; Hu and Fan, 1993). In human vascular tissue, B₁ receptor upregulation has been shown in atheroma lesions (Raidoo et al., 1997) and in the coronary vessel endothelium in human failing hearts (Liesma et al., 2005). Three single nucleotide polymorphisms of the human B₁ receptor gene (BDKRB1) have been associated with hypertension in a sample of American-Caucasians (Cui et al., 2005). While endogenous B₁ receptors mediate adaptative responses that are apparently beneficial in some animal models (limitation of hypertrophy and dysfunction following myocardial infarction, Xu et al., 2005; renal protection in SHR rats, Hagiwara et al., 2004; limitation of smooth muscle migration and mitosis post-angioplasty, Agata et al., 2000), the blockade of these receptors may be a legitimate therapeutic intervention for specific indications, such as inflammatory pain and inflammation (Marceau and Regoli, 2004), counterproductive angiogenesis, visceral ischemia (Souza et al., 2004) and perhaps sepsis (McLean et al., 1999).

Numerous peptide and nonpeptide antagonists selective for either B₁ or B₂ receptor subtypes have been produced (Leeb-Lundberg et al., 2005). The nonpeptide agents tend to be hydrophobic drugs suitable for oral dosing. The “second generation” peptide antagonists of the B₁ receptor may still be of therapeutic interest as hydrophilic agents suitable for intravenous injection/infusion or topical administration with limited systemic distribution (Fortin et al., 2005). An excellent example of this class of peptide is B-9958 (Lys-Lys-[Hyp³, CpG⁵, D-Tic⁷, CpG⁸]des-Arg⁹-BK; Larrivée et al., 2000), a high affinity and specific antagonist of the rabbit B₁ receptor. Previous work with an antagonist of a comparable design (B-10350) suggests that B-9958 may be a substrate for aminopeptidase N, which represents an inactivation pathway for
peptide B₁ receptor antagonists (Fortin et al., 2005). Novel analogs of B-9958 have been produced to circumvent the inactivation problem while keeping an acceptable affinity. In the course of these experiments, a novel analogue that unexpectedly also has the ability to block aminopeptidase N has been discovered.
Methods

**Peptide synthesis.** The kinin receptor antagonists B-9958 and derivatives (table 1) were synthesized, purified and characterized using general methods described elsewhere (Gera et al., 1996).

**Binding assay.** Rabbit aortic smooth muscle cells and human umbilical artery smooth muscle cells were cultured as described (Fortin et al., 2003b; 2005). The binding of \[^3\text{H}\]Lys-des-Arg\(^9\)-BK (PerkinElmer Life Sciences, Boston, MA; 69-80 Ci/mmol) to adherent intact smooth muscle cells from either species was evaluated as described (Fortin et al., 2005; except for the radioligand concentration, 0.5 nM in the present experiments) in cells maintained in the culture medium supplemented with fetal bovine serum and interleukin-1\(\beta\) (5 ng/ml, for the last 4 h). The assay was applied to evaluate binding competition by unlabelled drugs.

**Membrane preparations, enzymatic assay.** Membrane preparations enriched for aminopeptidase N were derived from primary lines of rabbit aortic or human umbilical artery smooth muscle cells as described (Fortin et al., 2005). Enzymatic assays, based on the chromogenic substrate L-alanine-\(p\)-nitroanilide (L-Ala-pNA, Sigma-Aldrich) and membranes as a source of aminopeptidase N were also performed as described, with some variations (10 \(\mu\)g membrane protein per tube; substrate concentration 0.3-2.5 mM). Colorless solutions of peptide ligands of the B\(_1\) receptor (agonist or antagonists, 1-50 \(\mu\)M) or the monoclonal antibody WM-15 (BD Pharmingen, 5 \(\mu\)g/ml) were used as competitors of L-Ala-pNA hydrolysis in some experiments. The active site-directed and highly specific WM-15 antibody (Ashmun et al., 1992)
has been previously used to ascribe catalytic activities present in biological samples to aminopeptidase N (Makrynikola et al., 1995; Kanayama et al., 1995). Michaelis-Menten enzyme kinetics and competitive inhibition $K_i$ values were evaluated using a computer program (Tallarida and Murray, 1987).

**HPLC separation of kinins in reaction mixtures.** Concentrated reaction mixtures composed of human smooth muscle cell membranes (100 µg/ml) suspended in phosphate buffered saline and containing Lys-des-Arg⁹-BK (50 µM, spiked with 10000 cpm of $[^3]H$Lys-des-Arg⁹-BK per tube), with or without aminopeptidase N inhibitors, were incubated for 4 h at 37°C. Ethanolic extracts of these containing immunoreactive kinins were evaporated to dryness and the residue of evaporation was dissolved in 200 µL of 5 mM KH$_2$PO$_4$, pH 3.0, 25% acetonitrile with 1.0% H$_3$PO$_4$. HPLC separation was obtained on a Agilent 1100 Series system (Agilent Technologies Canada, Mississauga, Ca) with a 2-sulfoethyl aspartamin column (PolySULFOETHYL A TM, The Nest Group Inc., Southboro, MA) using a linear gradient of KCl (0-300 mM) in 5 mM KH$_2$PO$_4$, 25% acetonitrile (v/v), pH 3.0, during 30 min. Samples were collected at a flow rate of 1 ml/min. The different eluted fractions were counted for radioactivity to assess the metabolic fate of Lys-des-Arg⁹-BK.

**Radioreceptor assay.** To evaluate their metabolic stability, some of the newly produced antagonists (400 nM) were incubated in the presence of smooth muscle membranes (100 µg/ml) from either species in phosphate buffered saline (pH 7.6) at 37°C for 60 min (reaction volume 100 µl). In duplicate, 25 µl of supernatant was transferred to the binding medium (975 µl, containing peptidase inhibitors) of confluent HEK 293 cells (24-well plates) that stably expressed
at high density the rabbit B1 receptor fused to yellow fluorescent protein (Sabourin et al., 2002). [3H]Lys-des-Arg⁹-BK (0.5 nM) was transferred to each well and the assay further run as described (Sabourin et al., 2002). The concentration of antagonist in unknown samples was determined using competition curves obtained with the authentic peptides and assuming that the metabolism of peptide antagonists will massively decrease or abolish their capacity to compete with the radioligand for the receptor binding. Controls consisted of peptide solutions incubated for 60 min at 37°C without source of enzyme (membranes).

**Immunofluorescence.** The indirect immunofluorescence detection of aminopeptidase N was performed using the monoclonal antibody WM-15 (BD Pharmingen; 5 µg/ml) in cultured smooth muscle cells from both species as generally described (Morissette et al., 2004).

**Contractility studies.** A local animal care committee approved the procedures based on rabbits. Rabbit aortic rings with intact endothelium were suspended under a tension of 2 g in 5 ml tissue baths containing oxygenated (95% O₂: 5% CO₂) and warmed (37°C) Krebs solution (Fortin et al., 2005). The procedure described in the latter report was precisely applied to measure the potency of peptides antagonists in the presence or absence of the aminopeptidase N inhibitor amastatin against the aminopeptidase N-resistant B1 receptor agonist des-Arg⁹-BK. The concentration-response curves for the contraction induced by this agonist were constructed 5.5 h from tissue mounting; pA₂ values were calculated as described (Fortin et al., 2005).

The reaction that cleaves angiotensin III into angiotensin IV is also reported to be catalyzed by aminopeptidase N (Robertson et al., 1992); to modelize this in the rabbit aorta contractility assay, cumulative concentration-effect curves were constructed for angiotensin II, III (des-Asp¹-
angiotensin II) and IV (des-(Asp¹, Arg²)-angiotensin II; Sigma-Aldrich) in separate tissues pre-equilibrated for 1 h as described (Fortin et al., 2003a; the first peptide was included for comparison). The same tissues were exposed to the AT₁ receptor antagonist losartan (100 nM, gift of Merck Research Laboratories) in the period 2-3 h post-mounting, and the curves were constructed again at time 3 h, a time point where control tissues show a great constancy of response relative to the time 1 h (Fortin et al., 2003a). The aims of these preliminary experiments were to clarify that the metabolism of angiotensin III into angiotensin IV is practically a functional inactivation and to confirm that all forms of angiotensin contract the rabbit aorta via the AT₁ receptor, as reported for the rat aorta (Li et al. 1995).

Other contractility studies involved agents unrelated to kinins: angiotensin II, angiotensin III and an agonist hexapeptide derived from the new amino terminus (NAT) sequence of the cleaved thrombin receptor (NAT₆-NH₂; gift from Dr. Guy Drapeau) (Godin et al., 1994). Cumulative concentration-response curves were constructed for these agents in the presence or absence of drugs believed to influence aminopeptidase N (applied 30 min before) in rabbit aortic rings pre-equilibrated for at least 2 h.
Results

Characterization of an aminopeptidase in human smooth muscle cell membranes. An aminopeptidase sensitive to amastatin is reported to mediate the major inactivation pathway for Lys-des-Arg⁹-BK in the human umbilical artery (Pelorosso et al., 2005). To further characterize this activity, we initially studied the presence of an ectoenzyme present in the human smooth muscle cell membranes using the conventional aminopeptidase substrate L-Ala-pNA (fig. 1). A 2.5 mM concentration of the substrate was efficiently hydrolyzed by smooth muscle cell membranes and this activity was nearly abolished by amastatin and extensively (78%) reduced by a monoclonal antibody (WM-15) known to block the aminopeptidase N activity with selectivity (Ashmun et al., 1992). Experiments performed using variable concentrations of the substrate showed that the enzyme that cleaves L-Ala-pNA exhibited a K_M of 0.61 mM (very similar to that found in the rabbit aortic smooth muscle cell membranes, Fortin et al., 2005). The hydrolysis of L-Ala-pNA was apparently inhibited by the B₁ receptor agonist Lys-des-Arg⁹-BK (10-50 µM), consistent with competition for the enzyme with the chromogenic substrate (fig. 1B; double reciprocal plot representation in fig. 1C; calculated Kᵢ of 47 µM). Human recombinant aminopeptidase P (an enzyme capable of hydrolyzing des-Arg⁹-BK) did not hydrolyze L-Ala-pNA at enzyme concentrations active on specific substrates (methods as in Molinaro et al., 2005).

The degradation of concentrated (50 µM) Lys-des-Arg⁹-BK by human smooth muscle cell membranes has been addressed in a more direct manner by exploiting HPLC separation of bradykinin-related peptides in reaction mixtures. Fig. 1D shows the separation of authentic standards in the chromatographic system employed. Bradykinin and lysyl-bradykinin cannot be formed from the hydrolysis of Lys-des-Arg⁹-BK; in addition, des-(Arg¹, Arg⁹)BK is not retained
in this chromatographic system. Fig. 1E shows the actual digestion of 50 µM Lys-des-Arg⁹-BK (spiked with the radioactive form of the same peptide) incubated for a long time (4 h). In the presence of concentrated membranes (100 µg/ml), very extensive degradation of Lys-des-Arg⁹-BK was recorded. However, its product of reaction with aminopeptidase N, des-Arg⁹-BK, did not accumulate accordingly, suggesting that the latter peptide is further degraded or that other peptidases process Lys-des-Arg⁹-BK into some other product(s) not retained in the column. Aminopeptidase N inhibitors amastatin and WM-15 protected a fraction of Lys-des-Arg⁹-BK from degradation.

The WM-15 monoclonal antibody reacted with a surface protein in human smooth muscle cell under the form of multiple small aggregates (fig. 2). Rabbit smooth muscle cells exhibited a comparable pattern but much less intense staining for the same antibody concentration (exposure time of several seconds in the microphotograph, fig. 2). The use and inhibitory effect of the antibody is not documented in this species; it did not reduce the hydrolysis of L-Ala-pNA by membranes isolated from rabbit smooth muscle cell (data not shown), suggesting that species is an important determinant of antibody affinity.

**Novel aminopeptidase-resistant analogues of B-9958.** Several analogues of B-9958 have been produced (structure and nomenclature in table 1). The main purpose of this series was to evaluate whether N-terminal protection against aminopeptidases could be achieved without important loss of receptor affinity (protection at the expense of affinity has been observed with N-acetylation, for instance, Fortin et al., 2005). A competition assay for the radioligand binding to smooth muscle cells was used to evaluate the affinity of the analogs (fig. 3, binding Kᵢ values in table 1). In both human and rabbit smooth muscle cells, B-9958 had the maximal affinity; the analogues
were 1.2-20-fold less potent in that respect, and some species-related differences were apparent. Nevertheless, the D-Lys isomer of B-9958 (B-10352) and its D-Arg analog (B-10356) were among the most potent of the novel peptides in both species.

The ability of kallidin-related peptides to compete for L-Ala-pNA hydrolysis by rabbit smooth muscle cell membranes has been previously interpreted as a substrate status (fig. 1; Fortin et al., 2005). B-9958, which possesses an unprotected L-Lys residue at its N-terminus, illustrates this relationship by competing for the reaction in membranes from both species (fig. 4; enzyme inhibition K_i in table 1). Its D-Lys isomer B-10352 has a higher K_i values than B-9958 in either species, indicating lower enzyme affinity in the competitive situation. However, the introduction of residues in D conformation, expected to abolish the susceptibility to aminopeptidase N hydrolysis, variably reduced the apparent affinity for the enzyme, with the lowest K_i values (highest affinity) for B-10356 in both species (0.9-2.2 µM). The D-Orn analogue of B-9958 (B-10354) has a low enzyme affinity in both species, but a peptide with a further modification of the N-terminal sequence (B-10358; D-Arg-D-Arg-Arg...) was associated with intermediate affinity for the rabbit enzyme only in the chromogenic substrate assay (fig. 4, table 1).

Several other synthetic ligands of the kinin receptors possess a N-terminal D-Arg residue, such as the widely used B_2 receptor antagonist icatibant (Hoe 140; D-Arg[Hyp\(^3\), Thi\(^5\), D-Tic\(^7\), Oic\(^8\)]-BK) or NPC 17731 (D-Arg[Hyp\(^3\), D-HypE(transpropyl)\(^7\), Oic\(^8\)]-BK. These peptides that share a D-Arg-Arg-Pro N-terminal sequence are fairly effective competitors of L-Ala-pNA for the smooth muscle cell aminopeptidase N preparations (K_i 4.0 µM for Hoe 140 in the rabbit, 6.7 µM in human), but not as potent as B-10356, indicating the probable role of the second Lys residue of
B-10356 for affinity.

**Radioreceptor assay.** Solutions of antagonists (400 nM) were submitted to 1 h incubation at 37°C with or without smooth muscle cell membranes and further diluted with radioligand assay buffer (see Methods). The radioreceptor assay showed that the final concentrations of the antagonists B-9958, B-10352 or B-10356 are in the vicinity of the theoretical value (10 nM) when incubated without membranes (fig. 5). In the presence of either human or rabbit cell membranes, only the concentration of B-9958 fell, supporting that the N-terminal modifications protect the two other peptides from inactivation by aminopeptidase N.

**Rabbit aorta contractility.** The functional importance of resistance to aminopeptidase was evaluated using the contractility of the fresh rabbit aorta for selected antagonists (fig. 6). The concentration-effect curve for an aminopeptidase N-resistant B₁ receptor agonist, des-Arg⁹-BK, was evaluated in the absence or presence of B-9958, B-10352 or B-10356; in half of the tissues, amastatin was also present. B-10352 and B-10356, despite exhibiting slightly lower affinities at the rabbit B₁ receptor in the binding assay, were more potent antagonists of des-Arg⁹-BK-induced contraction of the rabbit aorta than B-9958 in the absence of amastatin (apparent pA₂ values listed in fig. 6C, F, I). The latter aminopeptidase inhibitor, without effect on the contraction of the agonist or the tissue tone, potentiated considerably the rightward shift of the curves induced by B-9958, but not that induced by B-10352 or B-10356; this is parallel to an increased calculated pA₂ value (0.84 log unit) in the presence of amastatin only for B-9958.

In preliminary experiments on the pharmacology of angiotensin-related peptides, the relative potency of angiotensin III and IV were established in the rabbit aorta contractility assay (fig. 7).
the latter peptide being the reaction product of the former via the reaction catalyzed by aminopeptidase N. Angiotensin IV was about 250-fold less potent than angiotensin III to contract the tissue, which supports that aminopeptidase N practically inactivates the latter peptide. For comparison, the high affinity agonist angiotensin II has also been evaluated (fig. 7). Losartan (100 nM), a surmountable antagonist of angiotensin II in this preparation (Fortin et al., 2003a), displaced the concentration-effect of all three angiotensins to the right by a similar ~20-fold factor (fig. 7), supporting that they are all agonists of the AT1 receptors with varying potencies. If B-10356 is not a substrate of aminopeptidase N but inhibits with good affinity the hydrolysis of the chromogenic substrate, it may be a bona fide enzyme inhibitor comparable to amastatin. The latter agent is known to potentiate the contraction caused by either angiotensin III (des-Asp1-angiotensin II) or a soluble form of the new amino terminus hexapeptide from the cleaved thrombin receptor (NAT6-NH2) in the rabbit aorta (Godin et al., 1994; Robertson et al., 1992), findings that have been replicated in the present experiments (fig. 8A, C). The average of individual EC50 values from control tissues for angiotensin II was 60.0 ± 9.1 nM this series of experiments (n = 10). B-10356 (1 µM, introduced 30 min before the stimulants) significantly reduced the EC50 values (35.7 ± 7.3 nM, P<0.05, Mann-Whitney test), an effect qualitatively similar to but less intense than that of amastatin (3 µM) on the concentration-effect relationship of angiotensin III (EC50 9.5 ± 0.5, P<0.001 vs. controls). B-10352 was inactive in this respect (EC50 58.7 ± 7.4). The contractile effect of NAT6-NH2 on the rabbit aorta was potentiated by amastatin pretreatment (3 µM; fig. 8C). Again, B-10356 (1 µM) reproduced a part of this potentiation, but not B-10352. As reported (Robertson et al., 1992), amastatin had a minimal effect of the concentration-effect curve of angiotensin II (no significant changes in EC50 values),
and B-10356 was also inactive in this respect (fig. 8B).
Discussion

There are potentially several aminopeptidases sensitive to amastatin and capable of hydrolyzing L-Ala-pNA (Fortin et al., 2005; Albiston et al., 2004). Other membrane bound M1 family aminopeptidases that are inhibited by amastatin include aminopeptidase A (EC 3.4.11.7, selective for residues with acidic side chains) and adipocyte-derived leucine aminopeptidase (no EC designation yet; Albiston et al., 2004). We took a further step to identify aminopeptidase N as the major ectoenzyme that degrades B₁ receptor ligands, as the active site blocking anti-aminopeptidase N monoclonal antibody WM-15 was capable of extensively (78%) inhibiting the hydrolysis of the chromogenic substrate in human smooth muscle cell membranes. Further, the reaction of this substrate with aminopeptidase N was competitively inhibited by the B₁ receptor agonist Lys-des-Arg⁹-BK in human umbilical artery smooth muscle cells, supporting that it can inactivate this high affinity B₁ receptor agonist in the contractility assay based on the fresh artery (Pelorosso et al., 2005). Under relatively unfavorable conditions of a high substrate concentration and long reaction time, amastatin and WM-15 retained the ability to protect a fraction of the substrate Lys-des-Arg⁹-BK from degradation by human smooth muscle cell membranes (fig. 1E); we cannot rule out that other membrane peptidases hydrolyze this peptide in that experiment. The anti-aminopeptidase N antibody stained the surface of human cultured smooth muscle cells, but apparently did not react strongly with nor inactivate the rabbit form of the peptidase. However, as previously discussed, it is reasonable to ascribe the inactivation of B₁ receptor ligands to aminopeptidase N in the rabbit aorta (peptidase associated with membranes, low sensitivity to puromycin, high sensitivity to amastatin; Fortin et al., 2005).

The prominent role of aminopeptidase N in the inactivation of the optimal B₁ receptor agonist
Lys-des-Arg^9-BK is increasingly recognized. The B1 receptor antagonist of classical design Lys-[Leu^8]des-Arg^9-BK is also susceptible to aminopeptidase N inactivation in the rabbit aorta, as evidenced by its intense potentiation by amastatin and its capacity to compete for L-Ala-pNA hydrolysis by a relevant source of enzyme (Fortin et al., 2005). The importance of this pathway may be more variable for other peptide antagonists, as N-terminal protection has been incorporated in some later designs (e.g., R-954 = Ac-Orn-[Oic^2, (α-Me)Phe^5, D-βNal^7, Ile^8]des-Arg^9-BK; Neugebauer et al., 2002). The present work has been undertaken to investigate the susceptibility to aminopeptidase N of a high affinity (subnanomolar) and specific B1 receptor peptide antagonist, B-9958, and to eventually modify its structure to retain high affinity and gain peptidase resistance. The free amino terminus of B-9958 has been kept, as its acetylation in Ac-Lys-[Leu^8]des-Arg^9-BK affords protection relative to aminopeptidase N, but at the expense of an important loss of affinity (Fortin et al., 2005). The first residue has been replaced by a series of D-conformation residues, of which D-Lys and D-Arg (in B-10352 and B-10356, respectively) conferred the highest affinity at the human or rabbit smooth muscle cell (only a minor loss relative to B-9958) and complete resistance to inactivation in the presence of human or rabbit smooth muscle cell membranes (radioreceptor assay). Other substitutions (D-Orn, D-Arg-D-Arg) were not as favorable.

It is likely that the compact organization of the rabbit aortic tissue, which includes up to 30 concentric layers of smooth muscle cells, limits the diffusion of drugs from the bathing fluid to the site where the receptors are located at the surface of the relevant contractile cells. Thus, an active drug removal mechanism, such as the hydrolysis by aminopeptidase N, distorts the apparent affinity of either peptide agonists or antagonists for various receptors in the isolated aorta (Robertson et al., 1992; Godin et al., 1994; Fortin et al., 2005) because the rate of agonist
inactivation at the vicinity of receptors is not fully compensated by drug diffusion from the bathing fluid. Also, this applies only if the reaction product is much less potent than the substrate, which is the case in the rabbit aorta for des-Arg⁹-BK relatively to Lys-des-Arg⁹-BK (Marceau et al., 1998), for angiotensin IV relative to angiotensin III (fig. 7) and for NAT⁶-NH₂, a proven substrate of an amastatin-sensitive aminopeptidase from blood plasma in a previous study (Godin et al., 1994). The aortic contractility system may represent a useful simulation of in vivo peptide drug administration for addressing inactivation mechanisms.

To evaluate B₁ receptor antagonists, we use the agonist des-Arg⁹-BK in the isolated rabbit aorta; it has a lower potency than Lys-des-Arg⁹-BK at the rabbit B₁ receptor, but is resistant to aminopeptidase N and allows isolation of the enzyme susceptibility of the antagonists. Following predictions for N-protected peptides, B-10352 or B-10356 were not potentiated as antagonists of des-Arg⁹-BK-induced contraction by amastatin, unlike B-9958 which is nearly 7-fold more potent (on the pA² scale) in the presence of this aminopeptidase N inhibitor. As for its previously reported analogue B-10350 (Fortin et al., 2005), it is likely that B-9958 undergoes two cycles of aminopeptidase N hydrolysis with the successive removal of the two Lys residues parallel to a large loss of receptor affinity. The loss of capacity of B-9958 to compete with the radioligand for recombinant B₁ receptor binding after incubation with membranes is consistent with inactivation in the present experiments. In the contractility test, B-10352 and B-10356 are more potent antagonists than B-9958 in the absence of amastatin, showing that aminopeptidase N resistance more than compensated for the minor loss of receptor affinity at the rabbit B₁ receptor. The novel analogues retain the surmountable behavior of the parent compound (Larrivée et al., 2000).

An unexpected property arose in some of the analogs of B-9958 substituted by D-conformation
residues at the first position: they could compete for the hydrolysis of the chromogenic aminopeptidase N substrate, a property that was expected for aminopeptidase N substrates, but not for N-protected peptides (Fortin et al., 2005). The protected analogue with the highest aminopeptidase N affinities in both species, B-10356, was further investigated with the hypothesis that it may represent a bona fide enzyme inhibitor comparable to amastatin. Indeed, B-10356 (but not B-10352) potentiated contractile peptides unrelated to kinins, but known to be susceptible to aminopeptidase N degradation (angiotensin III and a thrombin receptor agonist hexapeptide) in a manner qualitatively similar to amastatin in the rabbit aorta. B-10356 was not inactivated in the presence of smooth muscle cell membranes, consistent with its resistance to aminopeptidase N (radioreceptor assay). Thus, B-10356 is both a B₁ receptor antagonist and an aminopeptidase N inhibitor at micromolar concentrations, unlike B-10352 which retains a purer profile (with ~10-fold less affinity for the aminopeptidase of rabbit cell membranes, and an even larger gap for the human enzyme). The D-Arg-Lys-Arg N-terminal sequence of B-10356 is similar to that of several other kinin receptor ligands, including the widely used B₂ receptor antagonist Hoe 140 (D-Arg-Arg…). However, the latter peptide has an affinity lower than that of B-10356 for aminopeptidase N in the substrate competition assay, and aminopeptidase inhibition may not be prominent when Hoe 140 is exploited in vivo at nanomolar concentrations to block B₂ receptors. These results also suggest that the Lys residue in the second position in B-10356 is important for aminopeptidase N affinity.

In the novel peptide series, B-10356 is the prototype of drugs that possess a dual mode of action. Speculatively, B₁ receptor blockade and aminopeptidase N inhibition may be additive in applications such as the suppression of undesirable angiogenesis (inflammatory, tumoral, diabetic), where agents with either mode of action have been proposed to be useful (Hu and Fan,
1993; Parenti et al., 2001; Bhagwat et al., 2001; Satoh, 2003; Lee et al., 2005). Under aminopeptidase N blockade, the degradation of Lys-des-Arg^9-BK should be reduced, but with little functional consequences, as the B_1 receptors would be also blocked; on the other hand, the peptidase has many other substrates which could account for other biological activities of its inhibitors (Albiston et al., 2004; Kanayama et al., 1995). B-10352 represents a water-soluble and more stable version of a high affinity B_1 receptor antagonist. B_1 receptor antagonists are a class of agents of great potential to treat inflammation and pain (Marceau and Regoli, 2004), but the study of locally applicable agents in this class may be warranted as the protective and adaptative significance of B_1 receptors is still largely undefined in pathology (see Introduction).

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**Figure legends**

**Fig. 1.** Characterization of aminopeptidase N from human smooth muscle cell membranes. A. Hydrolysis of L-Ala-pNA (2.5 mM) in the presence of 30 μg of membrane proteins as influenced by relevant aminopeptidase inhibitors (amastatin, WM-15 monoclonal antibody). Values are the mean ± s.e.m. of 2 determinations. B. Effect of L-Ala-pNA concentration on the reaction velocity, as modified by the presence of the B1 receptor agonist Lys-des-Arg⁹-BK (source of enzyme 10 or 30 μg smooth muscle cell membrane protein; results normalized as a % of the control velocity recorded with the 2.5 mM substrate concentration in each experiment; n = 2 to 4). C. Double reciprocal plot representation of the data from B: competition for the hydrolysis of L-Ala-pNA by Lys-des-Arg⁹-BK. D. HPLC separation of kinins: authentic standards. E. Use of HPLC separation to assess the metabolic fate of Lys-des-Arg⁹-BK (50 μM, spiked with the tritiated form of the same peptide) incubated at 37°C for 4 h in the presence of membranes (100 μg/ml), amastatin (10 μM) and/or WM-15 antibody (5 μg/ml).

**Fig. 2.** Immunofluorescence for aminopeptidase N at the surface of fixed but nonpermeabilized smooth muscle cells of rabbit aortic or human umbilical artery origins. Paired phase contrast and immunofluorescence views of the same fields (original magnification 200 ×) are shown. The primary antibody (WM-15, 5 μg/ml) stained human cells much more intensely; both cell types exhibit granular staining. There was no staining in the absence of the primary antibody (rabbit cells shown; data not shown for human cells).

**Fig. 3.** Competition of radioligand binding to B₁ receptors by unlabelled peptide antagonists. The
residual specific binding of 0.5 nM [³H]Lys-des-Arg⁹-BK to human (A) or rabbit (B) vascular smooth muscle cells expressing the natural B₁ receptor is presented; unlabelled drugs were co-incubated at the indicated concentrations with the radioligand. Values are the mean ± s.e.m. of two experiments composed of duplicate determinations.

**Fig. 4.** Double reciprocal plot representation of the effect of various receptor ligands (1 µM) on the hydrolysis of L-Ala-pNA (0.3-2.5 mM) by the smooth muscle cell membranes (A. human, B. rabbit). Before reciprocal transformation, velocity values were normalized as a % of the control recorded with the 2.5 mM substrate concentration in each experiment.

**Fig. 5.** Residual concentration of three peptide antagonists (initial concentration 400 nM) as determined by radioreceptor assay after 1 h incubation at 37°C with or without smooth muscle cell membranes of human (A) or rabbit origin (B) and dilution (1:40) into radioligand assay buffer. Values are the means ± s.e.m. of 3 separate experiments.

**Fig. 6.** Effects of antagonists on des-Arg⁹-BK-induced contraction of the rabbit isolated aorta. The concentration-effect curves recorded at time 5.5 h are shown and were constructed in the presence of an antagonist or its saline vehicle. Values are means ± s.e.m. of 3 determinations from different animals. A, B. B-9958 without or with amastatin (3 µM), respectively. C. Schild plot analyses derived from panel A and B data and based on the agonist EC₅₀ values from the averaged concentration-effect curves (DR, dose ratio = EC₅₀ in the presence of the antagonists divided by the control EC₅₀). Calculated pA₂ values ± s.e.m. (Tallarida and Murray, 1987) are reported in the figure. D, E. B-10352 (the D-Lys isomer of B-9958) without or with amastatin. F.
Schild plot analyses derived from panel D and E data. G, H. B-10356 (the D-Arg analog of B-9958) without or with amastatin. I. Schild plot analyses derived from panel G and H data.

**Fig. 7.** Concentration-effect relationship for the contraction induced in the rabbit isolated aorta by angiotensins II, III and IV in the absence or presence of the AT₁ receptor antagonist losartan (100 nM). Responses are expressed as a percent of the maximal effect of each peptide (for low potency agonists, angiotensin II 1-10 µM was used at the end of the curve construction to obtain a maximum). Values are means ± s.e.m. (n = 4).

**Fig. 8.** Potentiation of the contractile response induced by peptides unrelated to BK by drugs in the rabbit aorta. A. Angiotensin III. B. Angiotensin II. C. Thrombin receptor hexapeptide agonist (NAT₆-NH₂). Values are means ± s.e.m. of 10 determinations. A, B. See Results for statistics. C. * P<0.05, ** P<0.001 (Mann-Whitney test with controls for each agonist concentration).
Table 1. Aligned structure of B₁ receptor antagonists and some of their properties

<table>
<thead>
<tr>
<th>compound</th>
<th>sequence</th>
<th>Receptor affinity (Kᵢ, nM)</th>
<th>Enzyme affinity (Kᵢ, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>human</td>
<td>rabbit</td>
</tr>
<tr>
<td>B-9958</td>
<td>NH₃⁺- Lys</td>
<td>Lys</td>
<td>Arg</td>
</tr>
<tr>
<td>B-10352</td>
<td>NH₃⁺- D-Lys</td>
<td>Lys</td>
<td>Arg</td>
</tr>
<tr>
<td>B-10354</td>
<td>NH₃⁺- D-Orn</td>
<td>Lys</td>
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<td>NH₃⁺- D-Arg</td>
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<td>Arg</td>
</tr>
<tr>
<td>B-10358</td>
<td>NH₃⁺- D-Arg</td>
<td>D-Arg</td>
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</tr>
</tbody>
</table>

* NI: no inhibition

Abbreviations: CpG, α-cyclopentylglycine; Hyp, trans-4-hydroxyproline; Igl, α-(2-indanyl)glycine; Orn, ornithine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.
A. L-Ala-pNA 2.5 mM

B. Kinetics

C. Double reciprocal plot

D. HPLC traces of authentic standards

E. Digestion of Lys-des-Arg³-BK
No 1st antibody

CD13

rabbit

human
Fig. 3

A. Human $B_1$R (umbilical artery SMCs) ($n = 2$)

B. Rabbit $B_1$R (aortic SMCs) ($n = 2$)
A. Human umbilical artery SMC membranes (n = 3)

B. Rabbit aortic SMC membranes (n = 2-5)
A. Human SMC

- membranes
+ membranes

B. Rabbit SMC

concentration (nM)

fig. 5
A. B-9958

- ○ 0 (saline)
- ● 10 nM
- □ 100 nM
- ▪ 1 µM

B. B-9958 + amastatin

C. Schild plots from A and B

D. B-10352

E. B-10352 + amastatin

F. Schild plots from D and E

G. B-10356

H. B-10356 + amastatin

I. Schild plots from G and H

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fig. 7

The graph shows the effect of various concentrations of angiotensin on contraction (% maximal response). The x-axis represents the concentration of angiotensin in nM, ranging from 0.1 to 100,000 nM. The y-axis represents the contraction as a percentage of the maximal response. Different treatments are indicated by different symbols and lines:

- Open circles: Ang II
- Filled circles: Ang II + losartan
- Open squares: Ang III
- Filled squares: Ang III + losartan
- Open triangles: Ang IV
- Filled triangles: Ang IV + losartan

The graphs illustrate the inhibitory effect of losartan on the contraction induced by angiotensin.