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RESEARCH PAPER

# Effect of bradykinin metabolism inhibitors on evoked hypotension in rats: rank efficacy of enzymes associated with bradykinin-mediated angioedema

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**Background and purpose:** Inhibition of bradykinin metabolizing enzymes (BMEs) can cause acute angioedema, as demonstrated in a recent clinical trial in patients administered the antihypertensive, omapatrilat. However, the relative contribution of specific BMEs to this effect is unclear and confounded by the lack of a predictive pre-clinical model of angioedema.
**Experimental approach:** Rats were instrumented to record blood pressure and heart rate; inhibitors were infused for 35 min and bradykinin was infused during the last 5 min to elicit hypotension, as a functional marker of circulating bradykinin and relative angioedema risk.
**Key results:** In the presence of omapatrilat bradykinin produced dose-dependent hypotension, an effect abolished by $B_2$ blockade. In the presence of lisinopril (ACE inhibitor), but not candoxatril (NEP inhibitor) or apstatin (APP inhibitor), bradykinin also elicited hypotension. Lisinopril-mediated hypotension was unchanged with concomitant blockade of NEP or NEP/DPPIV (candoxatril + A-899301). However, hypotension was enhanced upon concomitant blockade of APP and further intensified in the presence of NEP inhibition to values not different from omapatrilat alone.
**Conclusions and implications:** We demonstrated that bradykinin is degraded *in vivo* with an enzyme rank-efficacy of ACE > APP ≫ NEP or DPPIV. These results suggest the effects of omapatrilat are mediated by inhibition of three BMEs, ACE/APP/NEP. However, dual inhibition of ACE/NEP or ACE/NEP/DPPIV elicits no increased risk of angioedema compared to ACE inhibition alone. Thus, novel BME inhibitors must display no activity against APP to avoid angioedema risk due to high prevalence of ACE inhibitor therapy in patients with diabetes and cardiovascular disease.
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**Keywords:** omapatrilat; bradykinin; hypotension; aminopeptidase P; neprilysin; angiotensin-converting enzyme; dipeptidyl peptidase IV; angioedema; blood pressure

**Abbreviations:** ACE, angiotensin-converting enzyme; APP, aminopeptidase P; AUC, area under the curve; $B_2$, bradykinin $B_2$ receptor; BK, bradykinin; BMEs, bradykinin-metabolizing enzymes; DPPIV, dipeptidyl peptidase IV; MAP, mean arterial blood pressure; NEP, neprilysin

## Introduction

We sought to demonstrate the utility of an *in vivo* rat depressor model to delineate the effects of inhibition of bradykinin (BK)-metabolizing enzymes (BMEs) for the determination of relative angioedema risk. Inhibition of BK metabolism in patients can manifest in acute episodes of angioedema, a life-threatening swelling beneath the skin occurring around the eyes, lips, hands, feet and throat, thought to be mediated by BK (Beltrami *et al*., 2006). Indeed, in a recent phase II clinical trial that enrolled over 25 000 patients omapatrilat, originally characterized as a selective dual angiotensin-converting enzyme (ACE) and neprilysin (NEP) inhibitor, elicited broadly superior antihypertensive therapy in patients compared with ACE inhibitors alone but

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also produced an increased risk of angioedema (Kostis *et al*., 2004).

Hence, in April 2000 Bristol-Myers Squibb voluntarily withdrew the new drug application for omapatrilat due to concerns regarding the incidence and severity of angioedema reported within the new drug application database (Tabrizchi, 2001). Although angioedema secondary to omapatrilat was initially believed to be mediated by increased circulating BK as a consequence of dual ACE/NEP blockade, recent evidence also suggests that omapatrilat is a potent inhibitor of aminopeptidase P (APP) (Sulpizio *et al*., 2005), another enzyme which may be involved in BK degradation. Thus, the perceived safety of novel inhibitors of the enzymes responsible for BK degradation has come into question especially when administered in patients on concomitant ACE inhibitor therapy (Jandeleit-Dahm, 2006). This suggests that there is a need to develop novel and efficacious BME inhibitors with enhanced *in vivo* selectivity (for example, no APP inhibitory activity) and hence, safety.

Angioedema is thought to be mediated by increases in circulating BK (Nussberger *et al*., 1998, 2002), a potent vasoactive peptide that is known to elicit a number of biological responses (Moreau *et al*., 2005). The kallikrein–kinin system is part of an endogenous metabolic cascade whereby activation produces the release of vasoactive kinins (BK-related peptides) that are implicated in many physiological and pathological processes and have been shown to play a role in the regulation of blood pressure, renal function and cardiac function (Moreau *et al*., 2005). However, the rank efficacy of ACE, NEP and APP to degrade BK *in vivo* and thus affect hypotension and angioedema are not fully understood. More recently, low dipeptidyl peptidase IV (DPPIV) enzyme activity, which can also degrade BK, was shown to predispose rats to ACE-inhibitor-mediated oedema (Byrd *et al*., 2007) and low DPPIV activity has also been shown to be depressed in patients with acute ACE-inhibitor-associated angioedema (Lefebvre *et al*., 2002). However, the relative risk of selective DPPIV inhibitors to produce angioedema has not been elucidated. The question of relative angioedema risk upon ACE, NEP, APP or DPPIV inhibition is further confounded by the lack of predictive pre-clinical *in vivo* models of angioedema. Indeed, while BK has been shown to produce a potent depressor response *in vivo* when degradation is inhibited (Kitamura *et al*., 1999), small (but physiologically relevant) changes in circulating BK are not easily measured by mass spectroscopy.

Thus, we developed a functional BK depressor model in the rat to assess the potential liability of inhibitors of BMEs to exacerbate BK-dependent hypotension *in vivo*. Results were compared with those of omapatrilat that has been demonstrated to produce angioedema in patients and which served as a positive control in the development of this *in vivo* model. The present data indicate that the effects of omapatrilat observed clinically are consistent with inhibition of APP concomitant with ACE and NEP inhibition, suggesting that novel BME inhibitors must display no activity against APP to avoid angioedema risk due to high prevalence of ACE inhibitor therapy in patients with diabetes and cardiovascular disease. Moreover, results from the present study suggest a path forward exists for the discovery and development of novel enzyme inhibitors targeting this pathway and dispel the myth that dual ACE/NEP inhibitors cannot be safely developed as novel therapies. Similarly, these data clarify the safety profile of DPPIV inhibitors and their hypothesized role in angioedema.

## Materials and methods

*Enzyme potency and selectivity assays*

NEP, NEP2, ACE and APP assays were performed at pH 7.4 (Johnson and Ahn, 2000; Alves, 2005; Molinaro, 2005), except for ECE1 which was performed at pH 6.5, due to its inactivity at pH 7.4 (Ahn *et al*., 1998). The reaction buffer for NEP and NEP2 contained 50 mM HEPES, 140 mM NaCl, 10 mM KCl, 0.01% BSA. The buffer for endothelin-converting enzyme contained 100 mM 2-(*N*-morpholino) ethanesulphonic acid, 140 mM NaCl, 10 mM KCl and 0.01% BSA. The buffer for ACE contained 100 mM Tris-HCl, 50 mM NaCl, 10 μM $ZnCl_2$, and the buffer for APP contained 100 mM HEPES and 0.01% BSA. Compounds were dissolved in 100% dimethyl sulfoxide (DMSO) at 10 mM and diluted to 1% DMSO in the assay. Assays were performed in 100 μl volume in black 96-well round-bottom plates at room temperature. Reactions were continuously monitored with excitation and emission wavelengths appropriate for each respective substrate. Enzyme velocity was determined from the linear part of the reaction. Inhibition constant, $K_i$, of compounds for NEP, NEP2 and ECE1 were determined using the method of Cornish-Bowden (1995) and $K_i$ of compounds for ACE and APP was calculated from $IC_{50}$ values and the Cheng-Prusoff equation (Cheng and Prusoff, 1973; Table 1).

In vivo *cardiovascular studies*

Male Sprague–Dawley rats were anaesthetized and instrumented to record mean arterial blood pressure (MAP) and heart rate as previously described (Kym *et al*., 2006). Briefly, male Sprague–Dawley rats (325–375 g) were anaesthetized with inactin (100 mg $kg^{-1}$ i.p.). MAP and heart rate were measured through a femoral artery catheter (PE50) connected to a pressure transducer (Transpac II; Abbott Labs). Two femoral vein catheters (PE50) were inserted and attached to a syringe pump to deliver 10 μl $min^{-1}$ of saline to maintain hydration and for drug delivery, respectively. After a stabilization period of at least 1 h, baseline MAP and heart rate values were recorded at 10-s intervals for 30 min. Subsequently, a slow i.v. infusion of vehicle or selected BME

**Table 1** $K_i$ (μM) of compounds against various metalloproteinases

| *Compounds* | *NEP* | *NEP2* | *ECE1* | *ACE* | *APP* |
|---|---|---|---|---|---|
| Candoxatrilat | 0.0032 | 0.044 | 6.5 | >10 | >10 |
| Lisinopril | >30 | >30 | >10 | 0.0012 | >10 |
| Omapatrilat | 0.00045 | 0.025 | 10 | 0.00064 | 0.25 |
| Apstatin | >30 | >30 | >30 | NT | 1.0 |

Abbreviations: ACE, angiotensin-converting enzyme; APP, aminopeptidase P; ECE1, endothelin-converting enzyme 1; NEP, neprilysin; NEP2, neprilysin 2; NT, not tested.
Mean values are presented; $n = 4$–15 per group.

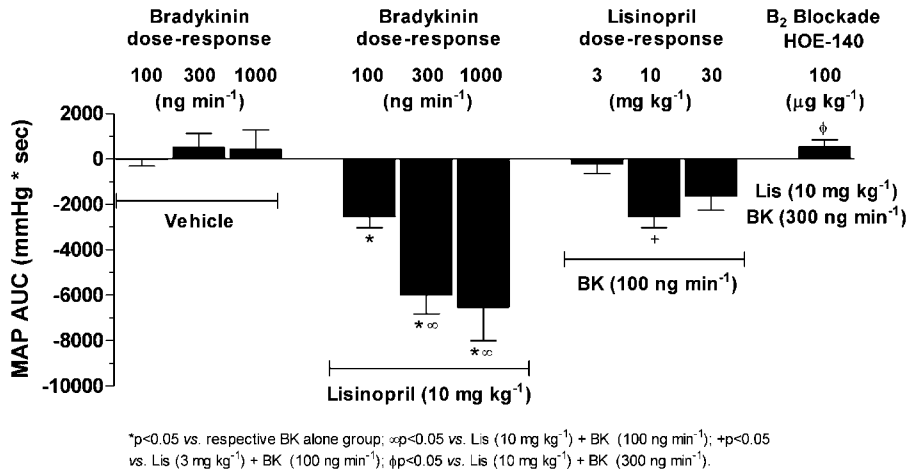


**Figure 1** Mean arterial blood pressure area under the curve (MAP AUC) was recorded during the last 5 min of the bradykinin (BK) infusion. BK alone produced no depressor effect. In the presence of lisinopril (Lis), BK produced dose-dependent reductions in MAP AUC that were completely abolished by the $B_2$ receptor blocker, HOE-140.

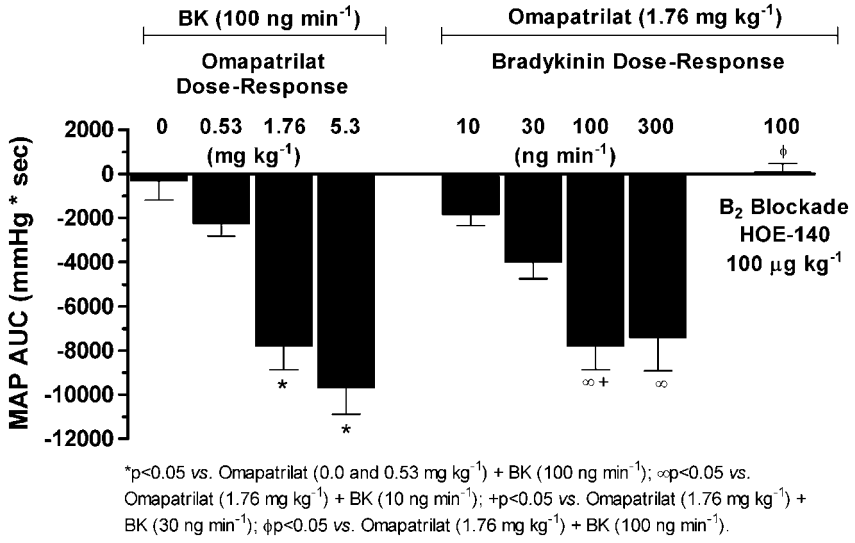


**Figure 2** Mean arterial blood pressure area under the curve (MAP AUC) was recorded during the last 5 min of the bradykinin (BK) infusion. In the presence of increasing doses of omapatrilat, BK produced dose-dependent reductions in MAP AUC that were completely abolished by the $B_2$ receptor blocker, HOE-140.

inhibitors (alone or in combination) was delivered over 35 min in a polyethylene glycol 400 vehicle at 0.5 ml $kg^{-1}$ 30 $min^{-1}$ to achieve steady-state plasma concentrations and BK was administered i.v. in a saline vehicle at 100 μl $min^{-1}$ for the last 5 min of the experimental protocol and the area under the curve (AUC) was calculated during this 5 min period. Rats were randomly assigned to one of twenty-six different groups ($n = 6$–12 per group; $n$ for individual groups are detailed in the Supplementary data).

In the first group of experiments (corresponding to Figure 1), BK and ACE inhibition dose–response was investigated with BK (100, 300 and 1000 ng $min^{-1}$) or lisinopril (3, 10 or 30 mg $kg^{-1}$) administered i.v. alone or in combination; HOE-140 (icatibant), a BK $B_2$ receptor blocker (100 μg $kg^{-1}$) was employed in a final group of animals to validate that the hypotensive effects of BK in the presence of ACE inhibition were wholly mediated by $B_2$ receptor stimulation; the dose of HOE-140 (100 μg $kg^{-1}$ i.v.) has been previously shown to abolish the depressor effect of Ang(1–7) in the presence of candesartan (Walters *et al.*, 2005).

In the second group of experiments (corresponding to Figure 2), BK and omapatrilat dose–response was investigated with BK (10, 30 or 100 ng $min^{-1}$) or omapatrilat (0.0, 0.53, 1.76 or 5.3 mg $kg^{-1}$) administered i.v. alone or in combination; HOE-140 (100 μg $kg^{-1}$) was employed in a final group of animals to validate that the hypotensive effects of BK in the presence of omapatrilat inhibition were wholly mediated by $B_2$ receptor stimulation.

In the third group of experiments (corresponding to Figure 3), using an optimal dose of BK (100 ng $min^{-1}$) as defined in the first two studies, the effect of single, double or even triple-enzyme inhibition was investigated: ACE (lisinopril; 10 mg $kg^{-1}$), NEP (candoxatril; 10 mg $kg^{-1}$) and APP (apstatin; 10 mg $kg^{-1}$). The effect of each compound alone,

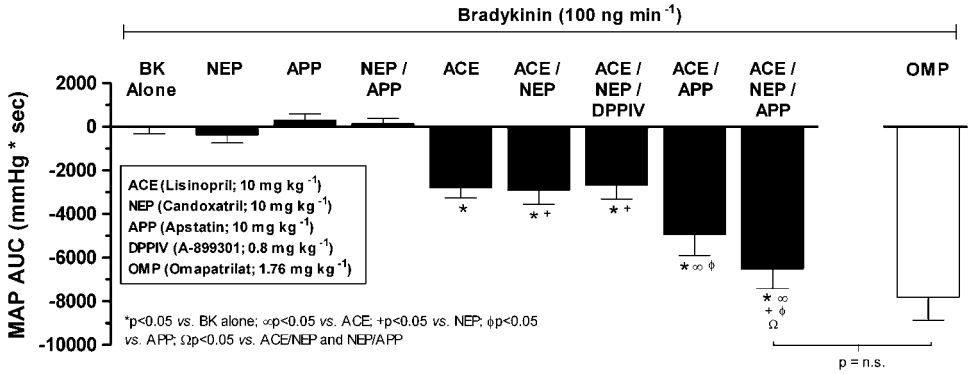


**Figure 3** Mean arterial blood pressure area under the curve (MAP AUC) was recorded during the last 5 min of the bradykinin (BK) infusion. BK alone or BK in the presence of inhibition of neprilysin (NEP) and/or aminopeptidase P (APP) produced no effect on MAP AUC. ACE inhibition decreased MAP AUC, an effect that was not enhanced by concomitant blockade of NEP or dipeptidyl peptidase IV (DPPIV). However, reductions in MAP AUC were intensified in the presence of concomitant ACE/APP blockade and were further enhanced in the presence of NEP inhibition to values that were not different from omapatrilat alone.

and in combination, was investigated, as was inhibition of all three enzymes in parallel. In a final study, the effect of DPPIV inhibition (A-899301; 0.8 mg kg$^{-1}$) in combination with inhibition of ACE (lisinopril; 10 mg kg$^{-1}$) and NEP (candoxatril; 10 mg kg$^{-1}$) was also delineated.

*Dose selection*

Doses of A-899301 and candoxatril in the present study were chosen to achieve complete and selective inhibition of DPPIV and NEP, respectively. Plasma concentrations of the DPPIV inhibitor, A-899301 (compound 30 in Backes *et al*., 2007), in the present study reached 0.165 ± 0.018 μg ml$^{-1}$ at the end of the 30 min infusion; A-899301 has been shown to be fully efficacious *in vivo* at 0.118 μg ml$^{-1}$ (Backes *et al*., 2007), a concentration remarkably similar to that achieved in the present study. Moreover, concentrations of A-899301 achieved in the present study would be expected to be selective for DPPIV ($K_i$ = 2.1 nM) rather than dipeptidyl peptidase 8 ($K_i$ = 4350 nM), dipeptidyl peptidase 9 ($K_i$ = 11 100 nM) and other dipeptidyl peptidases, including prolyl oligopeptidases ($K_i$ > 30 000 nM) and fibroblast activation protein α (seprase; $K_i$ > 30 000 nM). In the present study, the plasma concentration of candoxatrilat, the active metabolite of candoxatril, reached 7.82 ± 0.64 μg ml$^{-1}$ after a 10 mg kg$^{-1}$ 30-min infusion. This concentration is above those previously demonstrated to produce a modest antihypertensive effect in conscious spontaneously hypertensive rats (Gardiner *et al*., 2006), but it is a concentration at which selectivity for NEP would be expected, since even at vastly higher concentrations (up to 0.1 mM) candoxatrilat was shown to exert no inhibitory activity at other well-known peptidases, including ACE, carboxypeptidase A, leucine aminopeptidase, trypsin, chymotrypsin and renin (Northridge *et al*., 1989; Barclay *et al*., 1991).

*Statistical methods*

Within each of the experimental groups, differences in MAP AUC during the BK infusion were analysed by one-way ANOVA, Bonferoni post-test ($P$ < 0.05); only relevant comparisons were made in MAP AUC to maximize the statistical sensitivity of the assay.

*Materials*

Cloned human NEP and human NEP2 were purchased from R&D Systems (Minneapolis, MN, USA). Human ECE1 membrane preparation was isolated from a Chinese hamster ovary cell line expressing ECE1 (Dr M Schmidt, Neuroscience Discovery, Ludwigshafen, Germany). Cloned human ACE was purchased from BioMol (Plymouth Meeting, PA, USA), and secreted form of the membrane-bound human APP was obtained from a human embryonic kidney cell media transfected with cloned hAPP (1–657). 7-Methyloxycoumarin-4-yl-R-P-G-F-S-A-F-K-K(dinitrophenyl)-OH, a BK analogue, was kindly synthesized by Dr P Richardson (Abbott Labs) and used as the substrate for NEP, NEP2 and ECE1 (Johnson and Ahn, 2000). Aminobenzoic acid-F-R-K(dinitrophenyl)-F-OH, the substrate for ACE (Alves, 2005) was purchased from Bachem (King of Prussia, PA, USA). K(dinitrophenyl)-P-P-G-K(aminobenzoyl)-$NH_2$, the substrate for APP, was also synthesized by Dr P Richardson (Molinaro, 2005).

## Results

Enzyme selectivity ($K_i$ values) of lisinopril, candoxatrilat, apstatin and omapatrilat is shown in Table 1. Candoxitrilat, the active metabolite of candoxatril, displays high potency for NEP and NEP2 inhibition and weak activity against ACE, ECE1 and APP. The ACE inhibitor, lisinopril, shows high potency and selectivity for ACE with weak activity for NEP, NEP2, ECE1 and APP. Omapatrilat exhibits high potency for NEP, NEP2 and ACE, moderate strong activity against APP, but low activity against ECE1. Apstatin demonstrated moderate, but selective, activity against APP without effect at NEP, NEP2, ECE1 or ACE.

The functional depressor effects of each group within each study were based on the MAP AUC, which takes into account both peak reductions in MAP and reductions in MAP over time (5 min). Correlation analysis of the depressor effects of

each dosing group suggested that analyses of the results based on MAP AUC are comparable to mean maximal reductions in MAP during the 5 min BK infusion period (linear regression analysis and $r^2 = 0.95$; please see Supplementary data). For each experiment, baseline and 30 min post-treatment MAP and HR values as well as maximal changes in MAP/HR and MAP AUC during BK infusion are detailed in the Supplementary data.

The first group of experiments was performed to elucidate the optimal dose of both BK and lisinopril for subsequent studies and to determine if the effects observed in the model were wholly dependent upon BK $B_2$ receptor activation. In the presence of vehicle, BK alone (100, 300 or 1000 ng min$^{-1}$) produced little to no effect on blood pressure and thus no effect on MAP AUC (Figure 1). In the presence of the ACE inhibitor, lisinopril (10 mg kg$^{-1}$), BK produced a dose-dependent reduction in MAP AUC; however, reductions in MAP AUC were not enhanced as the concentration of BK increased from 300 to 1000 ng min$^{-1}$. Thus, 100 ng min$^{-1}$ BK, which elicited a submaximal depressor response, was employed in subsequent studies to elucidate whether inhibition of enzymes responsible for BK degradation could enhance the reductions in blood pressure produced by BK infusion. At 100 ng min$^{-1}$ BK, peak reductions in MAP AUC in the presence of lisinopril were achieved at 10 mg kg$^{-1}$. Although MAP AUC values in the 30 mg kg$^{-1}$ lisinopril group did not achieve statistical significance relative to BK at 100 ng ml$^{-1}$, values did show a downward trend and were not different from values in the 10 mg kg$^{-1}$ lisinopril group ($P > 0.05$, *t*-test) suggesting complete ACE inhibition was achieved at 10 mg kg$^{-1}$. Thus, 10 mg kg$^{-1}$ lisinopril was employed in subsequent studies. Finally, the BK $B_2$ receptor blocker, HOE-140, completely reversed hypotension produced by BK in the presence of lisinopril, suggesting that the effects of BK in this model are wholly mediated by $B_2$ receptor activation.

A second group of experiments was performed to delineate the effects of omapatrilat in the model. In the presence of optimal doses of BK (100 ng min$^{-1}$), omapatrilat produced a dose-dependent reduction in MAP AUC; peak reductions were observed at 1.76 and 5.3 mg kg$^{-1}$ and were not different between the two groups (Figure 2). We confirmed that the effects of omapatrilat in this model were dependent on increases in circulating BK; the effect of omapatrilat (1.76 mg kg$^{-1}$) on MAP AUC was reduced in the presence of lower concentrations of BK (10 and 30 ng min$^{-1}$) but was not enhanced when BK was infused at 300 ng min$^{-1}$ ($P > 0.05$). Moreover, the BK $B_2$ receptor blocker, HOE-140, completely reversed hypotension produced by BK in the presence of omapatrilat.

A final group of experiments was performed to delineate the contribution of each enzyme responsible for BK degradation in the production of the hypotensive response; selected enzyme inhibitors were studied alone and in combination (Figure 3). BK alone, or in the presence of candoxatril or apstatin, produced no effect on MAP AUC. Lisinopril decreased MAP AUC, an effect that was not enhanced by concomitant administration of candoxatril or candoxatril + A-899301. However, reductions in MAP AUC produced by lisinopril were enhanced in the presence of apstatin and further intensified in the presence of candoxatril to values that were not different from omapatrilat alone ($P > 0.05$, *t*-test).

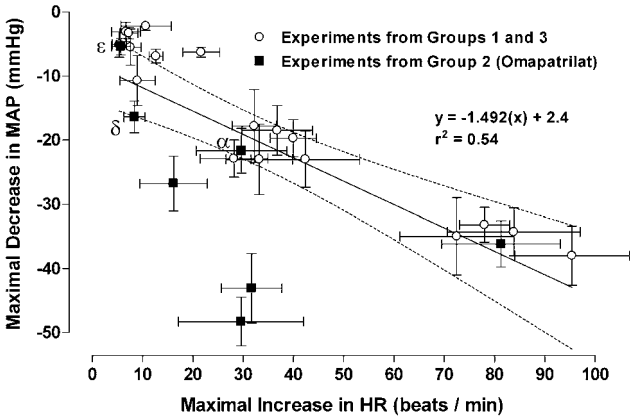


**Figure 4** Correlation analysis and 95% confidence intervals of the depressor effects as compared with maximal increases in heart rate during the 5 min bradykinin (BK) infusion period for experiments in the absence or presence of omapatrilat. Linear regression analysis suggested that reflex tachycardia was blunted in rats administered omapatrilat (effects outside 95% confidence interval) in three of four omapatrilat treatment groups with large changes in blood pressure, an effect not observed in the other treatment groups with select enzyme inhibitors or in the presence of a low dose of omapatrilat (0.53 mg kg$^{-1}$; denoted by α), low concentrations of BK (10 ng min$^{-1}$; denoted by δ), or omapatrilat-treated rats in the presence of $B_2$ receptor blockade with HOE-140 (denoted by ε).

To determine if changes in reflex-mediated tachycardia influenced the depressor response to the various BME inhibitors, maximal changes in blood pressure were plotted vs maximal increases in heart rate during the 5 min BK infusion (Figure 4). Linear regression analysis demonstrated that while most values fell within the 95% confidence interval, values for three of four groups of animals that received omapatrilat, at doses (1.76 and 5.3 mg kg$^{-1}$) that produced substantial reductions in blood pressure (>25%), exhibited blunted reflex tachycardia. However, in the presence of a low dose of omapatrilat (0.53 mg kg$^{-1}$), low concentrations of BK (10 ng min$^{-1}$) or $B_2$ receptor blockade with HOE-140 induced values to fall within the confidence interval.

## Discussion

These studies demonstrate the utility of an *in vivo* rat depressor model to delineate the effects of inhibition of BMEs for the determination of relative angioedema risk. We demonstrated that omapatrilat produces marked hypotension, an effect dependent upon BK $B_2$ receptor activation and consistent with inhibition of APP concomitant with both ACE and NEP blockade. Moreover, we clearly demonstrated in the rat that BK is degraded *in vivo* with an enzyme rank efficacy of ACE > APP ≫ NEP or DPPIV. Thus, our results suggest that novel BME inhibitors must display no activity against APP to minimize angioedema risk due to the high prevalence of ACE inhibitor therapy in patients with cardiovascular disease and in diabetic patients (Aguilar and Solomon, 2006).

Due to the complexity of measuring transient increases in plasma BK, a direct cause–effect relationship between this peptide and angioedema has been difficult to prove in pre-clinical models. However, several lines of evidence indicate that BK catabolism is central to the pathogenesis of ACE inhibitor-related angioedema in both pre-clinical models and patients (Han *et al*., 2002; Moreau *et al*., 2005; Beltrami *et al*., 2006). In support of the latter, it has been shown that angioedema exists in patients with defective control of BK generation (Agostoni and Cicardi, 2001; Bas *et al*., 2006) and is observed in patients taking ACE inhibitors with elevated plasma BK concentrations (Nussberger *et al*., 1998; Agostoni *et al*., 1999; Cugno *et al*., 2003).

Importantly, these results suggest that inhibition of NEP plays only a tertiary role to ACE and APP in BK degradation and subsequently in the depressor response to BK infusion. In support of this conclusion, we demonstrated that candoxatril alone elicited no hypotension in response to BK and did not exacerbate the effects of lisinopril when given in combination, suggesting that NEP probably contributes only minimally to omapatrilat-induced hypotension and angioedema. These results are consistent with those of Ishida *et al*. (1989a) who demonstrated *in vitro* that ACE was responsible for a large proportion of total plasma kininase activity and the contribution from NEP was negligible.

Data from other studies also suggest that omapatrilat is not a DPPIV inhibitor, indicating that omapatrilat-induced angioedema is independent of DPPIV inhibition (Sulpizio *et al*., 2005). In support of this, we demonstrated that inhibition of DPPIV with A-899301 concomitant with lisinopril and candoxatril does not exacerbate the depressor response produced by lisinopril alone suggesting, by extension, that DPPIV inhibition would not be expected to contribute to angioedema *in vivo*. In contrast, a recent publication by Byrd *et al*. (2007), in DPPIV-deficient rats, demonstrated that ACE inhibition with captopril resulted in greater peritracheal oedema than that induced by captopril treatment in normal rats, suggesting that DPPIV deficiency may predispose rats to oedema. In addition, there is similar evidence indicating increased angioedema risk in patients with low DPPIV levels (Lefebvre *et al*., 2002). Although the effect of concomitant pharmacological blockade of ACE and DPPIV on angioedema risk has not been investigated clinically, the present study demonstrates no exacerbated hypotension in lisinopril-treated rats in the presence of A-899301 at concentrations shown to inhibit fully DPPIV activity in rats (Backes *et al*., 2007).

Previous studies have demonstrated that APP contributes to the regulation of BK metabolism and that depressor responses to apstatin are exacerbated in the presence of ACE inhibition, an effect correlated with increases in measured concentrations of BK in the plasma (Kitamura *et al*., 1999). In the present study, we clearly demonstrated the importance of APP in the *in vivo* degradation of BK, second only to ACE in potency, and furthermore suggest that concomitant inhibition of both enzymes by omapatrilat is probably the primary mechanism responsible for the large depressor response in rats (and subsequent angioedema in patients); a finding consistent with increased susceptibility to angioedema in patients with low APP activity on ACE inhibitor therapy (Adam *et al*., 2002; Duan *et al*., 2005; Nikpoor *et al*., 2005). Thus, there is a need to develop novel BME inhibitors with better selectivity (for example, no APP inhibitory activity) to ensure patient safety from adverse events mediated by APP inhibition (Jandeleit-Dahm, 2006).

Interestingly, the novel dual ACE/NEP inhibitor, GW660511X, with no activity against APP, elicited no angioedema in a small clinical trial. However, this compound was not efficacious at inducing a reduction in blood pressure (Jandeleit-Dahm, 2006; Johnson *et al*., 2006). Thus, whether concomitant inhibition of APP is necessary to achieve robust blood pressure-lowering effects with ACE/NEP inhibitors is presently unknown. Regardless of the enzymes responsible for efficacy, in the present study we demonstrated that these novel dual inhibitors must be devoid of APP inhibitory activity, as the depressor risk associated with omapatrilat was probably mediated by concomitant inhibition of ACE, NEP and APP.

Prior to the present study the question of relative angioedema risk upon ACE, NEP, APP or DPPIV inhibition has been confounded by the lack of a predictive pre-clinical model. Although these results suggest that inhibition of ACE, NEP and APP is necessary to produce omapatrilat-like depressor responses, others have suggested in a different model, using a two-point and wide-ranging dose–response (0.1 and 30 mg kg$^{-1}$), that APP inhibition does not explain the increased plasma extravasation produced by omapatrilat in NEP-inhibited rats (Sulpizio *et al*., 2005). Unfortunately, APP activity was not determined in the latter study and the low dose of apstatin used in this model may have been insufficient to produce complete blockade of APP *in vivo* (Sulpizio *et al*., 2005). Further, these authors suggest that APP inhibition may indeed contribute to the increase in angioedema incidence in the presence of omapatrilat in patients (Sulpizio *et al*., 2005).

Interestingly, evidence from the present study suggests that hypotension elicited by omapatrilat (1.76 and 5.3 mg kg$^{-1}$) is due to a blunted reflex tachycardia. When the maximum increase in heart rate during the 5 min BK infusion was analysed and compared with the maximal reductions in MAP, the results suggest that reflex tachycardia was attenuated in three of four omapatrilat treatment groups that exhibited large changes in blood pressure (greater than 25%). However, this effect was not observed in the other treatment groups with enzyme-selective antagonists or in the presence of low-dose omapatrilat (0.53 mg kg$^{-1}$), low-dose BK (10 ng min$^{-1}$) and was not present in omapatrilat-treated rats (1.76 mg kg$^{-1}$) in the presence of $B_2$ receptor blockade with HOE-140. Although the mechanism of this blunted baroresponsiveness is not known, a few noteworthy possibilities exist that may explain this observation. These include omapatrilat-induced elevations of BK or substance P in the nucleus tractus solitarii (NTS) and increases in peripheral atrial natriuretic peptide (ANP), all of which may subsequently modulate baroreflex function (Hall *et al*., 1989; Lasher *et al*., 1990; Volpe, 1992; Caligiorne *et al*., 1996; McClean *et al*., 2000).

Indeed, Caligiorne *et al*. (1996) have demonstrated in the rat that microinjections of BK into the NTS of anaesthetized rats elicit an increase in vagal efferent activity and a decrease

in sympathetic activity, effectively modulating the central cardiovascular control, whereas others have suggested that BK may inhibit the carotid sinus baroreflex in anaesthetized rats via vascular endothelial NO release (Wu and He, 1999). However, if increases in BK in the NTS are responsible for the blunted baroreflex responsiveness in the presence of omapatrilat, it is curious that this effect was not observed in the present study in the presence of triple-enzyme inhibition with lisinopril, candoxatril and apstatin that also elicited a large depressor response. Another explanation may relate to omapatrilat-induced inhibition of NEP localized within the NTS (Lasher *et al*., 1990). Whereas NEP inhibition would be expected to increase BK within the NTS, NEP is also responsible for the breakdown of substance P to substance P (1–7) (Hall *et al*., 1989). This substance modulates the gain of the baroreceptor reflex and effectively serves as a neuromodulator/neurotransmitter in baroreceptor afferent neurons (Helke and Seagard, 2004). However, although omapatrilat exhibits high tissue penetration (Backlund *et al*., 2001), evidence for NEP inhibition in the brain by omapatrilat has not been demonstrated (Kubota *et al*., 2003), suggesting that this effect may result from the peripheral, rather than central, actions of omapatrilat. Interestingly, it has been demonstrated that omapatrilat elicits increases in ANP in patients (McClean *et al*., 2000). Also, experimental observations in several animal species and in humans suggest that ANP resets the baroreflex control of heart rate in a way that favours bradycardia, opposes cardioacceleration and interferes with reflexes controlling vascular tone (Volpe, 1992). Although it has been suggested that this influence of ANP on the baroreflex control of circulation may be important in short-term cardiovascular adaptations (Volpe, 1992), whether this mechanism came into play in the present study is unknown.

Although angiotensin I is traditionally considered the main physiological substrate for ACE ($K_m$ approximately 16 μM), ACE also inactivates BK ($K_m$ approximately 0.18 μM) by hydrolysing two separate bonds on its C-terminal end transforming BK into its final inactive product, BK[1–5] (Inokuchi and Nagamatsu, 1981; Oshima *et al*., 1985; Erdos, 1990; Moreau *et al*., 2005). In the present study, inhibition of APP alone produced no effect on BK-induced depressor responses despite conversion of BK to the inactive metabolites BK[2–9] and BK[2–8] by APP, an effect probably explained by the relatively high $K_m$ for BK ($K_m$ approximately 75 μM) relative to that of ACE (Moreau *et al*., 2005). Like ACE and APP, NEP also inactivates BK to an inactive metabolite, BK[1–7] and BK[1–4] (Moreau *et al*., 2005). However, in the present study inhibition of NEP produced little or no effect on BK-induced reductions in blood pressure, whereas ACE inhibition produced clear hypotension. This effect may be explained by the observation that NEP is the main enzyme responsible for the metabolism of kinins in the kidney, whereas in plasma, unlike ACE and APP, NEP does not play a significant role in BK metabolism (Decarie *et al*., 1996; Moreau *et al*., 2005). Thus, the activity of both ACE and APP in the plasma probably contributed to the exacerbated depressor response to dual enzyme inhibition relative to that produced by sole inhibition of either enzyme alone in the present study. Moreover, the lack of effect of DPPIV inhibition on blood pressure in the present study in the presence of concomitant NEP and ACE blockade is probably because DPPIV degrades the APP BK metabolite, BK[2–9], rather than BK itself, to BK[4–9] (Lambeir *et al*., 2003). Thus, although DPPIV activity is depressed in patients with acute ACE-inhibitor-associated angioedema (Lefebvre *et al*., 2002), the results from the present study suggest that DPPIV inhibition, concomitant with NEP and ACE, would be expected to produce no increased risk of angioedema compared with ACE inhibition alone.

Tissue kininases are also critical in the metabolism of BK and subsequent hypotensive response in the face of kininase blockade. The wide distribution of tissue kallikreins and kininases suggests an autocrine or paracrine function, inasmuch as they act principally in the local environment (Marcondes and Antunes, 2005). Also, the *in vivo* metabolism of kinins by tissue kininases has been probed in a number of species and it has been shown that the tissue kallikrein system contributes to the maintenance of arterial pressure homoeostasis (Marcondes and Antunes, 2005). Indeed, Ishida *et al*. (1989b) demonstrated, in subtotally nephrectomized rats, that kidney ACEs as well as other tissue peptidases are critical in kinin metabolism. Moreover, Johnston *et al*. (1982) demonstrated, in anaesthetized dogs, that ACE/kininase inhibition with captopril increases local tissue concentration of kinins contributing to hypotension. Thus, these studies in concert with the present results suggest that effects on both the plasma and tissue kininogen–kallikrein–kinin systems mediate the disruptions in pressure homoeostasis under conditions of kininase blockade.

The contributions of carboxypeptidase-N and -M to the hypotensive response elicited by BK infusion were not elucidated in the present study. Although these enzymes represent only a minor metabolic pathway for inactivation of BK under normal conditions *in vivo* (Ishida *et al*., 1989b; Moreau *et al*., 2005), these enzymes may play a more prominent role in BK metabolism (to des-Arg9-BK) in the presence of ACE inhibition (Skidgel, 1988) and reduced metabolism of des-Arg9-BK may be responsible for angioedema produced by ACE blockade (Blais *et al*., 1999; Molinaro *et al*., 2002). Also, as a potential limitation to the present study, it should be noted that some angioedema observed clinically may be independent of BK, such as that observed in a small percentage of patients on angiotensin receptor blockers (Cicardi *et al*., 2004) and immune-related angioedema mediated by histamine (Agostoni and Cicardi, 2001; Nussberger *et al*., 2002). Finally, the doses of the antagonists used in the present study to elicit hypotension may be different from those able to increase vascular permeability and as such it is possible that, although NEP or DPPIV inhibition produces no effect on blood pressure, they may still influence vascular permeability (Sulpizio *et al*., 2005).

In the present study, we demonstrated that the BK $B_2$ receptor antagonist, HOE-140 (icatibant), can fully prevent the depressor effects of omapatrilat *in vivo* supporting the concept that BK $B_2$ receptor blockers have an important clinical role in the control of BK-dependent angioedema in patients (Moreau *et al*., 2005). Indeed, in clinical studies $B_2$ receptor blockade with icatibant has been shown to be

effective in treating acute hereditary angioedema and it has been suggested that icatibant may be a viable option in the treatment of patients with angioedema induced by ACE inhibitors or other kinins (Bas *et al*., 2006; Bork *et al*., 2007).

In summary, we demonstrated that in the presence of ACE inhibition in rats, breakdown of BK *in vivo* is carried out by non-ACE pathways, predominately APP, with only a modest contribution from NEP and with little to no subsequent degradation by DPPIV. These results suggest that dual inhibition of ACE and NEP with or without concomitant DPPIV inhibition would be expected to produce no increased risk of angioedema compared with ACE inhibition alone. Moreover, these results also demonstrated that BK is degraded *in vivo* with an enzyme rank efficacy of ACE$>$ APP$\gg$NEP or DPPIV and suggest that novel BME inhibitors must display no activity against APP to avoid any additional risk of angioedema, especially given the high prevalence of ACE inhibitor therapy in patients with cardiovascular disease and in diabetics (Aguilar and Solomon, 2006). Moreover, the results from the present study suggest a path forward exists for the discovery and development of novel enzyme inhibitors targeting this pathway and dispel the myth that dual ACE/NEP or DPPIV inhibitors pose a significant safety risk when moved into clinical development.

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## Conflict of interest

At the time the studies were performed all authors were employees of Abbott Laboratories.

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