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Potency and selectivity of RXP407 on human, rat, and mouse angiotensin-converting enzyme

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Abstract

By screening phosphinic peptide libraries, we recently reported the discovery of RXP407 (Ac-Asp-PheY(PO_2-CH_2)$_L$Ala-Ala-NH_2), a potent N-domain-selective inhibitor of recombinant human angiotensin-converting enzyme (ACE). Preliminary studies to evaluate the *in vivo* activity of RXP407 in rat led us to suspect possible differences in the binding property of RXP407 between human and rat ACE. The aim of the present study was thus to determine the potency of RXP407 toward rat and mouse ACEs, as compared to non-recombinant human ACE, and to assess the efficacy of this inhibitor in discriminating between the N- and C-domains of these ACE enzymes. By comparing the ability of RXP407 to block purified somatic and germinal ACE from mice, RXP407 was shown to be a potent N-domain-selective inhibitor of mouse somatic ACE, a behavior similar to that observed with human somatic ACE. In contrast, RXP407 appeared less potent toward purified ACE from rat and furthermore was unable to block ACE activity present in crude rat plasma. This study demonstrated that for further evaluation of the *in vivo* efficacy of RXP407, mice rather than rats should be used as the animal model. Thus, following the change in the Ac-S-D-K-P plasmatic levels, after i.v. injection of RXP407 to mice, will permit the potency and selectivity of this novel ACE inhibitor to be assessed.

Keywords: Zinc-metalloproteinase; Phosphinic; ACE; Ac-S-D-K-P

1. Introduction

ACE is a zinc metallopeptidase that plays a key role in cardiovascular homeostasis by generating the potent vasoconstrictor hormone angiotensin II from angiotensin I and degrading the vasodilator bradykinin peptide hormone [1]. ACE is characterized by the presence of two homologous domains (the N- and C-domains), each bearing a functional zinc-dependent active site [2,3]. These two active sites hydrolyze angiotensin I and bradykinin with approximately the same efficiency. In contrast, AcSDKP, a negative regulator of hematopoietic stem cell differentiation and proliferation, was observed to be hydrolyzed *in vitro* 50 times faster by the N-domain of human recombinant ACE (based on k_{cat}/K_m values), compared to the C-domain [4]. Furthermore, in man ACE was demonstrated to be the main enzyme responsible for the metabolism of AcSDKP [5]. These novel findings have stimulated studies aimed at identifying new ACE inhibitors able to block only one of the two active sites of this enzyme, the N-domain [6]. By screening phosphinic peptide libraries, we discovered the RXP407 phosphinic peptide, which is able to differentiate the two ACE active sites of human recombinant ACE and acts as a potent N-domain-specific inhibitor of this enzyme [7]. Interestingly, despite the presence of peptide bonds in this compound, all the RXP407 i.v. dose injected in rat was recovered in urine with the inhibitor intact, showing that this phosphinic peptide inhibitor is not metabolized *in vivo* [7]. However, preliminary investigation of the *in vivo* RXP407 activity in this animal model provided disappointing results.

This led us to critically examine the potency (IC_{50} and K_i values) of this novel ACE inhibitor toward somatic ACE purified from blood plasma of rat and mouse, as compared to human ACE purified from plasma. The efficacy of RXP407 in blocking ACE activity present in crude plasmas from human, rat, and mouse was also evaluated. Finally, in

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Abbreviations: ACE, angiotensin-converting enzyme; AcSDKP, Ac-Ser-Asp-Lys-Pro; RXP407, Ac-Asp-PheY(PO_2-CH_2)$_L$Ala-Ala-NH_2; and EDC, (*N*-ethyl-*N*-3-diethylaminopropyl)3-ethylcarbodiimide hydrochloride.

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the absence of N- and C-domain mutants for mouse ACE, the N- and C-domain selectivity of RXP407 toward somatic ACE from mice was assessed indirectly by comparing inhibition profile curves obtained with somatic and testicular ACE from mice. This study leads us to conclude that the mouse model appears more suitable than the rat model for the evaluation of the *in vivo* activity of RXP407.

2. Materials and methods

2.1. Materials

Human blood plasma was obtained from the Centre National de Transfusion Sanguine. Rat and mouse plasmas were prepared from blood samples taken by retro-orbital sinus puncture from male Wistar rats and female cbaj mice. Testicular and lung ACE preparations were obtained from the same animals. Mca-Ala-Ser-Asp-Lys-DpaOH substrate was synthesized according to Knight *et al*. [8]. Lisinopril was from Sigma. RXP407 and its tritiated form were synthesized according to Dive *et al*. [7]. All peptides were purified by reversed-phase HPLC (Vydac, 218TP1022 column). Peptide purities were checked by analytical HPLC (Vydac, 218TP104 column) and mass spectroscopy.

2.2. Affinity column

An affinity chromatography matrix was prepared by coupling lisinopril to Sepharose by a modification of the original procedure described by Bull *et al*. [9]. An appropriate spacer group (suberic acid) was chosen to insure a sufficient length between lisinopril and the Sepharose matrix. Suberic acid 0.1 M pH 5.6 (5 mL) (Aldrich) was activated with 5 mL of EDC (0.1 M pH 7.3) (Aldrich), with magnetic stirring for 15 min. This solution was then mixed with 5 mL of Sepharose EAH4B matrix (Pharmacia) overnight at room temperature. The resulting resin (suberic acid spacer-linked Sepharose) was rinsed by extensive washing with water. Unreacted amino groups of the resin were blocked by 5 mL of EDC-activated acetic acid 0.1 M pH 4.6, with magnetic stirring for 4 hr. The free carboxylates of the spacer-linked resin were activated with an equimolar ratio of EDC (5 mL, 10 mM, pH 4.8) for 1 hr and then coupled to lisinopril (5 mL, 0.1 M pH 5.2) by gentle agitation on a rotator for 3 days at room temperature. The affinity gel was washed extensively with water and stored in 0.1 M HEPES pH 7.5, 0.3 M KCl, 0.1 mM $ZnSO_4$, containing 0.03% sodium azide.

2.3. Purification of ACE

Tissue homogenization and detergent solubilization of rat and mouse ACE from lung were performed as described by Pantoliano *et al*. [10]. Blood plasma was treated and testicular ACE prepared according to Lanzillo *et al*. [11]. ACEs were purified from these preparations by affinity chromatography, using lisinopril affinity resin as described above and a published loading procedure [12]. Immobilized ACE was eluted with 50 mM sodium carbonate pH 9.5, instead of free lisinopril [13,14]. Final separation of the 110-kDa testicular ACE isoform from the larger isoform (170 kDa) was achieved by gel filtration, using a TSK G 3000SW (0.75 cm i.d. $\times$ 60 cm, TosoHaas) gel filtration column. ACEs purified by these methods appeared as homogenous single bands on SDS/PAGE.

2.4. Enzyme assays

Enzyme assays were performed at 25° in 50 mM HEPES pH 6.8, 200 mM NaCl, and 10 μM $ZnCl_2$, using the Mca-Ala-Ser-Asp-Lys-DpaOH peptide as a fluorogenic substrate. Continuous assays were performed by recording the fluorescence increase at 390 nm (λ_{ex} = 340 nm) induced by the cleavage of the Mca substrate by ACE, using black, flat-bottomed, 96-well, non-binding 999999 surface plates, (Corning Costar Fce). Fluorescence signals were monitored using a Biolumin 960 photon counter spectrophotometer (Molecular Dynamics) equipped with a temperature device control and a plate shaker. Solutions of purified ACE were titrated using calibrated lisinopril solutions. The ACE concentrations for experiments with purified enzymes and plasmas (dilution by a factor 4) were chosen so as to remain well below 10% of substrate utilization and observe initial rates. The kinetic parameters K_m and k_{cat} for the hydrolysis of the Mca substrate by purified ACE from human, rat, and mice plasmas were determined using the direct linear plot method [15]. These values were obtained by assuming that the two active sites of these enzymes cleaved this substrate with similar catalytic efficiency, as already observed for human recombinant ACE [7].

2.5. Inhibition studies

In the absence of RXP407 inhibitor, the steady-state rate of Mca-Ala substrate hydrolysis by ACE is reached instantaneously and remains constant over the time scale monitored [7]. In the presence of RXP407, there is a time-dependent decrease in the steady-state rate, which is a function of the inhibitor concentration, suggesting that RXP407 behaves as a slow-binding inhibitor. Preincubation experiments indicated that, given the inhibitor concentration range, stable ACE inhibition requires incubation of the enzyme and inhibitor for at least 45 min [7]. Such a duration precludes the observation of the equilibrium before depletion of the substrate. Thus, inhibition studies with RXP407 were performed by equilibrating increasing concentrations of RXP407 with ACE (45 min at 4°) and then initiating the reaction by adding the substrate to determine the residual free enzyme concentration. Incubations were performed at 4° to maintain full ACE activity during the incubation time.

Due to the slow dissociation of RXP407 from ACE[1], it was possible to neglect the effect of substrate addition on the equilibrium position and to observe a stable steady-state rate on the time scale of monitoring. Experiments with lisinopril were conducted in the same manner, except that ACE and lisinopril were incubated overnight at 4°. RXP407 inhibition of the hydrolysis of AcSDKP peptide by ACE was performed using tritiated AcSDKP. Labeled AcSDKP peptide (25 μM) was incubated for 15 min at 25° with ACE in a final volume of 100 μL of buffer, in the absence (control) or presence of increasing concentrations of RXP407. Experiments were stopped by acidification, and the products of the reaction were analyzed by reversed-phase HPLC, coupled to a radioactivity detector. All the inhibition experiments were performed in 50 mM HEPES pH 6.8, 200 mM NaCl, and 10 μM $ZnCl_2$.

2.6. Determination of [^{3}H]RXP407 binding constant K_d by equilibrium dialysis

These experiments were performed on ACE purified from rat and mouse lung preparations and from recombinant human enzyme. Dialysis was carried out at 4° using two-chamber equilibrium dialysis units. Each chamber had a 0.05-mL capacity. Dialysis membranes (Hoefer Scientific Instruments) had a molecular mass cut-off of 12,000–13,000 Da. One compartment was loaded with ACE enzyme in 0.05 mL 50 mM HEPES pH 6.8, 200 mM NaCl, 0.04% BSA. The other compartment was loaded with [^{3}H]RXP407, diluted in the same buffer. K_d values were measured at one enzyme concentration and at 8 different inhibitor concentrations. In order to insure the observation of equilibrium, incubations were carried out overnight at 4°, with continuous shaking. Aliquots of 0.01 mL were taken from each compartment and counted in scintillation vials after adding 5 mL of Lumaflow II scintillation fluid (EGG, Berthold). All measurements were performed in triplicate. Enzyme activities in the dialysis unit after overnight incubation at 4° were measured to confirm the absence of enzyme inactivation. In control experiments, one compartment was loaded with [^{3}H]RXP407 and the other with buffer only. These experiments confirmed the equal distribution of RXP407 between the two compartments under these conditions and measured non-specific binding of RXP407. The K_d values of [^{3}H]RXP407 for the different ACE enzymes were determined by non-linear regression using the equation: $[EI] = ½ [(I_t + E_t + K_d) - [(I_t + E_t + K_d)^2\text{-}4\ E_t\ I_t]^{1/2}]$, assuming that at the RXP407 concentrations used, mainly the N-domain active site of ACE interacts with RXP407.

[1] Cotton J, Cuniasse P, Vazeux G, Dive V. Manuscript in preparation.

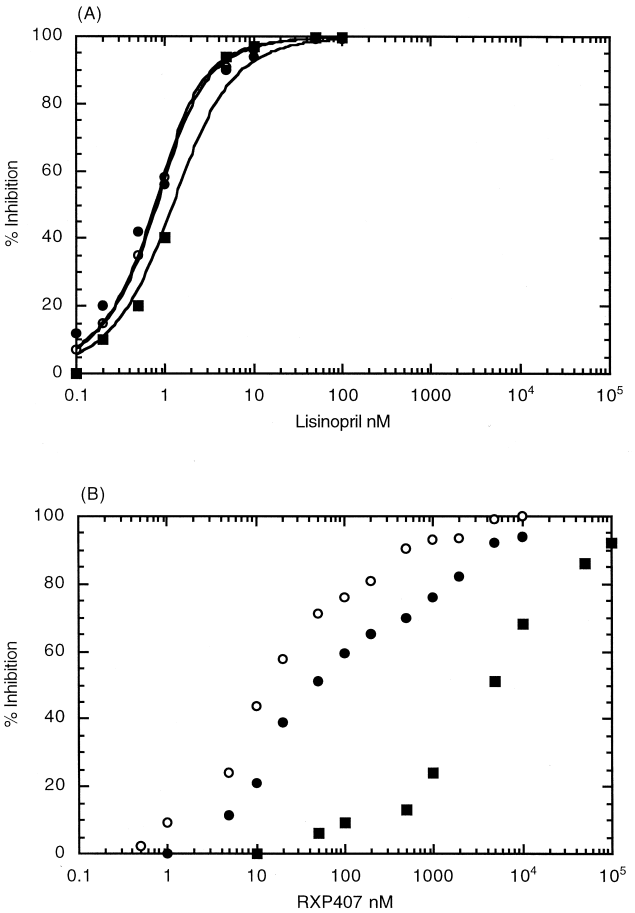

Fig. 1. Inhibition profiles of purified ACE from human (filled circles), mouse (open circles) and rat (squares) blood plasma by lisinopril (A) and RXP407 (B). The substrate used was Mca-Ala-Ser-Asp-Lys-Dpa (25 μM) in 50 mM HEPES buffer pH 6.8, 200 mM NaCl, 10 μM $ZnCl_2$, 25°. The concentrations of human, mouse, and rat ACEs were 0.8, 1, and 1.5 nM, respectively.

3. Results

3.1. Activity of purified ACEs from human, rat, and mouse blood plasma

Purified rat, mouse, and human ACEs, as obtained from blood plasma by lisinopril affinity column purification, were enzymatically characterized using a novel fluorogenic synthetic substrate (Mca-Ala-Ser-Asp-Lys-Dpa, Mca-Ala), partially based on the sequence of Ac-Ser-Asp-Lys-Pro. The cleavage of this substrate by these ACE enzymes, occurring exclusively between aspartic acid and lysine (data not shown), produced a marked fluorescence signal that could be used to monitor ACE activity with high accuracy. Lisinopril fully and potently blocked the degradation of this substrate by the three ACEs (Fig. 1A). Thus, in these preparations, only ACE activity contributed to Mca-Ala substrate cleavage. As shown in Table 1, the Mca-Ala substrate was efficiently cleaved by the three ACEs. The k_{cat} and K_m kinetic parameters for the hydrolysis of this substrate by rat, mouse, and human ACEs were similar.

Table 1
Kinetic parameters for the hydrolysis of Mca-Ala-Ser-Asp-Lys-Dpa by purified ACE from human, mouse, and rat plasmas

ACE source	Eo (nM)	S range (μM)	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (10^6 M^{-1}, sec^{-1})
Human	1	[2–40]	68	26	0.38
Mouse	0.7	[2–40]	59	19	0.31
Rat	1.3	[2–40]	67	18	0.27

Assays were carried out in 50 mM HEPES pH 6.8, 200 mM NaCl, 10 μM $ZnCl_2$, 25°.

E^o, enzyme concentration. S range, substrate concentration range.

3.2. Potency of RXP407 in inhibiting purified mouse, rat, and human ACEs

The RXP407 inhibition profiles of human, mouse, and rat ACEs purified from plasma, using Mca-Ala as a substrate, are reported in Fig. 1B. According to these curves, RXP407 displayed similar potency toward mouse and human ACEs. In contrast, much higher concentrations of RXP407 were required to fully block rat ACE. Direct binding experiments performed with tritiated RXP407 confirmed the particular behavior of this inhibitor toward rat ACE. In fact, the K_d values estimated from these experiments showed that RXP407 displayed almost the same potency toward human and mice ACEs, but was one order of magnitude less potent with rat ACE (Table 2).

3.3. Efficacy of RXP407 in inhibiting ACE activity in crude plasma

The degradation of the Mca-Ala substrate by crude human and rat plasma was fully blocked by lisinopril (Fig. 2A), suggesting that in these plasmas only ACE activity cleaves this substrate. As compared to pure ACE (Fig. 1A), only slightly higher lisinopril concentrations were required to block ACE activity in these crude plasmas. In plasma from mice, full inhibition of Mca-Ala cleavage by lisinopril was not achieved. Thus, in addition to a major cleavage by ACE, one or more other proteolytic activities contained in this plasma contributed to the proteolysis of this substrate. Despite this minor non-specific degradation, the efficacy of RXP407 in mouse plasma could be assessed with the Mca-Ala substrate. The curves reported in Fig. 2B demonstrated that RXP407 inhibited the ACE activity of crude human and mouse plasma with similar potency. However, as compared to the curves reported for pure ACE (Fig. 1B), these profiles were shifted to the right side of the inhibitor concentration scale. Thus, the efficacy of RXP407 in blocking human and mouse ACE activity in crude plasma was lowered 25-fold as compared to pure ACE. In crude rat plasma, 100 μM RXP407 only inhibited 12% of ACE activity. This observation contrasts with the 92% inhibition of pure rat ACE produced by the same RXP407 concentration (Fig. 1B).

Table 2
Dissociation constants (K_d) determined by equilibrium dialysis for the binding of tritiated RXP407 to purified ACE from human, mouse, and rat

ACE source	E_0 nM	I_0 nM	K_d nM
Human	1	[1–100]	9 ± 3
Mouse	6	[1–200]	23 ± 5
Rat	3	[1–400]	190 ± 10

Assays were carried out in 50 mM HEPES pH 6.8, 200 mM NaCl, 10 μM $ZnCl_2$, 4°.

I^o, inhibitor concentration.

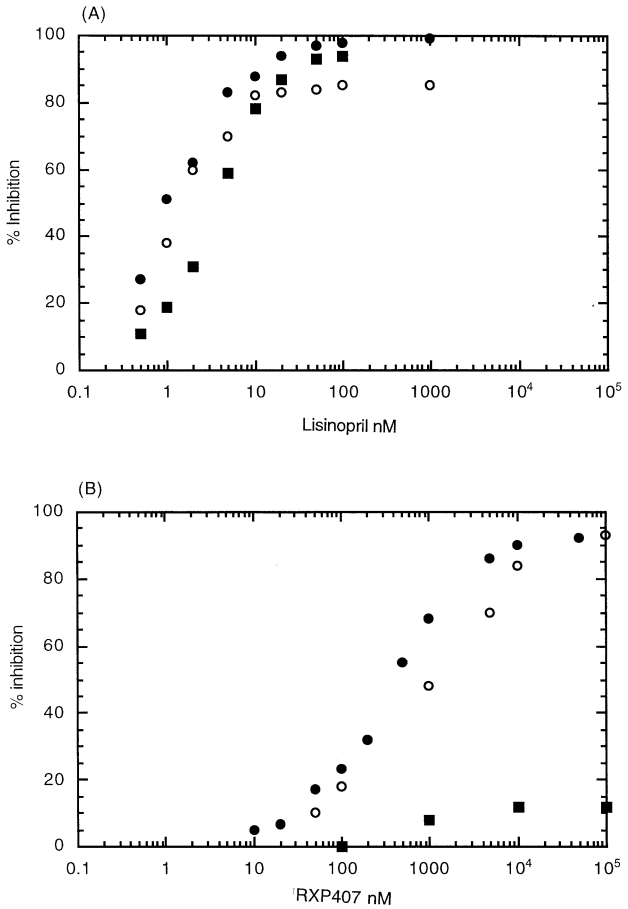

Fig. 2. Inhibition profiles of ACE activity present in crude plasma from human (filled circles), mouse (open circles), and rat (squares) by lisinopril (A) and RXP407 (B). Experimental conditions were as described in Fig. 1.

3.4. Potency of RXP407 with purified testicular ACE from mice

As compared to somatic ACE, the testicular ACE isoform contains only one active site, which corresponds to the C-domain of somatic ACE [13,16]. Thus, testicular ACE could be used to determine the C-domain potency of RXP407 and, indirectly, to deduce the N- or C-domain selectivity of this inhibitor toward somatic ACE. The profile of RXP407 inhibition of testicular ACE from mice, using

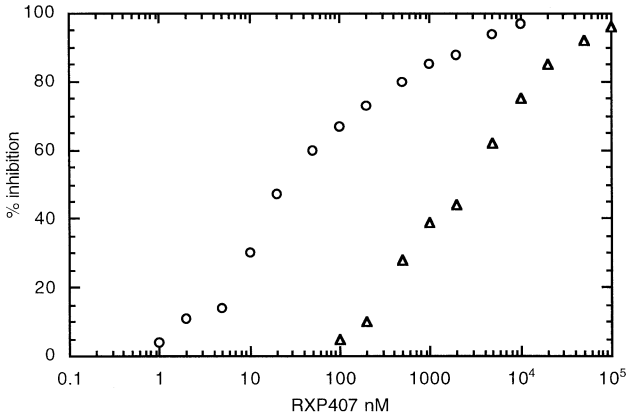

Fig. 3. Inhibition profiles of purified somatic (open circles) and germinal (open triangles) ACEs from mice by RXP407. Experimental conditions were described in Fig. 1.

Mca-Ala as substrate, revealed that RXP407 was a weak inhibitor of this ACE isoform, with an IC_{50} value of 2–3 μM (Fig. 3). This inhibition profile differed greatly from that observed with mouse somatic ACE (Fig. 3). The simplest explanation for this difference is that the 20-nM IC_{50} seen for the inhibition of somatic ACE by RXP407 reflects the high affinity of this inhibitor for the N-domain of somatic mouse ACE.

3.5. Inhibition of AcSDKP hydrolysis by RXP407

The regulatory peptide AcSDKP is a physiological N-domain-specific substrate of human somatic ACE [4,5]. Indeed, based on k_{cat}/K_m values, this substrate was shown to be cleaved *in vitro* 50 times faster by the N-domain of somatic recombinant human ACE than by the C-domain. The RXP407 inhibition profile of purified ACE from human plasma, using AcSDKP as substrate, indicated that 100 nM RXP407 fully inhibited ACE (Fig. 4). This was in contrast

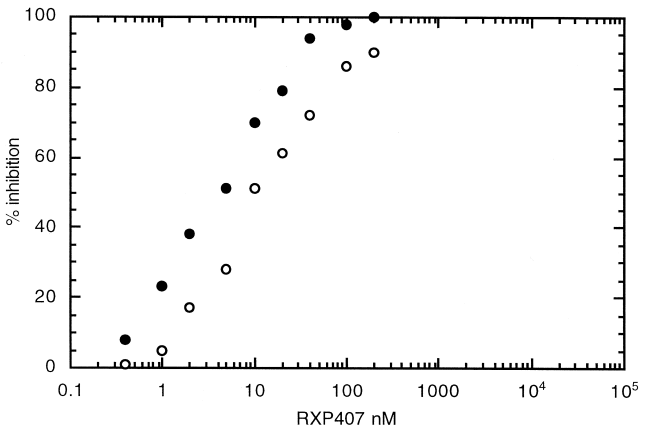

Fig. 4. Inhibition profiles of purified ACE from human (filled circles) and mouse (open circles) plasma by RXP407. The substrate used was AcSDKP (25 μM) in 50 mM HEPES buffer pH 6.8, 200 mM NaCl, 10 μM $ZnCl_2$, 25°.

with the inhibition profile obtained with the Mca-Ala substrate (Fig. 1B), which showed that 100 nM RXP407 inhibited 80% of human ACE, complete inhibition only being observed at 10 μM RXP407. This was due to the fact that the Mca-Ala substrate, as a mixed substrate of human ACE, was cleaved by both the N- and C-domains. Thus, full inhibition of the cleavage of this substrate by RXP407 requires blocking of both the N- and C-domains. In contrast, inhibition of the ACE N-domain by RXP407 was sufficient to block the degradation of AcSDKP, an N-domain-specific substrate of human ACE. Very similar results were obtained with ACE purified from mouse plasma when comparing the inhibition profiles of RXP407, as recorded with the Mca-Ala and AcSDKP substrates (Figs. 1A and 4). These results support the notion that RXP407 behaves as an N-specific inhibitor of ACE from mice and that AcSDKP is also an N-domain-specific substrate for the mouse enzyme.

4. Discussion

RXP407 was recently reported to be a potent N-domain-specific inhibitor of human recombinant ACE [7]. The inhibition profile reported in the present study for ACE purified from human plasma can be superimposed on that observed with the recombinant human ACE [7]. In this previous work, we showed conclusively that the biphasic inhibition profile of recombinant human ACE with RXP407 indicates that the Mca-Ala substrate used in this study is cleaved by the two active sites. This profile could be observed only if the difference in cleavage efficiency of the substrate by the two active sites was below a factor of 10. In addition, we showed that, with a mixed substrate, the range of inhibitor concentration necessary for a full inhibition of the enzyme is proportional to the relative affinity of the inhibitor for the two sites. The inhibition profile by RXP407 observed for ACE purified from human plasma was characterized by the need to use at least a four-order range of inhibitor concentration to observe a 10% to 90% inhibition. The different affinity of RXP407 for the two ACE active sites accounts for this very broad inhibition profile. By comparison, a two-order range of concentration was sufficient to block human ACE from 10% to 90% with lisinopril, a compound that blocked the two ACE active sites with similar affinities (Fig. 1A). According to the above remarks, the results reported for purified ACE from mouse plasma strongly suggest that RXP407 is able to differentiate the two active sites of mouse ACE (Fig. 1B) as it does the two human ACE active sites. Based on the weak potency exhibited by RXP407 with testicular ACE from mice, we conclude that RXP407 is a potent N-specific inhibitor of mouse somatic ACE. The K_d value of 23 nM determined for the binding of RXP407 to somatic ACE from mice thus represents the binding constant corresponding to the interaction of RXP407 with the N-domain of this enzyme. This K_d value is in reasonable agreement with the IC_{50} value of

10 nM observed for the inhibition of AcSDKP hydrolysis by RXP407 (Fig. 4).

In contrast, when tested on purified ACE from rat, RXP407 appeared 10 times less potent (K_d value of 190 nM). The RXP407 inhibition profile of rat ACE, using Mca-Ala as substrate, was also very particular, as compared to the profiles obtained with mouse and human ACEs (Fig. 1B). The first part of the inhibition profile of rat ACE (from 10 to 500 nM) probably represents the binding of RXP407 to the N-domain, while the second part may reflect the interaction of the inhibitor with the C-domain. Applying this hypothesis, an IC_{50} value of 200 nM might be estimated from the first part of the inhibition profile, a value that fits very well with the K_d value determined from direct binding experiments. However, to explain the shift in the inhibition profile observed with the rat ACE as compared to the profiles from mouse and human ACEs, one must assume that the Mca-Ala substrate is not a mixed substrate of rat ACE, in contrast with what was observed for mouse and human ACEs. Such a shift in the inhibition profile is expected in the case of a substrate being better cleaved by the C- than the N-domain of ACE. Indeed, we previously demonstrated that the inhibition profile of a two-active-site enzyme like ACE by RXP407 is highly dependent on the relative substrate specificity with which these two active sites cleave the substrate used for the inhibition titration [7].

From the inhibition profiles obtained with crude human and mouse plasma, IC_{50} values of 0.4 and 1 μM can be estimated for human and mouse ACE, respectively. These values, as compared to those determined using purified ACE, indicate a significant binding of RXP407 to protein components of these plasmas. Surprisingly, even at a 100-μM concentration, RXP407 was unable to significantly block the ACE activity present in crude rat plasma. This inactivity of RXP407 cannot be accounted for by a particular metabolism of this inhibitor in rat plasma, as RXP407 is completely metabolically stable *in vivo* in the rat [7]. Another possible explanation could be the presence of a prominent unspecific binding of RXP407 to plasma protein components of rat. This possibility is supported by the observation of important differences in plasma protein composition between rat and mouse [17].

Whatever the molecular interactions responsible for the lack of RXP407 potency when tested on crude rat plasma, different results in the present study argue against *in vivo* evaluation of RXP407 in rats. Instead, mice appear to be a good model to demonstrate whether RXP407 is active *in vivo*. In this respect, our results demonstrate that RXP407 is able to potently block the degradation of AcSDKP by mouse ACE and support the view that AcSDKP is almost cleaved by the N-domain of mouse ACE. Thus, it should be possible with the mouse model to assess whether an efficient control of AcSDKP metabolism can be achieved by blocking only the N-domain of mice ACE with RXP407. Such a property of RXP407 may find an important application, as Ac-SDKP administration has been shown to increase survival in mice treated with sublethal doses of cytotoxic agents [18,19]. Under RXP407 administration, it would be of great interest to verify whether the free C-domain is still able to cleave peptides such as angiotensin I and bradykinin in a normal fashion. This kind of experiment would contribute to the examination of the exciting hypothesis that *in vivo* the two active sites of ACE might control different physiological functions, such as blood pressure and hematopoiesis. Experiments in mice are currently underway to investigate these proposals.

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