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Dual inhibition of angiotensin-converting enzyme and neutral endopeptidase by the orally active inhibitor mixanpril: A potential therapeutic approach in hypertension

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ABSTRACT **In the treatment of cardiovascular disease, it could be of therapeutic interest to associate the hypotensive effects due to the inhibition of angiotensin II formation with the diuretic and natriuretic responses induced by the protection of the endogenous atrial natriuretic peptide (ANP). Investigation of this hypothesis requires an orally active compound able to simultaneously inhibit angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP), which is involved in renal ANP metabolism. Such compounds have been rationally designed by taking into account the structural characteristics of the active site of both peptidases. Among them, RB 105, *N*-[(2*S*,3*R*)-2-mercaptomethyl-1-oxo-3-phenylbutyl]-(*S*)-alanine, inhibited NEP and ACE with K_i values of 1.7 ± 0.3 nM and 4.2 ± 0.5 nM, respectively. Intravenous infusion of RB 105 in conscious spontaneously hypertensive rats prevented the pressor response to exogenous angiotensin I and potentiated the natriuretic response to ANP. Infusion of RB 105, at 2.5, 5, 10, 25, and 50 mg/kg per hr decreased blood pressure dose-dependently in conscious catheterized spontaneously hypertensive rats and increased diuresis and natriuresis. Infusion of RB 105 as a bolus of 25 mg/kg followed by 25 mg/kg per hr similarly decreased blood pressure and increased natriuresis in three different models of hypertension (renovascular, deoxycorticosterone acetate–salt, and spontaneously hypertensive rats). Mixanpril, a lipophilic prodrug of RB 105 (ED_{50} values when given orally to mice, 0.7 mg/kg for NEP; 7 mg/kg for ACE), elicited dose-dependent hypotensive effects of long duration in spontaneously hypertensive rats after oral administration [−37 mmHg for 50 mg/kg twice a day (1 mmHg = 133 Pa) and is therefore the first dual NEP/ACE inhibitor potentially useful for clinical investigations.**

Blood pressure and fluid volume homeostasis are critically dependent on regulatory peptides such as angiotensin II, which has vasoconstrictive properties, and atrial natriuretic peptide (ANP), which induces diuresis, natriuresis, and slight vasodilatation. Other peptides, such as bradykinin and endothelin, may also play a role in cardiovascular homeostasis (review in ref. 1). The metabolism of these peptides is mainly controlled by two enzymatic systems, angiotensin-converting enzyme (ACE, EC 3.4.25.1) and neutral endopeptidase (NEP, EC 3.4.24.11, also called neprilysin) (review in ref. 2). ACE belongs to the enzymatic cascade of the renin–angiotensin system and releases the vasoconstrictor peptide angiotensin II from the inactive precursor, angiotensin I. NEP inactivates ANP (review in ref. 3), and bradykinin is metabolized by ACE and NEP at their endothelial and epithelial sites, respectively (4). The modulation of the circulating levels of these various endogenous peptides may therefore be an efficient way of treating various cardiovascular diseases. Selective inhibitors of ACE (5, 6) are clinically useful for the treatment of hypertension (7) and congestive heart failure (8). To increase their efficiency, these inhibitors are generally associated with classical diuretics, which can, however, evoke secondary effects such as activation of pressor systems and kaliuresis (7).

Selective inhibitors of NEP have been developed with the aim of delaying ANP degradation (9–12) and their efficiency in protecting endogenous ANP has been demonstrated in various animal models of hypertension (10–13). In humans they have significant diuretic and natriuretic effects (14–17) with no potassium loss (14, 16). However, these renal effects are not accompanied by significant changes in blood pressure or left ventricular hemodynamic load (14).

Taken together, these results suggested the therapeutic interest of a simultaneous inhibition of ACE, to avoid angiotensin II formation, and of NEP, to potentiate ANP action. Accordingly, the association of selective inhibitors of both enzymes has been tested in various models of hypertension in rats, and a potentiation of their respective effects has been observed (18, 19). Nevertheless, for reasons of bioavailability, pharmacokinetic parameters, and toxicity, it was more interesting to develop mixed inhibitors of NEP and ACE. The first dual inhibitor of NEP and ACE, *N*-(2-mercaptomethyl-3-phenylpropanoyl)-L-leucine was described >10 years ago (20–22). This compound, also designated SQ 28133, has been found to elicit depressor activity in both deoxycorticosterone acetate (DOCA)–salt hypertensive rats and spontaneously hypertensive rats (SHRs) after i.v. administration at high doses (100–300 mg/kg) (18). Another mixed inhibitor of NEP and ACE, alatriopril, has been also studied after i.v. administration in anesthetized rodents (23). This compound induces responses corresponding to ANP protection (diuresis, natriuresis, cGMP excretion) but does not significantly modify arterial pressure.

In this paper, we describe a dual inhibitor of NEP and ACE that is able to reduce blood pressure and to increase sodium excretion in different models of hypertension. This molecule, RB 105, *N*-[(2*S*,3*R*)-2-mercaptomethyl-1-oxo-3-phenylbutyl]-(*S*)-alanine (Fig. 1), designed by a rational approach including molecular modeling, inhibits NEP and ACE with K_i values in the nanomolar range. After i.v. administration, RB 105 elicits the vascular and renal effects resulting from inhibition of both ANP metabolism and angiotensin II formation in conscious rats. Furthermore, mixanpril (Fig. 1), a prodrug of RB 105 which has an improved bioavailability allowing simultaneous inhibition of endothelial ACE (lung) and epithelial NEP (kidney), decreases blood pressure in SHRs after oral administration.

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Abbreviations: ACE, angiotensin-converting enzyme; NEP, neutral endopeptidase; ANP, atrial natriuretic peptide; SHR, spontaneously hypertensive rat; HACBO-Gly, *N*-[4-(hydroxyamino)-1,4-dioxo-2-(phenylmethyl)butyl]glycine; DOCA, deoxycorticosterone acetate.

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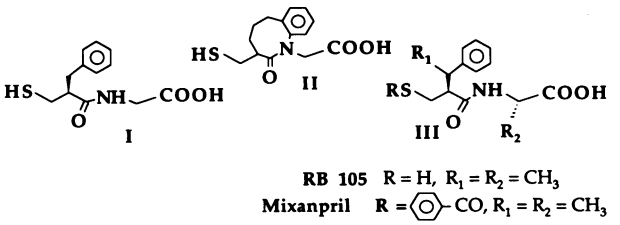

FIG. 1. Formulas of the NEP inhibitor thiorphan (I), the ACE inhibitor 3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1*H*-1-benzazocine-1-acetic acid (II), and the mixed NEP/ACE inhibitor RB 105 (III).

MATERIALS AND METHODS

Chemicals. ^{3}H-labeled [DAla^2,Leu^5]enkephalin (32 Ci/mmol; 1 Ci = 37 GBq) and *N*-[4-(hydroxyamino)-1,4-dioxo-2-(phenylmethyl)butyl]glycine ([^{3}H]HABCO-Gly) (39 Ci/mmol) were from Dositek. Cbz-Phe-His-Leu (Cbz, carbobenzoxy) was from Bachem. [^{3}H]Trandolaprilate (66 Ci/mmol) was a gift of Roussel UCLAF. The synthesis of the optically pure mixed inhibitor RB 105, *N*-[(2*S*,3*R*)-2-mercaptomethyl-1-oxo-3-phenylbutyl]-(*S*)-alanine, its three stereoisomers, and the prodrug analog mixanpril will be described elsewhere.

Assays for *in Vitro* NEP and ACE Inhibition. NEP was purified to homogeneity from rabbit kidney (24). K_i values were determined with ^{3}H-labeled [DAla^2,Leu^5]enkephalin (K_m = 20 μM) as substrate (25). NEP activity was measured in urine with the same substrate. Enzymatic studies on ACE were performed by using mouse lung membranes with Cbz-Phe-His-Leu (K_m = 50 μM) as substrate (26).

Assays for *in Vivo* NEP and ACE Inhibition in Mice. After oral administration of various doses of the prodrug mixanpril, ED_{50} values were determined by competition experiments using i.v. administered [^{3}H]trandolaprilate (0.5 μCi) for ACE (27) and [^{3}H]HACBO-Gly (1 μCi) for NEP (28). Fifteen minutes after injection of the tritiated probes, the mice were sacrificed and the kidneys (for NEP) or lungs (for ACE) were taken and quickly homogenized in 50 mM Tris/HCl buffer (pH 7.4) at 4°C. The homogenate was filtered and the radioactivity was measured by liquid scintillation counting. Captopril (1000 equivalents) in the case of lung ACE and retrothiorphan (29) (10,000 equivalents) for kidney NEP were used to determine nonspecific binding.

The time course of the inhibition was obtained by the same procedure, by using a single dose of mixanpril (26 μmol/kg) and various times (from 30 min to 18 hr) before injection of the radiolabeled probes.

Responses to Angiotensin I Injection and Exogenous ANP Infusion in SHRs. Angiotensin I was injected i.v. at 100, 150, 200 and 250 ng/kg every 15 min into vehicle-treated controls and after 30 min of RB 105 i.v. infusion (bolus of 25 mg/kg followed by 25 mg/kg per hr). ANP was infused into SHRs at 300 ng/kg per min during RB 105 i.v. infusion (bolus of 25 mg/kg followed by 25 mg/kg per hr); for comparison, SHRs were treated similarly with vehicle instead of ANP.

Dose–Response Curve of i.v. RB 105 in SHRs. A dose–response curve for natriuresis and blood pressure following i.v. infusion of RB 105 (0.25, 5, 10, 25, and 50 mg/kg) was established in 12- to 14-week-old male SHRs (n = 5–8 per dose) (Iffa Credo). The experimental protocol was carried out in unanesthetized, conscious animals as described (19). After a baseline period of 1 hr, RB 105 or its vehicle was administered by injection plus infusion. The electrolyte concentrations of fresh urine samples were measured with an ion-selective electrode (Beckman) and expressed as μmol/min.

Pharmacological Effects of i.v. RB 105 in Normotensive and Hypertensive Rats. The effects of RB 105 infusion (bolus of 25 mg/kg followed by 25 mg/kg per hr) were compared in normotensive 12- to 13-week-old male Wistar rats (n = 10) and in three models of hypertension: DOCA–salt hypertensive rats (n = 10), renovascular 1C,2K hypertensive rats (n = 10), and SHRs (n = 12). DOCA–salt hypertensive rats were obtained 4 weeks after unilateral nephrectomy and subcutaneous implantation of a deoxycorticosterone acetate pellet (200 mg/kg of body weight) in male Wistar rats (200–220 g). The drinking water was supplemented with 1% NaCl and 0.2% KCl. Renovascular hypertension was induced by placing a clip (diameter, 0.20 mm) on the left renal artery of male Wistar rats (150 g); the left kidney remained untouched (1-C, 2-K Goldblatt model). These rats were used 4 weeks after surgery.

Pharmacological Effects of Orally Administered Mixanpril in SHRs. The effects of the prodrug mixanpril were assessed after chronic oral administration in SHRs (n = 5). The animals had free access to tap water and food. Mixanpril (or its vehicle) was administered by gavage twice a day for 4 days at increasing concentrations (2.5, 5, 25, and 50 mg/kg). Blood pressure was measured every day, 2 hr after gavage, by the tail-cuff method.

Statistical Methods. Results are expressed as mean ± SEM and comparisons were performed by two-way and one-way analysis of variance followed by Scheffe F test. Correlation coefficients were obtained by the least-squares method.

RESULTS

Synthesis. The synthesis of the mixed inhibitor RB 105, *N*-[(2*S*,3*R*)-2-mercaptomethyl-1-oxo-3-phenylbutyl]-(*S*)-alanine (**4c**) and of its three stereoisomers **4a**, **4b**, and **4d** is summarized in Fig. 2 and will be reported elsewhere. The absolute configuration of the isomers was obtained by enantioselective synthesis.

***In Vitro* Inhibitory Potency.** The inhibitory potencies of the four stereoisomers **4a–4d** were tested on both NEP and ACE (Table 1). As already observed (30), inhibition of NEP was not greatly dependent of the stereochemistry of each asymmetric carbon, since the K_i values were in the range 0.7–2.3 nM. For ACE inhibition, the K_i values were particularly sensitive to the absolute configuration of the α-carbon of the butanoyl moiety. Compound **4c** (RB 105), with the 2*S*, 3*R* configuration, showed good inhibition of both enzymes.

***In Vivo* Inhibition of Lung ACE and Kidney NEP in Mice.** The *in vivo* inhibitory potencies of mixanpril were 0.7 mg/kg for kidney NEP and 7 mg/kg for lung ACE, respectively (Fig. 3). The time course of peptidase inhibition was determined for a single dose of mixanpril, 10 mg/kg, which gave about 80% and 100% inhibition of ACE and NEP, respectively, at 30 min. A significant inhibition (≈50% of ACE and ≈90% of NEP), was

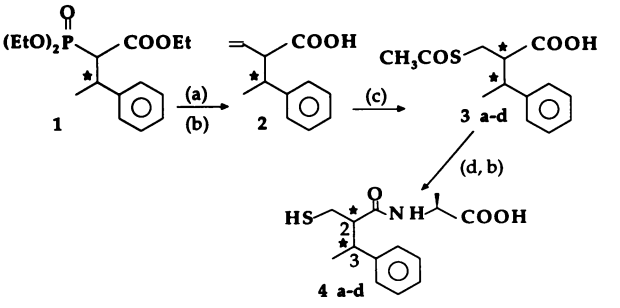

FIG. 2. Scheme for the synthesis of the mixture of the four stereoisomers of *N*-(2-mercaptomethyl-1-oxo-3-phenylbutyl)-(*S*)-alanine. DCC, dicyclohexylcarbodiimide HOBT, 1-hydroxybenzotriazole.

Table 1. Inhibitory potencies on NEP and ACE of the four stereoisomers of *N*-(2-mercaptomethyl-1-oxo-3-phenylbutyl)-(*S*)-alanine

Stereoisomer*	K_i, nM	
	NEP	ACE
4a (2*R*,3*R*)	2.3 ± 0.4	80 ± 10
4b (2*S*,3*S*)	2.1 ± 0.4	16 ± 4
4c (2*S*,3*R*)	1.7 ± 0.3	4.2 ± 0.5
4d (2*R*,3*S*)	0.7 ± 0.1	95 ± 10

See Fig. 2.

still observed 4 hr after mixanpril administration, indicating a rather long duration of action of the dual inhibitor (data not shown). In the same conditions the inhibition of ACE and NEP by alatriopril was 20% and 40%, respectively (data not shown).

Responses to Angiotensin I Injection and Exogenous ANP Infusion in SHRs. Pressor responses after i.v. injection of angiotensin I at 100, 150, 200, and 250 ng/kg were completely inhibited by RB 105 at 25 mg/kg (data not shown). The effect of ANP infusion at 300 ng/kg per min on sodium urinary excretion was potentiated by RB 105 infusion from 21 ± 4 μmol of Na^+ per min with ANP alone to 30 ± 2.5 μmol of Na^+ per min with ANP plus RB 105 ($P < 0.03$).

Dose–Response Curves of Antihypertensive and Diuretic Responses Induced by i.v. RB 105 in SHRs. RB 105 was administered as a bolus of 0, 2.5, 5, 10, 25, or 50 mg/kg followed by infusions of the same doses per hour. A maximal decrease in blood pressure was obtained 90 min after injection (Fig. 4*A*). Only doses of 25 and 50 mg/kg significantly ($P < 0.01$) decreased blood pressure as compared with control rats. A maximal effect on natriuresis was obtained at 30 min with all doses of RB 105 tested (Fig. 5*A*). Kaliuresis did not change throughout the experiment with various doses of RB 105 (data not shown).

Effects of RB 105 on Blood Pressure and Natriuresis in Normotensive and Hypertensive Rats. RB 105 was administered as a bolus of 25 mg/kg followed by infusion at 25 mg/kg per hr to normotensive, deoxycortison acetate-induced hypertensive rats, renovascular hypertensive rats, and SHRs. Administration of RB 105 decreased blood pressure in normotensive rats from 30 min to the end of the experiment. Treatment with RB 105 decreased blood pressure in hypertensive rats even more than in normotensive rats, but the decrease in blood pressure was not significantly different among the types of hypertensive rats (Fig. 4*B*). Natriuresis was not modified in vehicle-treated SHRs, whereas it increased significantly in normotensive and in all hypertensive rats after RB 105 administration (Fig. 5*B*).

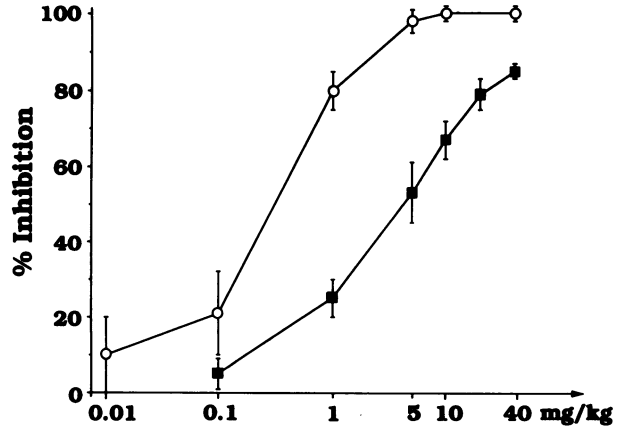

FIG. 3. Dose–inhibition curves of kidney NEP (○) and lung ACE (■) after oral administration of mixanpril in mice. Inhibition was determined by competition with tritiated ligands: [^{3}H]trandolaprilate for ACE and [^{3}H]HACBO-Gly for NEP.

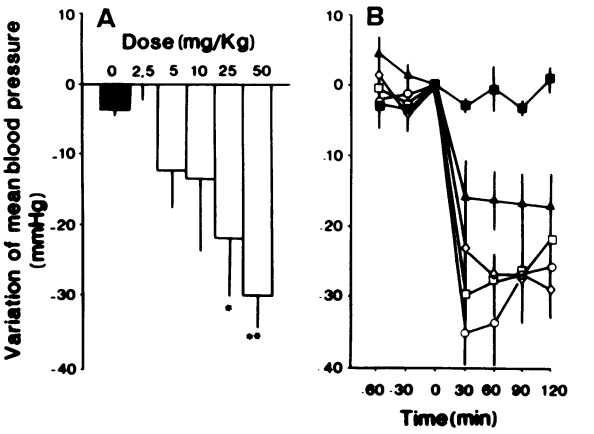

FIG. 4. Variation in mean blood pressure observed in response to RB 105 treatment. (*A*) Variation in the mean blood pressure observed 90 min after i.v. bolus plus infusion of various doses of RB 105 in conscious SHRs ($r = 0.7$, $P < 0.001$). (*B*) Significant decrease ($P < 0.001$) in mean blood pressure observed in three rat models of hypertension [DOCA–salt (◇), SHR (□), and renovascular hypertension (○)] and in normotensive Wistar rats (▲) in response to i.v. RB 105 administered as a bolus of 25 mg/kg followed by infusion at 25 mg/kg per hr. ■, Vehicle-treated SHRs. (1 mm Hg = 133 Pa.)

Effects of Oral Administration of Mixanpril in SHRs. SHRs were treated with mixanpril at various oral doses twice a day. Blood pressure decreased progressively and dose-dependently during the experiment. A maximal decrease was observed with the dose of 50 mg/kg (−37 mm Hg, $P < 0.001$) (Fig. 6). The calculated ED_{50} was ≈10 mg/kg. Mixanpril (50 mg/kg) was associated with 98% inhibition of urinary NEP activity (data not shown).

DISCUSSION

The design of mixed NEP/ACE inhibitors was facilitated because these enzymes belong to the group of zinc ectopeptidases, whose mechanism of action has been determined

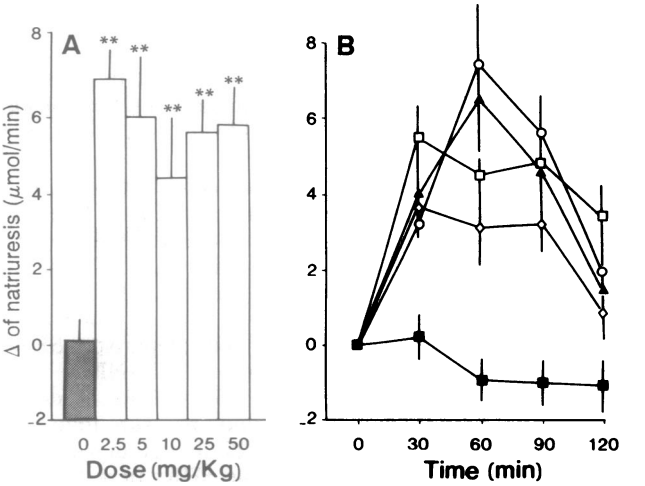

FIG. 5. Variation in natriuresis due to RB 105 treatment. (*A*) Effects on natriuresis induced by increasing doses of RB 105 i.v. injected in conscious SHR as compared with vehicle-treated rats ($P < 0.001$ by analysis of variance). (*B*) Increase in natriuresis observed in three models of hypertension [DOCA–salt (◇), SHR (□), and renovascular (○)] and in normotensive Wistar rats (▲) in response to i.v. infusion of RB 105 (25 mg/kg) and compared with vehicle-treated SHRs (■) ($P < 0.001$ by analysis of variance).

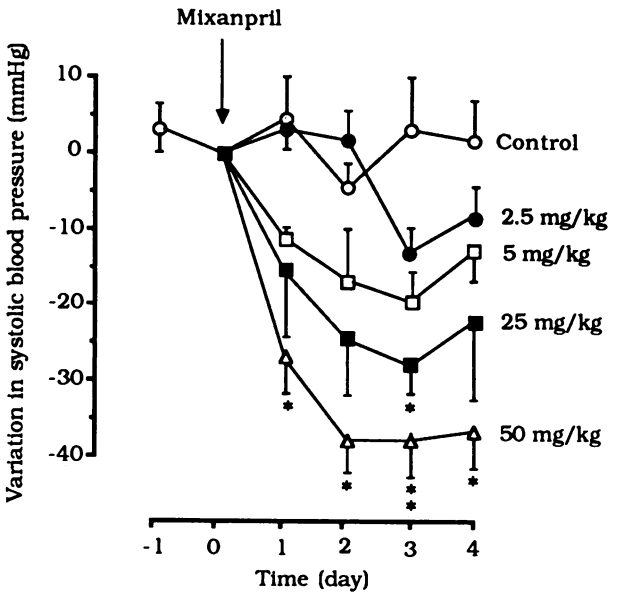

FIG. 6. Dose-dependent ($r = 0.6$, $P < 0.01$) effect of orally administered mixanpril on blood pressure in SHRs. Mixanpril or its vehicle was administered by gavage twice a day at the indicated doses and systolic blood pressure measured by the tail-cuff method 2 hr after oral administration.

from both crystallographic data (31) and molecular biology experiments (32–34). Moreover, a large number of selective inhibitors have allowed the analogies and the differences in the active site of the two enzymes to be determined (22).

Inhibitors interacting with the S_1' and S_2' subsites[§] of NEP and ACE and bearing a sulfydryl group as a zinc chelator, such as captopril for ACE and thiorphan for NEP, were selected as models for dual NEP/ACE inhibition. Furthermore, highly efficient and selective ACE inhibitors, such as 3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1*H*-benzazocine-1-acetic acid (IC_{50} = 4 nM) (35) (compound II in Fig. 1) have been obtained by introducing cyclic constraints in the structure of captopril. To increase the affinity for both NEP and ACE, various conformational restrictions aimed at reducing the degree of freedom of the dual inhibitor were introduced in a structure able to fit optimally the active site of both enzymes.

Taking into account the fact that the active site of NEP is relatively large, but does not accept an imino acid in the P_2' position[§] of the substrate (22), acyclic hydrophobic constraints were introduced on the benzyl moiety of thiorphan. Introduction of a methyl group in the β position of this residue led to the best superimposition with both the putative biologically active conformation of thiorphan in the active site of NEP (36) and the constrained benzolactam II structure obtained from molecular modeling studies (48).

Furthermore, an important step in designing a dual inhibitor of NEP and ACE was to select a molecule able to reach the endothelial and renal epithelial targets at the same time. The chosen compound possesses L-alanine as the C-terminal residue and corresponds to the 2*S*, 3*R* isomer of *N*-[2-(mercaptomethyl)-1-oxo-3-phenylbutyl]-(*S*)-alanine (RB 105) (K_i on ACE, 4.2 ± 0.5 nM; K_i on NEP, 1.7 ± 0.3 nM). The hydrophobicity of this compound was further improved by introduction of the lipophilic benzoyl moiety as a mercapto-protecting group, leading to mixanpril, which was active after oral administration in mice at low doses and had a long duration of action.

In vivo studies show that acute administration of RB 105 induces a dose-dependent decrease in blood pressure, with a significant effect for a bolus injection of 25 mg/kg followed by infusion at 25 mg/kg per hr, and an increase in natriuresis whatever the doses tested in SHRs. Administration of a specific ACE inhibitor plus a specific NEP inhibitor induces hypotensive and natriuretic effects in SHRs (19), with a large hypotensive response resulting from a potentiation between the two inhibitors (18, 19). Likewise, the hypotensive and natriuretic effects obtained with the mixed inhibitor RB 105 are related to the concomitant inhibition of both ACE and NEP. In our experiment there was no dose dependency for the natriuretic effect. This could be due to the route of administration, leading to an early renal filtration of the compound with immediate inhibition of renal epithelial NEP. However, the large decrease in blood pressure with the highest doses of RB 105 (25 and 50 mg/kg) could also have blunted the natriuretic effect. Seymour *et al.* (18) have shown that i.v. administration of large doses of SQ 28,133 produces a hypotensive response in SHRs and Gros *et al.* (23) have reported that i.v. injection of alatrioprilate potentiates the natriuretic response to salt load and blocks the pressor response to exogenous angiotensin I in anesthetized rats. Nevertheless, none of these compounds were shown to produce both the vascular and renal responses expected to result from dual inhibition of NEP and ACE.

In this study, a comparison between SHRs and the other models of hypertension showed that there was no difference for the hypotensive and the natriuretic response with RB 105 at 25 mg/kg in the different models. The natriuretic effect in the three models is likely to be related to NEP inhibition, and the hypotensive effect is probably due to NEP inhibition in deoxycortisone acetate-induced hypertensive rats but related to ACE inhibition in renovascular hypertensive rats (19). It has been shown (10, 11, 19) that acute NEP inhibition alone can decrease blood pressure in the deoxycortisone acetate-induced hypertensive model, whereas ACE inhibition cannot modify blood pressure in this renin-independent model of hypertension. In contrast, blockade of the renin angiotensin system by ACE inhibition or by angiotensin II antagonism (37) is particularly efficient in decreasing blood pressure in the renovascular, angiotensin-dependent model of hypertension. Therefore the hypotensive effect of dual inhibition does not seem to be model-restricted. Hence, these results demonstrate that coinhibition of ACE and NEP is efficient for decreasing blood pressure and inducing natriuresis whatever the experimental model of hypertension, dependent or not, on the renin–angiotensin system or salt overload.

Oral administration of the prodrug mixanpril elicits a long duration and dose-dependent hypotensive response in SHRs and an inhibition of urinary NEP activity, showing that a mixed inhibitor administered orally can be efficient on both vascular wall and renal targets and thus could represent a therapeutic alternative for the treatment of this disorder. These favorable vasodilatator and renal effects could also be beneficial in other cardiovascular diseases, such as heart failure (38). On the other hand, because angiotensin II has been shown to be a growth factor-like molecule (39, 40), and ANP (41, 42) and kinin-induced endothelial nitric oxide (43, 44) have been reported to act as antiproliferative agents for smooth muscle cells, mixed inhibitors could have beneficial effects in vascular disease (45) involving hypertrophy or proliferation of smooth muscle cells.

Note: An orally active dual NEP/ACE inhibitor has been reported by Flynn *et al.* (46). This compound decreases blood pressure in the same range as mixanpril, but there is no indication of the activity of this compound in the kidney.

[§]The nomenclature used for the amino acid residues (P) of the substrate and to name the subsites (S) of the active site is that of Schechter and Berger (47).

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