Central Antihypertensive Effects of Orally Active Aminopeptidase A Inhibitors in Spontaneously Hypertensive Rats

Yannick Marc, Ji Gao, Fabrice Balavoine, Annie Michaud, Bernard P. Roques, Catherine Llorens-Cortes

Abstract—Brain renin-angiotensin system hyperactivity has been implicated in the development and maintenance of hypertension. We reported previously in the brain that aminopeptidase A and aminopeptidase N are involved in the metabolism of angiotensin II and angiotensin III, respectively. By using in vivo specific and selective aminopeptidase A and aminopeptidase N inhibitors, we showed that angiotensin III is one of the main effector peptides of the brain renin-angiotensin system, exerting a tonic stimulatory control more than blood pressure in hypertensive rats. Aminopeptidase A, the enzyme generating brain angiotensin III, thus represents a potential target for the treatment of hypertension. We demonstrated here the antihypertensive effects of RB150, a produg of the specific and selective aminopeptidase A inhibitor, EC33, in spontaneously hypertensive rats, a model of human essential hypertension. Oral administration of RB150 in conscious spontaneously hypertensive rats inhibited brain aminopeptidase A activity, demonstrating the central bioavailability of RB150 and its ability to generate EC33 into the brain. Oral RB150 treatment dose-dependently reduced blood pressure in spontaneously hypertensive rats with an ED50 of 30 mg/kg, lasting for several hours. This decrease in blood pressure is partly attributed to a decrease in sympathetic tone, reducing vascular resistance. This treatment did not modify systemic renin-angiotensin system activity. Concomitant oral administration of RB150 with a systemic renin-angiotensin system blocker, enalapril, potentiated the RB150-induced blood pressure decrease achieved in <2 hours. Thus, RB150 may be the prototype of a new class of centrally active antihypertensive agents that might be used in combination with classic systemic renin-angiotensin system blockers to improve blood pressure control. (Hypertension. 2012;60:00-00.) ● Online Data Supplement

Key Words: aminopeptidase A inhibitors ■ blood pressure ■ brain renin-angiotensin system ■ spontaneously hypertensive rats ■ hypertension

Despite the availability of >75 antihypertensive agents, high blood pressure (BP) remains difficult to control. Many patients will require 2 or even 3 drugs to control their BP.1 Resistant hypertension to ≥3 antihypertensive drugs (including a diuretic) occurs in 15% of the hypertensive population. The current antihypertensive agents are also less effective in patients of African ancestry17,20 or those with diabetes mellitus or renal insufficiency.12 Consequently, there is a need to develop new classes of antihypertensive agents with different mechanisms of action to improve BP control and the associated cardiovascular risks.

It has been suggested that there may be a central component involved in hypertension based on the sympathetic hyperactivity observed in the early stages of this pathology.7 Indeed, overactivity of the brain renin-angiotensin system (RAS) has been implicated in the development and the maintenance of hypertension in several types of experimental and genetic hypertension animal models, such as spontaneously hypertensive rats (SHRs), deoxycorticosterone acetate (DOCA)-salt hypertensive rats,8–10 or transgenic mice overexpressing both human angiotensinogen and human renin.11,12 This results in the brain in an increased angiotensinogen gene expression,13 elevated renin and angiotensin II (Ang II) and angiotensin III (Ang III) levels,14,15 an upregulation of Ang II turnover,9 an overexpression of Ang II receptors,16 and an increased sympathetic neuron activity17 or arginine-vasopressin (AVP) release.18 In contrast, plasma renin activity (PRA) used as a marker of the activity of the systemic RAS is normal in the SHR model, whereas it is depressed in DOCA-salt rats and increased in transgenic animals.19–21
We reported previously\(^2\) that, in the brain RAS, aminopeptidase A (APA; EC 3.4.11.7), a membrane-bound zinc metalloprotease, removes in vivo the N-terminal aspartate of Ang II (Ang 1-8) to generate Ang III (Ang 2-8), whereas aminopeptidase N (EC 3.4.11.2), another membrane-bound zinc metalloprotease, removes the N-terminal arginine of Ang III to generate angiotensin IV (Ang 3-8; Figure 1). By using specific and selective APA and aminopeptidase N inhibitors, EC33 and PC18, respectively,\(^2\) we demonstrated that Ang III is one of the main effector peptides of the brain RAS in the control of AVP release and BP.\(^{20,22,25-27}\) Brain Ang III exerts a tonic stimulatory effect on the control of BP,\(^{20,22,25-27}\) neuron activity by measuring BP after ganglionic blockade.\(^{20,22,25-27}\) Therefore, the inhibition of central but not peripheral APA with EC33 reduces brain Ang III levels leading to a decrease in BP in DOCA-salt rats\(^{20,22,25-27}\) and SHRs.\(^{29}\) Therefore, brain APA constitutes a promising therapeutic target for the treatment of certain forms of hypertension, which justifies the development of potent and selective APA inhibitors as centrally acting antihypertensive agents. However, because EC33 does not cross the blood-brain barrier (BBB),\(^{25,29}\) a systemically active prodrug of EC33, 4,4'-dithio (bis[3S]-3-aminobutyl sulfonic acid); RB150\(^{20,29}\) was designed. RB150 is a dimer of EC33, generated by creating a disulfide bond (Figure 1). RB150 does not inhibit APA activity, because in this molecule the thiol group of EC33 is engaged in a disulfide bridge and is unable to interact with the zinc atom present in the APA active site and essential for APA catalytic activity.\(^{30,31}\) However, this bridge allows RB150 injected systemically to cross the BBB and enter the brain. Once in the brain, the disulfide bridge of RB150 is cleaved by brain reductases generating 2 active molecules of EC33, which block brain APA activity.\(^{20,29}\) Accordingly, RB150 given by oral route to conscious DOCA-salt rats was shown to enter the brain, induce a sustained inhibition of brain APA activity, and block brain Ang III formation, thereby normalizing BP.\(^{20,29}\) In this model, RB150 also decreased plasma AVP levels, increased diuresis, and induced a mild natriuresis, without affecting kaliuresis, thereby reducing blood volume participating to BP decrease.\(^{29}\) Thus, RB150 could represent the prototype of a new class of centrally acting antihypertensive agents. For this purpose, this study was designed to extend the findings obtained with RB150 in the DOCA-salt rat to other hypertension models, such as the SHR, to further validate the therapeutic potential of brain APA targeting in cardiovascular diseases. We thus assessed in SHRs whether RB150 given by oral route crosses the BBB, inhibits brain APA activity, and significantly lowers BP. With the aim to better understand in SHRs the mechanism of action of RB150 on BP, we investigated the effects of RB150 oral administration both on blood volume by measuring water intake, diuresis, and urinary electrolytes and on sympathetic neuron activity by measuring BP after ganglionic blockade. In parallel, we also assessed the effects of RB150 administration on the systemic RAS activity by measuring PRA. Finally, we studied in SHRs whether concomitant oral administration of RB150 with a systemic RAS blocker, enalapril, could be useful for normalizing BP.

**Methods**

All of the procedures in this study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar-Kyoto (WKY) rats and SHRs were randomly assigned to oral saline, RB150 (from 15 to 150 mg/kg), enalapril (3 mg/kg), or RB150 (100 mg/kg) plus enalapril (1 mg/kg) treatment groups. Another set of rats were treated with hexamethonium (HEX; 20 mg/kg, IP) alone or combination with RB150 (150 mg/kg, PO). Toxicity studies were performed with Sprague-Dawley rats and with Beagle dogs. We performed the following experimental procedures: APA enzymatic activity measurement, BP measurement, urine and electrolyte output and fluid consumption, determination of PRA, toxicity studies, and statistical analysis. For an expanded methods section, see the online-only Data Supplement.

**Results**

**Inhibitory Effect of Oral RB150 Administration on Brain APA Activity in Conscious SHRs**

Brain APA activity in SHRs was 2.2 times significantly higher than that in normotensive WKY rats (91.8±3.3 versus 41.8±0.6 nmol of GluβNA hydrolyzed per milligram of protein per hour, respectively; \(P<0.01\); Figure 2A). Oral RB150 administration (100 mg/kg) in SHRs significantly inhibited brain APA activity (by 31%) after 3.5 hours compared with SHRs receiving PO saline (63.4±2.9 versus 91.8±4.3 nmol of GluβNA hydrolyzed per milligram of protein per hour; \(P<0.01\)). The brain APA activity in RB150-treated SHRs did not reach the control levels measured in normotensive WKY rats given saline PO (63.4±2.9 versus 41.8±0.6 nmol of GluβNA hydrolyzed per milligram of protein per hour; \(P<0.05\); Figure 2A). However, RB150 treatment reduced by 58% the increase in APA activity in SHRs compared with normotensive WKY rats (\(Δ_P\) versus \(Δ_Q\); Figure 2A). As shown previously,\(^{29}\) the brain APA activity of normotensive WKY rats was not modified by the PO RB150 administration (15 mg/kg; 43.7±0.6 versus 45.1±4.1 nmol of GluβNA hydrolyzed per milligram of protein per hour for RB150 and saline, respectively).
The oral administration of saline in conscious SHRs, used as an internal control, did not significantly alter MABP or HR, regardless of the time elapsed between administration and recording (between 0 and 24 hours). Interestingly, 5 hours after oral administration, RB150 (15 mg/kg) had no significant effect on MABP or HR in conscious normotensive WKY rats (Table S1). The maximal decrease in MABP (−32.2±8.0 mm Hg) was observed at a dose of 150 mg/kg. The hypotensive effect for each dose studied (from 15 to 150 mg/kg) began 2 to 3 hours after administration, was maximal between 5 and 6 hours, and disappeared after 24 hours (Figure 2C). The oral administration of saline in conscious SHRs, used as an internal control, did not significantly alter MABP or HR, regardless of the time elapsed between administration and recording (between 0 and 24 hours). Interestingly, 5 hours after oral administration, RB150 (15 mg/kg) had no significant effect on MABP or HR in conscious normotensive WKY rats (Table S1). The maximal decrease in MABP (−32.2±8.0 mm Hg) was observed at a dose of 150 mg/kg. The hypotensive effect for each dose studied (from 15 to 150 mg/kg) began 2 to 3 hours after administration, was maximal between 5 and 6 hours, and disappeared after 24 hours (Figure 2C).

Effects of Concomitant Acute Oral Administration of RB150 With Enalapril on BP and HR in Freely Moving SHRs

Concomitant oral administration of RB150 (100 mg/kg) with enalapril (1 mg/kg) significantly and markedly decreased MABP (Figure 3) without significantly altering HR (not shown) in conscious SHRs. The BP decrease (−16.4±3.08 mm Hg) is already significant 1 hour after administration (Figure 3). A maximal decrease in MABP (−40.7±3.83 mm Hg) was observed 6 hours after administration. For each time, the hypotensive effect induced by the combination of RB150 plus enalapril was significantly different from the hypotensive effects of each compound administered alone. This was particularly illustrated 1 hour after administration; in fact at this time, RB150 at the higher dose of 150 mg/kg or enalapril at the dose of 3 mg/kg did not induce any significant BP decrease in SHRs (Figure 3).
Effects of Acute Oral RB150 Administration and Ganglionic Blockade on BP and HR in Freely Moving SHRs

Acute IP injection of HEX (20 mg/kg; n = 7) in conscious SHRs elicited rapid, reversible decreases in MABP (Figure 4A and 4B) accompanied by decreases in HR (data not shown). Maximal decreases in MABP (−46.5 ± 4.8 mm Hg) and HR (−45.1 ± 22.4 bpm) were observed 20 minutes after the injection (Figure 4B). The next day, SHRs were submitted to acute oral treatment with RB150 (150 mg/kg), and a maximal decrease in MABP (−24.1 ± 2.6 mm Hg) was observed 6 hours after treatment without any significant modification of HR (data not shown). The absolute levels of BP in RB150- or saline-treated SHRs, 6 hours after oral administration, were 141.5 ± 2.9 and 162.2 ± 3.7 mm Hg, respectively. At this time, HEX (20 mg/kg; IP) treatment significantly decreased BP in RB150-treated rats (124.8 ± 7.8 mm Hg), as well as in saline-treated rats (115.7 ± 6.8 mm Hg), during the entire period of BP recording (60 minutes). RB150 pretreatment decreased by 64% the maximal hypotensive effect observed after ganglionic blockade with HEX (−16.7 ± 8.1 mm Hg in HEX + RB150-treated SHRs versus −46.5 ± 4.8 mm Hg in HEX + saline-treated SHRs) and delayed it by 10 minutes (Figure 4B).

Effects of Oral Administration of RB150 on PRA in WKY Rats and SHRs

As shown in Figure S1, no significant difference in PRA was observed between saline-treated WKY rats and SHRs (3.15 ± 0.13 versus 2.63 ± 0.37 ng of angiotensin I produced per milliliter of plasma per hour; n = 8 in each group). The PRAs of the different experimental groups were comparable to those reported previously. Moreover, oral administration of RB150 (100 mg/kg; n = 8) in SHRs did not have any significant effect on PRA as compared with SHRs receiving an oral saline administration (3.30 ± 0.39 versus 3.15 ± 0.13 ng of angiotensin I produced per milliliter of plasma per hour; Figure S1).

Effects of Oral RB150 Administration in Conscious SHRs on Water Intake, Diuresis, Natriuresis, and Kaliuresis

The values measured for water intake, diuresis, and urinary electrolytes in WKY rats and SHRs are consistent with previous studies. As shown in Figure S2, in normotensive WKY rats, oral treatment with RB150 (50 mg/kg; n = 10) did not modify significantly water intake, diuresis, natriuresis, and kaliuresis for a 5-hour analysis as compared with oral saline treatment. Although water intake and natriuresis were similar in saline-treated WKY rats and SHRs (Figure S2A and S2C), diuresis and kaliuresis were significantly lower in saline-treated SHRs as compared with saline-treated WKY rats (Figure S2B and S2D). In SHRs receiving oral RB150 (50 mg/kg; n = 5) administration, no significant change for water intake, diuresis, natriuresis, or kaliuresis for a 5-hour analysis was observed as compared with SHRs receiving saline (Figure S2). The oral administration of RB150 (150 mg/kg; n = 5) in SHRs did not induce any significant change in water intake (29.0 ± 2.3 Versus 28.8 ± 0.3 mL/24 hours), diuresis (0.4 ± 0.04 versus 0.5 ± 0.06 mL/h), natriuresis (0.2 ± 0.02 versus 0.3 ± 0.06 mEq/5 hours), or kaliuresis (0.4 ± 0.04 versus 0.4 ± 0.06 mEq/5 hours), for a 5-hour analysis as compared with SHRs (n = 10) receiving an oral saline administration.

Toxicity Studies With RB150 in the Rat and the Dog

RB150 administered daily by oral route for 28 consecutive days at the doses of 125 and 550 mg/kg did not induce mortality or major signs of toxicity in Sprague-Dawley rats. When administered at 1000 mg/kg, although the cause of the death of 1 male and 1 female remain unclear, RB150 did not induce any sign of toxicity (Table S2). In Beagle dogs, RB150 administered daily by the oral route for 28 consecutive days at the doses of 125, 500, and 1000 mg/kg did not induce mortality or major signs of toxicity except diarrhea observed at the doses of 500 and 1000 mg/kg in male and female dogs (Table S2). Nevertheless, diarrhea was not associated with body weight loss. For both animal species, body weight, food intake, drinking behavior, diuresis, and
This study describes a new pharmacological approach for lowering BP in SHRs. This approach is based on the oral administration of the APA inhibitor produg, RB150, that was shown previously to block in DOCA-salt rats the brain RAS activity. After its oral administration in SHRs, RB150 crosses the gastrointestinal barrier, enters the brain, and generates 2 active molecules of the specific and selective APA inhibitor EC33. The latter blocks brain APA activity, preventing brain Ang III formation, and induces a marked BP decrease in conscious SHRs without changing HR. The RB150-induced BP decrease is in part mediated by an inhibition of sympathetic neuron activity, because the decrease in BP induced by ganglionic blockade with HEX is highly reduced by RB150 pretreatment. In contrast, oral RB150 treatment does not modify systemic RAS activity. However, both blocking the activity of the brain and systemic RAS by concomitant oral administration of RB150 with enalapril potentiates the RB150-induced BP decrease in SHRs.

Overactivity of the brain RAS was shown to play a critical role in mediating hypertension in SHRs. In several brain nuclei, APA activity was significantly higher in SHRs than in WKY rats. In agreement with these data, the present study showed that APA enzymatic activity in the whole brain of SHRs was like that in DOCA-salt rats, increased by 2.2 times as compared with brain APA activity in normotensive rats. This strongly suggests a participation of APA in the RAS hyperactivity of the SHR brain. Blockade of brain angiotensin receptors in SHRs and pharmacological interference with brain angiotensin formation decreases arterial BP to normal levels. We showed previously that the blockade of endogenous brain Ang III formation by the ICV injection of the APA inhibitor EC33 alone markedly decreased BP in alert SHRs. In contrast, EC33 given alone by IV route, even at a high dose, did not modify BP in alert SHRs. Together, these data show that the blockade of the formation of brain but not systemic Ang III by EC33 is responsible for the decrease in BP.

In such a context, APA inhibitors able to cross the BBB after oral administration and to block the brain RAS activity appear as innovative antihypertensive agents. We, thus, developed RB150, an orally active produg of EC33, which enters the brain, inhibits brain APA activity, and blocks brain Ang III formation.

Discussion

This study shows that oral administration of RB150 (from 125 to 1000 mg/kg per day, PO) during 28 days had no effect on systolic BP in Beagle dogs, as shown in Table S3. Taken together, the no-observed adverse effect level after 28-day repeated oral administration corresponded with ≥550 mg/kg in the rat and 1000 mg/kg in the dog.

Figure 5. Conversion of angiotensin (Ang) II to Ang III in the brain by the zinc metalloprotease aminopeptidase A (APA). Structure of the APA inhibitor EC33 and its corresponding produg RB150, consisting of 2 molecules of EC33 linked by a disulfide bridge. After PO administration, the bridge enables RB150 to cross the blood-brain barrier and to penetrate the brain. In the brain, the disulfide bridge of RB150 is cleaved by reductases releasing 2 active molecules of EC33, inhibiting APA activity. Consequently, brain Ang II is not cleaved into Ang III, which normally exerts in brain structures (PVN, SON, PPIt, NTS, and RVLM) a stimulatory action on the control of blood pressure. This results in a BP decrease via a decrease in arginine-vasopressin (AVP) release and sympathetic neuron activity. Ang II indicates angiotensin II; Ang III, angiotensin III; APA, aminopeptidase A; NTS, nucleus of the solitary tract; PPIt, posterior pituitary; PVN, paraventricular nucleus; SFO, subfornical organ; SON, supraoptic nucleus; RVLM, rostral ventrolateral medulla.
activity found in SHRs after oral RB150 administration with the dose-response inhibition curve obtained after the ICV administration of EC33\(^{20}\) suggests that 0.1% of the prodrug penetrates the brain. This value is 10 times lower than that obtained in DOCA-salt rats after similar RB150 oral administration\(^{20}\) and may be attributed to a more permeable BBB in DOCA-salt rats compared with that of SHRs because of the presence of mineralocorticoid and salt in drinking water, as shown previously.\(^{17,38}\) The inhibition of brain APA activity in conscious SHRs after oral administration of RB150 (100 mg/kg corresponding with a concentration of 80 nmol in brain) resulted in a 27-mm Hg decrease in BP, equivalent to that observed with EC33\(^{20}\) (150 nmol, ICV). This hypotensive effect was dose dependent with an ED\(_{50}\) of 30 mg/kg. The dose of 150 mg/kg PO induced a maximal 37-mm Hg decrease in BP. The RB150-induced decrease in BP was large and long lasting, but HR was not significantly affected, suggesting that the sensitivity of the baroreflex was decreased by brain Ang III, consistent with the findings of Lin et al\(^{39}\) on the involvement of endogenous angiotensins in tonic baroreflex suppression. Our results highlight the efficiency of oral RB150 administration to inhibit brain APA activity and to flex suppression. Our results highlight the efficiency of oral RB150 administration to inhibit brain APA activity and to decrease BP several hours without changing HR in SHRs, as shown previously in DOCA-salt rats.\(^{20,29}\) The RB150 hypotensive effect was effective only at higher doses in the SHR (ED\(_{50}\), 30 mg/kg) compared with the DOCA-salt rat (ED\(_{50}\), 1 mg/kg), presumably because of a less favorable pharmacokinetics profile of RB150 in the SHR. Nevertheless, toxicology, safety pharmacology, and pharmacokinetics studies performed in animals have demonstrated that RB150 is well tolerated both in rats (no-observed adverse effect level, 550 mg/kg) and dogs (no-observed adverse effect level, 1000 mg/kg) at doses significantly superior to the dose showing antihypertensive activity in rats. RB150 was also shown to have a better bioavailability in dogs than in rats (data not shown).

We also found that RB150 (50 mg/kg) was as efficient as the angiotensin I–converting enzyme inhibitor enalapril (3 mg/kg), which is currently used clinically for treatment of hypertension. Both compounds showed 20- to 26-mm Hg reductions in BP. Moreover, RB150 (100 mg/kg, PO) in SHRs did not have any significant effect on PRA compared with SHRs receiving oral saline, suggesting that RB150 does not modify the systemic RAS activity. Consequently, we can hypothesize that synergy and improvement in BP control of hypertensive patients can be found by combining RB150 with an angiotensin I–converting enzyme inhibitor or an Ang II type 1 receptor antagonist, because both the brain and the systemic RAS would be inhibited. In agreement with this hypothesis, we showed presently in SHRs that concomitant oral administration of RB150 with enalapril potentiated the RB150-induced decrease in BP as compared with BP decrease induced by RB150 or enalapril alone. This was particularly well illustrated at short-time treatment; whereas RB150 at the dose of 100 mg/kg or enalapril at the dose of 1 mg/kg did not induce any significant change in BP 1 hour after oral administration, a significant hypotensive effect (~16.4±3.08 mm Hg) occurred with the combination of the 2 compounds.

The brain RAS controls BP via 3 different mechanisms\(^{40}\), (1) activating sympathetic neuron activity in the rostral ventrolateral medulla; (2) synaptic inhibition of the baroreflex in the nucleus of the tractus solitarius; and (3) increase in the release of AVP from the posterior pituitary into the blood circulation (Figure 5). All of these structures were known to contain APA activity, Ang II type 1 receptor binding sites, and angiotensinergic nerve terminals.\(^{41}\) It is now well established that SHRs exhibit an increase in sympathetic neuron activity, which participates in the development and maintenance of hypertension in this model.\(^{17}\) Consistent with the mechanism of action of the brain RAS in BP control, RB150 given PO to SHRs seems to decrease sympathetic neuron activity, as shown by the significant decrease of the hypotensive effect of HEX, a ganglionic blocker,\(^{42}\) in RB150-pretreated SHRs as compared with saline-pretreated SHRs, although vascular effects cannot be totally excluded. However, recent data obtained in collaboration with the laboratory of Huang et al\(^{43}\) strengthened this conclusion by showing that ICV infusion of RB150 during 28 days in rats postmyocardial infarction drastically reduced the electric activity of the sympathetic renal nerve, attenuating sympathetic overactivity occurring in this model. RAS hyperactivity observed in SHR brain, partly because of a greater APA activity\(^{34,44}\) in brain structures known to control sympathetic neuron activity (paraventricular nucleus, nucleus of the tractus solitarius, and rostral ventrolateral medulla), results in an increased sympathetic tone\(^{10,45}\) and may account for the efficiency by which APA inhibitors decreased sympathetic tone and BP in this model.

In rodents, Ang III injected ICV but not Ang II was shown to increase systemic AVP release, resulting in a reduced diuresis.\(^{22}\) In contrast to DOCA-salt rats,\(^{29}\) no significant change was observed in diuresis in SHRs after oral RB150 administration. This is probably because of the fact that no increase in vasopressinergic neuron activity occurs in this genetic model of hypertension.\(^{46}\)

**Perspectives**

RB150 is the first orally active molecule able of inhibiting brain RAS without affecting systemic RAS activity. RB150 strongly reduces BP several hours after a single administration, without changing HR in an experimental model of essential hypertension, as in an experimental salt-dependent model of hypertension. Centrally acting APA inhibitors may, therefore, constitute a new class of antihypertensive agents. They could contribute alone or in combination with a systemic RAS blocker to improve BP control and reduce cardiovascular risks in patients. RB150 appears as the first prototype of this potentially novel class of drugs and so far as a very promising drug candidate. Because RB150 (also named QGC001) has obtained the approval from the French health authorities for the first clinical trial in humans, future clinical investigations with this compound in healthy volunteers and patients should bring a better understanding of the role of APA and brain RAS in the development and the maintenance of hypertension in humans.

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ics Corp and from the Institut National de la Santé et de la Recherche Médicale.

Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- RB150/QGC001 is the prototype of a new class of centrally acting antihypertensive agents, contributing to improve BP. Its action is based on a new concept of brain enzyme inhibition.

**What Is Relevant?**

- RB150 is a very promising drug candidate for treatment of hypertension. It received on January 5, 2012, the approval from health authorities for the first clinical trial in humans.

**Summary**

RB150 alone or in combination with enalapril induces a strong reduction of BP in SHRs by inhibiting brain and/or systemic RAS activity.
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CENTRAL ANTIHYPERTENSIVE EFFECTS OF ORALLY ACTIVE AMINOPEPTIDASE A INHIBITORS IN SPONTANEOUSLY HYPERTENSIVE RATS

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ONLINE SUPPLEMENT

Supplemental Methods

Drugs
EC33 and RB150 were synthesized and prepared as described previously.\(^1^,\(^2\) The ACE inhibitor, enalapril (MK-421), was supplied from Merck Sharp and Dohme research Lab. Bestatin was purchased from Sigma and L-Glutamyl-\(\beta\)-naphthylamide (Glu\(\beta\)Na) was purchased from Bachem (Bunderdorf, Switzerland).

Animals
Male normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR), weighing 250 to 350g, were obtained from Charles River Laboratories (L'Arbresle, France) and animals were kept under artificial light (12-hours light/12-hours dark cycle) with a normal standard diet and water given \textit{ad libitum}. Toxicity studies were performed with 7 weeks Sprague-Dawley rats (weighing 170 to 205 g for males and 130 to 150 g for females) obtained from Charles River Laboratories (L'Arbresle, France) and with 8 to 10 months Beagle dogs (weighing 5.7 to 9.4 kg on the day of randomization) obtained from Harlan France (Gannat, France). The experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Aminopeptidase A (APA) Enzymatic Activity Measurement
\textit{In vitro} APA enzymatic activity was determined using recombinant mouse APA routinely produced in the laboratory and Glu\(\beta\)Na as a synthetic substrate under conditions of initial velocity, as previously described.\(^2^,\(^3\)
For the brain APA enzymatic activity measurement, rats were deprived of food for 12 hours before a 300 µL oral administration of saline or RB150 (100 mg/kg). Brains were collected 3.5 hours later and one half of the brain was homogenized by sonication in 6 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.4).\(^1^,\(^3\) Bestatin, a nonselective aminopeptidase inhibitor, was added at a concentration (1 µmol/L) unable to inhibit APA activity.\(^3^,\(^4^,\(^5\)) Assays were performed in presence or absence of EC33 (5 µmol/L).

Surgical Methods and BP Recording.
Twelve weeks male SHR were anesthetized with an intraperitoneal (IP) injection of pentobarbital sodium (60 mg/kg, Centravet). A catheter was implanted in the femoral artery.\(^6^,\(^7\) The femoral arterial catheter was brought under the skin and emerged at the nape of the neck. After surgery, each rat was given 0.1 mL penicillin-streptomycin (50,000 units/mL, IP, Sigma) and allowed to recover for at least 24 hours before the experiment. Saline, RB150 (15 to 150 mg/kg) or enalapril (3 mg/kg) were orally administered (300 µL) in conscious, unrestrained rats. After treatment, mean arterial BP (MABP) and heart rate (HR) were monitored continuously for 6 hours on the first day of experiment. Another recording of 1 hour was performed 24 hours after drug administration. Each experiment was monitored by using a COBE CDX III pressure transducer (Phymep, Paris, France) connected to the MacLab system (Phymep) consisting of MacLab hardware unit and chart software running on a Macintosh computer. MABP and HR were calculated by the BP signal.\(^7\)
For the experiments performed to evaluate the effects of ganglionic blockade after pretreatment with RB150 in SHR, baseline MABP and HR were first recorded for 1
hour. Then, hexamethonium chloride (HEX, Sigma, St Louis, MO, USA), a ganglionic blocker, was injected by IP route (20 mg/kg in saline) under a volume of 1 mL/kg. MABP and HR were recorded for 60 minutes. Twenty four hours post-administration, baseline MABP and HR were again monitored in the same SHR for 1 hour, then, RB150 (150 mg/kg) was given orally and MABP and HR were recorded for 5 or 6 hours, time corresponding to the maximal decrease in MABP observed after RB150 treatment alone. At this time, HEX (20 mg/kg, IP) was injected and changes in MABP and HR were recorded during the following 60 min. The contribution of the sympathetic nervous system to the maintenance of arterial BP of both groups was evaluated by the variation in MABP ($\Delta$MABP) and HR ($\Delta$HR) measured after HEX injection alone or combination of RB150 and HEX injection.

For the experiments performed to evaluate the effects of concomitant administration of RB150 with enalapril in SHR, baseline MABP and HR were first recorded for 1 hour. Then, saline, RB150 (100 mg/kg), enalapril (1 mg/kg) or RB150 (100 mg/kg) plus enalapril (1 mg/kg) were orally administered, MABP and HR were recorded for 6 hours. Another recording of 1 hour was performed 24 hours after drug administration.

**Urine and Electrolyte Output and Fluid Consumption Measurements**

Normotensive WKY and hypertensive SHR were individually housed in metabolic cages (Tecniplast). After acclimatization over a period of 3 days, each rat was deprived of food for 12 hours. Then, fluid consumption, urinary excretion of water, and electrolytes were measured after saline or RB150 (50 and 150mg/kg) PO administration. Urinary Na$^+$ and K$^+$ concentrations were determined using a flame photometer (model PFP7/C, JENWAY).

**Determination of Plasma Renin Activity**

Twelve-week-old male WKY and SHR were deprived of food 12 hours before a 300 µL oral administration of saline or RB150 (100 mg/kg). For plasma renin activity (PRA) measurement, 5 hours after treatment, trunk blood (6-7 mL) was collected into chilled tubes containing 0.35 mL of 0.3M EDTA (pH 7.4) on ice and centrifuged at 1,600 x g at 4°C for 15 min. Plasma (3.5 mL) was then stored at -20°C until assayed. 0.25 mL of plasma were added to 0.215 mL of 0.2M phosphate buffer (pH 6.5) containing 1.5% EDTA, 25 µL of 0.3M HCl and 10 µl of PMSF (25mg/mL). The incubation lasted 2 hours at 37°C. The reaction was stopped on ice and aliquots of 25 and 50 µl of the incubation medium were transferred into tubes for immediate AngI radioimmunoassay (RIA). PRA was determined by measuring released AngI, as described by Menard and Catt, and the results were expressed as ng of AngI produced per mL of plasma per hour.

**Toxicity studies with RB150 in the rat and the dog**

Rats were housed in groups of 5 or 3 (separated by sex) in cages of standard dimensions with sawdust bedding. RB150 (125, 550, 1000 mg/kg) or its vehicle (i.e. Water for Irrigation, Baxter) was administered to rats for 28 consecutive days by the oral route in a volume of 10 mL/kg. Dogs were housed individually in standard size pens (2.25 m$^2$). RB150 (125, 500, 1000 mg/kg) was administered to dogs for 28 consecutive days by the oral route in a non gastro-resistant capsule (TORPAC, size 12). Control dogs were given the empty capsule under the same conditions as dogs dosed with RB150. For both animal species, mortality was recorded twice a day during the treatment period; general observations were performed before the first dosing and once a day during the treatment period; functional and neurobehavioral
tests and body temperature were also assessed before the first dosing and on D28. Animals were weighed on the day of randomization, on D-1 and every 7 days during the study. Food consumption was measured weekly for each treatment cage. Water consumption was measured daily for each treatment cage. Blood for chemistry analysis was taken from all animals at the end of the treatment period. Urine collection was performed individually in metabolism cages for a period of about 16 hours, at pretreatment and at the end of treatment period. Quantitative estimation of sodium, potassium and creatinine was performed.

**Data Analysis and Statistics**

Data are presented as means ± SEM and comparisons among groups of APA activity, water intake, urine flow rate, urinary electrolytes, plasma renin activity were performed by one-way analysis of variance followed by Bonferroni’s Multiple Comparison tests. Baseline MABP and HR (values obtained 1 hour before PO administration), MABP and HR after RB150 oral administration were made with paired or unpaired Student’s t test. A factorial two-way analysis of variance (ANOVA) was performed to test the interaction of time and treatment on the different variables of ∆MABP. The treated groups and the saline group within each treatment were compared at each time by a factorial 1-way ANOVA and Fisher PLSD test. Differences were considered significant if P value was < 0.05.
References


## Table S1

<table>
<thead>
<tr>
<th>Rats</th>
<th>MABP, mmHg</th>
<th>HR, bpm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>After <em>per os</em> administration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (RB150, 15mg/kg)</td>
<td>105.2 ± 4.2</td>
<td>100.7 ± 4.2&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR Saline</td>
<td>164.7 ± 4.6</td>
<td>161.5 ± 4.2&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR (RB150, 15mg/kg)</td>
<td>165.7 ± 7.4</td>
<td>147.5 ± 6.3&lt;sup&gt;*&lt;/sup&gt;†</td>
</tr>
<tr>
<td>SHR (RB150, 50mg/kg)</td>
<td>173.2 ± 3.8</td>
<td>150.3 ± 4.1†</td>
</tr>
<tr>
<td>SHR (RB150, 150mg/kg)</td>
<td>183.0 ± 5.2</td>
<td>145.8 ± 6.5†</td>
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<tr>
<td>SHR (enalapril, 3mg/kg)</td>
<td>179.6 ± 6.3</td>
<td>153.1 ± 6.9†</td>
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</tbody>
</table>

<sup>ns</sup> *P*>0.05 vs basal values

* *P*<0.05 vs basal values

† *P*<0.001 vs WKY receiving RB150 (15mg/kg)

### Table S1.

Maximal changes in MABP and HR after oral administration of RB150 or enalapril in conscious WKY and SHR rats.
Table S2. Toxicity studies with RB150 in the rat and the dog. Animals were treated daily during 28 days with RB150 (125, 550 or 1000mg/kg, PO) and different parameters were measured as described in supplemental methods.

(1) Percent differences at end of dosing period (from D0 and D28) are shown.
(2) Variations expressed in percentage in relation to values of the control group at end of dosing period are shown. * $P<0.01$, when compared with control group; Statistical significance is based on actual data (not on the percent differences). ND: Not determined; M: Male, F: Female; BP: Blood Pressure; F.C: Food Consumption; W.C: Water Consumption; n: number of animals in each group.

### DOGS

<table>
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<tr>
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<th>M + F (n=10)</th>
<th>M + F (n=10)</th>
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<tbody>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Body Weight (1)</td>
<td>+ 6%</td>
<td>+ 5%</td>
<td>+ 6%</td>
<td>+ 5%</td>
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<tr>
<td>F.C (1)</td>
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</tr>
<tr>
<td>W.C (1)</td>
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<td>- 11%</td>
</tr>
<tr>
<td>Urine volume (1)</td>
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<td>+ 21%</td>
<td>- 21%</td>
<td>- 20%</td>
</tr>
<tr>
<td>Urine osmolality (1)</td>
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<td>+ 22%</td>
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### RATS

<table>
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<tr>
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<tr>
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<td>-</td>
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<td>-</td>
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<td>1/10</td>
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<tr>
<td>F.C (1)</td>
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<td>- 4%</td>
<td>+ 9%</td>
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<tr>
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<td>+ 2%</td>
<td>+ 14%</td>
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<td>- 14%</td>
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<tr>
<td>Urine osmolality (2)</td>
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<td>+ 16%</td>
<td>+ 26%*</td>
<td>+ 20%</td>
<td>+ 33%*</td>
<td>+ 20%</td>
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Table S2. Toxicity studies with RB150 in the rat and the dog. Animals were treated daily during 28 days with RB150 (125, 550 or 1000mg/kg, PO) and different parameters were measured as described in supplemental methods.
Table S3

<table>
<thead>
<tr>
<th>Treatment</th>
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<tr>
<td>Vehicle</td>
<td>Mean ± SEM</td>
<td>161±8</td>
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<tr>
<td></td>
<td>n</td>
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<td>RB150 (125mg/kg/day, PO)</td>
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<tr>
<td></td>
<td>P</td>
<td>NS</td>
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<tr>
<td>RB150 (500 mg/kg/day, PO)</td>
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<td></td>
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<td>RB150 (1000 mg/kg/day, PO)</td>
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Table S3. Effects of Chronic Oral RB150 Administration for 28 days on Systolic BP in Beagle dogs. Results are expressed in mmHg; n: number of animals; Vehicle: empty capsule; NS: Non Significant corresponding to P>0.05. Analysis of variance for repeated measurements with Dunnett's test; %: variation expressed in percentage in relation to predose values. NA: not applicable.
Figure S1. Effects of RB150 on plasma renin activity. PRA was determined by measuring released AngI after oral RB150 (100mg/kg) administration in SHR. The results were expressed as ng of AngI produced per mL of plasma per hour. Values are expressed as Mean±SEM of 10 animals individually analyzed for each condition. No statistical difference (ANOVA) was observed between groups.
Figure S2. Effects of oral administration of RB150 on water intake, urinary excretion of water, natriuresis and kaliuresis in WKY and SHR. After 3 days of acclimatization to metabolic cages, WKY (white bar) and SHR (grey bar) were treated with RB150 (50 mg/kg) or saline. Then, they were returned to the metabolic cages for the measurement of water intake (A), diuresis (B), natriuresis (C), and kaliuresis (D) over a 5-hours period. Mean±SEM of 5 to 10 animals individually analyzed for each condition; ANOVA; * P <0.05, WKY receiving saline vs SHR receiving saline.