Development and characterization of novel potent and stable inhibitors of endopeptidase EC 3.4.24.15

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Solid-phase synthesis was used to prepare a series of modifications to the selective and potent inhibitor of endopeptidase EC 3.4.24.15 (EP24.15), *N*-[1-(*R,S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP), which is degraded at the Ala-Tyr bond, thus severely limiting its utility *in vivo*. Reducing the amide bond between the Ala and Tyr decreased the potency of the inhibitor to 1/1000. However, the replacement of the second alanine residue immediately adjacent to the tyrosine with α -aminoisobutyric acid gave a compound (JA-2) that was equipotent with cFP, with a K_i of 23 nM. Like cFP, JA-2 inhibited the closely related endopeptidase EC 3.4.24.16 1/20 to 1/30 as potently as it did EP24.15, and did not inhibit the other thermolysin-like endopeptidases angiotensin-converting enzyme,

endothelin-converting enzyme and neutral endopeptidase. The biological stability of JA-2 was investigated by incubation with a number of membrane and soluble sheep tissue extracts. In contrast with cFP, JA-2 remained intact after 48 h of incubation with all tissues examined. Further modifications to the JA-2 compound failed to improve the potency of this inhibitor. Hence JA-2 is a potent, EP24.15-preferential and biologically stable inhibitor, therefore providing a valuable tool for further assessing the biological functions of EP24.15.

Key words: enzyme activity, fluorescent kinetic assay, metallo-peptidase.

INTRODUCTION

Endopeptidase EC 3.4.24.15 (EP24.15) is a 75 kDa neutral metalloendopeptidase that is widely distributed in cells and tissues throughout the body. It exists primarily in a soluble form that accounts for approx. 80 % of the total enzyme activity, the remaining activity being attributable to a minor membrane-associated component [1].

Although the three-dimensional structure of EP24.15 has yet to be determined, both its amino acid sequence and its sensitivity to chelators of metal ions classify EP24.15 as a member of the thermolysin-like family of metalloendopeptidases. This family of peptidases includes angiotensin-converting enzyme (ACE), endothelin-converting enzyme (ECE), neutral endopeptidase (NEP) and endopeptidase EC 3.4.24.16 (EP24.16). The active sites of these proteins contain a Zn^{2+} ion that participates in the catalytic process [2] and has the classical His-Glu-Xaa-Xaa-His motif typical of zinc metalloproteases [3].

In vitro, EP24.15 rapidly degrades a number of important bioactive peptides, including gonadotrophin-releasing hormone, bradykinin and neurotensin, suggesting a role in the physiological regulation of neuropeptide metabolism and hence an involvement in brain and endocrine functions. To help to establish the precise role of an enzyme in neuropeptide activation and/or deactivation, it is necessary to prove that its inhibition *in vivo*, or in intact cells, leads to changes in the concentration of the intact neuropeptide as well as changes in the biological responses dependent on the neuropeptide [4]. Such a demonstration requires the use of potent, selective and, importantly, biologically stable inhibitors.

So far, relatively few inhibitors of EP24.15 have been reported. One of the most potent, selective and frequently used inhibitors is *N*-[1-(*R,S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate (cFP), a protected tripeptide containing a free *N*-carboxymethyl group capable of interacting with the active-site Zn^{2+} of EP24.15 [4]. This compound displays an apparent K_i for EP24.15 of 16 nM [4]. A role for EP24.15 in the control of blood pressure was first suggested by Genden and Molineaux in 1991 [5]. They reported that the intravenous infusion of the phenylalanine-substituted analogue of this inhibitor (cFP-AAF-pAB, in which pAB stands for *p*-aminobenzoate) produced an immediate decrease in mean arterial pressure in normotensive rats and that this marked decrease was almost abolished by a kinin receptor antagonist. Their results suggested a direct involvement of EP24.15 in the inactivation of endogenous and exogenous bradykinin and further supported a role for EP24.15 in the regulation of blood pressure.

Unfortunately, cFP-AAF-pAB and cFP-AAF-pAB (K_i 27 nM) were later shown to be unstable *in vivo*, being rapidly degraded to form a potent ACE-inhibiting metabolite (cFP-Ala-Ala) [6–8]. This suggested that the action of cFP *in vivo* was attributable to the inhibition of ACE rather than of EP24.15, thus confounding the Genden–Molineaux data and leaving it unclear whether EP24.15 was involved in the control of blood pressure.

Although other peptide-based specific inhibitors of EP24.15 have been reported [9] and preliminary studies with whole brain homogenates suggest stability (F. Checler, personal communication), their true stability *in vivo* and thus their utility have yet

Abbreviations used: ACE, angiotensin-converting enzyme (EC 3.4.15.1); Aib, α -aminoisobutyric acid; Boc, *t*-butoxycarbonyl; cFP, *N*-[1-(*R,S*)-carboxyl-3-phenylpropyl]-Ala-Ala-Tyr-*p*-aminobenzoate; DTT, dithiothreitol; DIPEA, di-isopropylethylamine; DMF, *N,N*-dimethylformamide; ECE, endothelin-converting enzyme; EP24.15, endopeptidase EC 3.4.24.15; EP24.16, endopeptidase EC 3.4.24.16; HBTU, 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; IS-MS, ion spray MS; JA-1, *N*-[1-(*R,S*)-carboxy-3-phenylpropyl]-Ala-Ala-[ψ CH₂NH]-Tyr-*p*-aminobenzoate; JA-2, *N*-[1-(*R,S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate; NEP, neutral endopeptidase (EC 3.4.24.11); pAB, *p*-aminobenzoate; PAM, 4-(oxymethyl)phenylacetamidomethyl; QFS, quenched fluorescent substrate; TFA, trifluoroacetic acid, Z, benzyloxycarbonyl.

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to be formally demonstrated. Given the potential role of EP24.15 in neuropeptide regulation, there is a clear need for the development of potent, selective and stable inhibitors. Such inhibitors would help to verify the role of EP24.15 in biological processes and might establish EP24.15 as a therapeutic target.

In the present study we have used cFP as a molecular template and have synthesized a series of compounds in which the scissile bond (Ala-Tyr) has been modified in an effort to block biological hydrolysis. These compounds have been evaluated for the desired characteristics of stability, potency and selectivity. One of these compounds, *N*-[1-(*R,S*)-carboxy-3-phenylpropyl]-Ala- α -aminoisobutyric acid (Aib)-Tyr-*p*-aminobenzoate (JA-2), fulfils all of these characteristics.

EXPERIMENTAL

Materials

Recombinant rat EP24.15 and recombinant rat EP24.16 were generously provided by J. L. Roberts and M. J. Glucksman (Fishberg Center, Mt. Sinai Hospital, New York, NY, U.S.A.) and E. Ferro (University of São Paulo, São Paulo, Brazil), respectively. The endopeptidase 24.15 inhibitor cFP was synthesized (Dr. J. Boublik and L. Lakat) at the Baker Institute by the method of Chu and Orlowski [2]. The specific quenched fluorescent substrate (QFS) 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl) was synthesized by AUSPEP (Parkville, Victoria, Australia). Aminomethylated resin was purchased from NovaBiochem (Alexandria, New South Wales, Australia). ACE inhibition studies were performed by Dr. Siew Yeen Chai (Howard Florey, Melbourne, Australia). ECE and NEP inhibition studies were performed by Kay Barnes (University of Leeds, Leeds, U.K.). Dithiothreitol (DTT) was purchased from Calbiochem (Alexandria, New South Wales, Australia). All other reagents were purchased from Sigma unless stated otherwise.

Inhibitor synthesis

The compounds *N*-[1-(*R,S*)-carboxy-3-phenylpropyl]-Ala-Ala-[ψ CH₂NH]-Tyr-*p*-aminobenzoate (JA-1) and JA-2 were both assembled by solid-phase synthesis starting with 4-(Boc-*p*-aminobenzoyl-OCH₂)-PAM resin [in which Boc stands for *t*-butoxycarbonyl and PAM stands for 4-(oxymethyl)phenylacetamidomethyl].

4-(Boc-*p*-aminobenzoyl-OCH₂)-PAM resin

4-(Boc-*p*-aminobenzoyloxymethyl)-phenylacetic acid was prepared from Boc-*p*-aminobenzoic acid by the method of Tam et al. [10], in 13% overall yield. 4-(Boc-*p*-aminobenzoyloxymethyl)-phenylacetic acid (0.52 g, 1.25 mmol) was dissolved in *N,N*-dimethylformamide (DMF) (2.7 ml) together with 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.47 g, 1.25 mmol) and di-isopropylethylamine (DIPEA) (0.65 ml, 3.75 mmol). This solution was stirred for 1 min at room temperature, added to aminomethylated resin (substitution value 0.75 mmol/g; 1.60 g, 1.2 mmol) and the mixture was agitated for 5 h. The extent of coupling was monitored by the negative ninhydrin test and indicated that coupling was 94% complete. Unreacted amino groups on the resin were capped by acetylation with acetic anhydride/DIPEA for 10 min and the resin was washed and dried. This procedure gave the 4-(Boc-*p*-aminobenzoyl-OCH₂)-PAM resin (2.0 g) with a theoretical substitution value of 0.71 mmol/g based on the coupling yield as monitored by the negative ninhydrin test.

JA-1

4-(Boc-*p*-aminobenzoyl-OCH₂)-PAM resin (0.35 g, 0.25 mmol) was deprotected at the N-terminus by shaking the resin twice for 1 min with trifluoroacetic acid (TFA) (5 ml); the resin was neutralized with a 50% (v/v) solution of DIPEA in DMF. The resin was washed with DMF and to it was added the symmetrical anhydride of Boc-Tyr(2-Br-Z)-OH (1 mmol) (in which Z stands for benzyloxycarbonyl), formed by dissolving Boc-Tyr(2-Br-Z)-OH (0.98 g, 2 mmol) in DMF (2 ml), adding 1,3-diisopropylcarbodi-imide (156 μ l, 1 mmol) and stirring the solution at room temperature for 5 min. After 16 h at room temperature, the resin was washed with DMF and deprotected with TFA (twice for 1 min). The reductive amination was performed by adding to the resin a solution of Boc-Ala-H (0.172 g, 1 mmol), synthesized by the method of Fehrenz and Castro [11], in dry THF (4 ml) together with acetic acid (40 μ l) and sodium cyanoborohydride (93 mg, 1.5 mmol). This solution was shaken at room temperature for 30 min, after which a negative ninhydrin test showed the reaction to be complete. The resin was again deprotected with TFA (twice for 1 min) and coupled with Boc-Ala-OH (0.19 g, 1 mmol) by dissolving the amino acid in 5 M HBTU in DMF (2 ml, 1 mmol) and adding DIPEA (258 μ l, 1.5 mmol). The final reductive amidation was achieved by dissolving the free acid of 4-phenyl-2-oxobutyric acid (356 mg, 2 mmol) in dry THF (4 ml) together with DIPEA (344 μ l, 2 mmol) and sodium cyanoborohydride (124 mg, 2 mmol). This mixture was stirred with the resin for 16 h. The resin was subsequently washed with DMF, methanol and dichloromethane and air-dried; the peptide was cleaved from the resin by using HF with *p*-cresol as scavenger. The crude peptide was purified by reverse-phase HPLC to give the title compound as a white powder (30 mg, 21% overall yield based on the initial substitution value of the resin). Ion spray-MS (IS-MS), *M* + *H* 591; analytical HPLC, 4%/min gradient, retention time 13.9 min.

JA-2

Boc-Tyr(2-Br-Z)-OH was coupled to the PAM resin as described above. Boc-Aib-OH (0.20 g, 1 mmol) and Boc-Ala-OH (0.19 g, 1 mmol) were sequentially coupled with HBTU/DIPEA and the final reductive amidation with 4-phenyl-2-oxobutyric acid was performed as described for the synthesis of JA-1. Cleavage with HF and purification by preparative reverse-phase HPLC afforded the title compound as a white powder (35 mg, 23% overall yield). IS-MS, *M* + *H* 732 (purified as the TFA salt). A pure sample of each diastereomer could be isolated by reverse-phase HPLC with a 1%/min linear gradient. Analytical HPLC (2.66%/min linear gradient), retention time for the first eluting diastereomer 29.3 min; retention time for the second eluting diastereomer, 29.9 min. ¹H NMR for first eluting diastereomer (H₂O to ²H₂O, 8:2) δ 9.55, br. s., 1H, Aib-NH; 8.67, br. s., 1H, PABA-NH; 7.85, d, 2H, *J* = 8.5 Hz, PABA-ArH; 7.85, d, *J* = 7.2 Hz, Tyr-NH; 7.4, d, 2H, *J* = 8.5 Hz, PABA-ArH; 7.34–7.21, m, 5H, cFP-ArH; 7.12, d, 2H, *J* = 8.3 Hz, Tyr-ArH; 6.8, d, 2H, *J* = 8.5 Hz, Tyr-ArH; 4.58, m, 1H, Tyr- α H; 3.90, q, 1H, *J* = 6.8 Hz, Ala- α H; 3.62, t, 1H, *J* = 6.3 Hz, cFP- α H; 3.11–3.06, m, 2H, Tyr- β H; 2.71–2.58, m, 2H, cFP- β H; 2.09, m, 2H, cFP- γ H; 1.52, d, 3H, *J* = 7.0 Hz, Ala- β H; 1.41, s, 3H, Aib- β H; 1.33, s, 3H, Aib- β H. ¹H NMR for second eluting diastereomer (H₂O to ²H₂O, 8:2) δ 9.41, br. s., 1H, Aib-NH; 8.85, br. s., 1H, PABA-NH; 8.10, d, 1H, *J* = 7.4 Hz, Tyr-NH; 7.81, d, 2H, *J* = 8.6 Hz, PABA-ArH; 7.45, d, 2H, *J* = 8.4 Hz, PABA-ArH; 7.26–7.03, m, 5H, cFP-ArH; 7.12, d, *J* = 8.3 Hz, Tyr-ArH; 6.81, d, *J* = 8.3 Hz, Tyr-ArH; 4.50, m, 1H, Tyr- α H; 3.95, q, 1H, *J* = 6.9 Hz, Ala- α H; 3.33, t, 1H, *J* = 6.2 Hz, cFP- α H; 3.21, m, 1H, Tyr- β H;

3.05, m, 1H, Tyr- β H; 2.58–2.43, m, 2H, cFP- β H; 1.95, m, 2H, cFP- γ H; 1.46, d, 3H, $J = 7.0$ Hz, Ala- β H; 1.37, s, 3H, Aib- β H; 1.22, s, 3H, Aib- β H.

^1H NMR spectra were recorded on a Bruker ARX 500 spectrometer. Proton assignments were determined by two-dimensional NMR experiments (double-quantum-filtered COSY and TOCSY). Preparative-scale reverse-phase HPLC separations were performed on Waters Delta-Pak PrepPak C_{18} 40 mm $\times$ 100 mm cartridges (100 Å pore size). Analytical reverse-phase HPLC was performed on a Vydac 218TP5415 C_{18} column with a linear gradient of mixtures of 0.1 % (v/v) TFA in deionized water (solvent system A) and 0.1 % TFA in 10 % deionized water/90 % acetonitrile (solvent system B). Mass spectra were obtained on a triple-quadrupole mass spectrometer (PE SCIEX API III) equipped with an ion-spray (pneumatically assisted electrospray) atmospheric-pressure ionization source.

Biological data

All biological data were generated with the diastereomeric mixtures of each inhibitor.

Inhibition studies

The inhibition of EP24.15 was determined with a specific QFS, 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl), similar to that described by Tisljar et al. [12]. Recombinant EP24.15 (0.5 μg) was assayed in duplicate in 2.5 ml of 25 mM Tris-buffered saline, pH 7.4, containing 125 mM NaCl and 0.1 mM DTT. Inhibitor (0.003–3.0 μM) was added to the reaction mix before substrate (0.56 μM in DMSO) to a final concentration of 4.5 μM ; the assay tubes were incubated at 37 °C for 30 min. Reactions were stopped by the addition of 25 μl of 100 mM ZnCl_2 and the reaction tubes were left to reach room temperature. Fluorescence was read on a Perkin-Elmer LS-5 luminescence spectrometer (excitation, 314 nm; emission, 418 nm). The reaction was linear up to 60 min; all kinetic measurements were determined at less than 10 % substrate degradation. Inhibition of recombinant EP24.16 was determined as for EP24.15 with the omission of the DTT from the reaction mix. K_i values for JA-2 and cFP were determined from the IC_{50} at each substrate concentration by using the relationship $\text{IC}_{50} = K_i(1 + [\text{S}]/K_m)$. The K_m had been determined as 19.8 μM by a Lineweaver–Burk analysis of initial velocities measured in triplicate at a range of QFS concentrations (1.1–140 mM) [13]. ACE (human plasma) was assayed by using the fluorimetric ACE method, as described by Friedland and Silverstein [14], which measures the degradation of hippuryl-His-Leu. JA-2 was added to a final concentration of 0.5 mM. ECE (from membranes of Chinese hamster ovary cells) was assayed by an HPLC method with the synthetic peptide, based on the minimal cleavage sequence of Big Endothelin {[Phe²²]big ET-1(18–34)} DIIWFN-TPEHVPYGLG-NH₂, at 200 μM final concentration as substrate as described by Sansom et al. [15]. JA-2 was added to a final concentration of 100 μM . NEP (COS-1 cell membranes) was assayed by an HPLC method with 150 μM [D-Ala², Leu⁵]enkephalin as a substrate, monitoring the formation of the Tyr-D-Ala-Gly product at 214 nm [16]. JA-2 was added to a final concentration of 100 μM .

Inhibitor stability studies

Soluble and membrane extract of sheep kidney, lung, liver, pituitary and hypothalamus were prepared by homogenization in 3 vol. wet weight of ice-cold 50 mM Tris/HCl, pH 7.4, followed by ultracentrifugation (100 000 g for 1 h at 4 °C). The soluble

fraction was removed; the pellet was resuspended in fresh Tris buffer and recentrifuged. The second pellet was resuspended in 1 ml of Tris buffer containing 1 % (v/v) Triton X-100 (BDH, Kilsyth, Australia), incubated for 1 h at 4 °C with frequent vortex-mixing, and recentrifuged. The supernatant containing the solubilized membranes was removed and the pellet was discarded. JA-2 and cFP were incubated with soluble (40 μl) and membrane (20 μl) preparations at 37 °C for 0, 2, 6, 12, 24 and 48 h. An aliquot equivalent to 5 μg of each inhibitor was taken at each time point and the reaction was stopped by the addition of methanol/0.1 % (v/v) TFA to a final methanol concentration of 80 % (v/v) and the supernatant was dried by vacuum centrifugation (Speed-Vac; Savant). Samples were analysed with a Waters HPLC system comprising two 510 pumps, a model 680 gradient controller, a U6K injector and a model 441 UV absorbance detector. Each sample was injected on a radially compressed Waters C_{18} Nova Pak reverse-phase column (8 mm $\times$ 10 cm). Solvent A was 0.08 % (v/v) TFA; solvent B was 70 % (v/v) acetonitrile containing 0.08 % (v/v) TFA. Separation was achieved with a linear gradient of 3–100 % solvent B over 30 min at a flow rate of 1 ml/min. The absorbance of eluted peaks was measured at 214 nm.

RESULTS

In these studies, the strategy for the development of biologically stable inhibitors of EP24.15 has focused on the synthesis of derivatives of the selective and potent inhibitor cFP. Because cFP-Ala-Ala-Tyr-pAB is degraded *in vivo* at the Ala-Tyr bond to give cFP-Ala-Ala, its stability *in vivo* could theoretically be enhanced by either replacing this scissile amide bond with a non-cleavable replacement or protecting this amide bond from hydrolysis (Figure 1A). The first compound that we synthesized

Figure 1 Chemical structures of EP24.15 Inhibitors

(A) cFP; (B) JA-1; (C) JA-2.

Figure 2 Reverse-phase HPLC analysis of cFP[Tyr] and JA-2 after incubation with sheep kidney membranes

Inhibitor was incubated with solubilized kidney membrane preparation (20 μ l) at 37 $^{\circ}$ C. At each time point, an aliquot equivalent to 5 μ g of inhibitor was taken and the reaction was stopped by the addition of methanol/0.1% (v/v) TFA to a final methanol concentration of 80% (v/v). Samples were dried under vacuum before analysis by reverse-phase HPLC.

was a reduced amide derivative of cFP, JA-1. In this compound, the carbonyl group of the amide bond between Ala and Tyr is absent; only a methylene group is present (Figure 1B). This inhibitor was fully stable on incubation with sheep kidney membranes, an enriched source of the proteolytic activity known to degrade cFP [17], under the assay conditions described in the Experimental section. Unfortunately, the potency of this compound towards EP24.15 was less than 1/1000 that of cFP (results not shown).

The replacement of the alanine residue immediately adjacent to the tyrosine residue with Aib generated the compound JA-2 (Figure 1C). Aib is very similar to Ala, except that the α -hydrogen of Ala has been replaced with a methyl group. The incorporation of α -methyl amino acids in peptide sequences is known to impart increased proteolytic stability to the amide bonds immediately adjacent to the residue [18].

To assess the biological stability of JA-2, the compound was incubated with sheep tissue extracts. JA-2 remained intact after 48 h of incubation with both membrane and soluble fractions of kidney, lung, heart, liver, hypothalamus and pituitary. Figure 2 shows the HPLC chromatograms of JA-2 after 2, 4 and 48 h of incubation with the kidney membranes. This profile is typical of all extracts examined. In contrast, degradation of cFP was evident after 2 h, with complete degradation at 4 h of incubation in all extracts tested. Figure 2 also shows the degradation of cFP by kidney membranes and is representative of the other samples tested.

It was clearly important to ensure that potency had not been sacrificed for stability, as with JA-1. Figure 3 shows the inhibition curve of EP24.15 with both JA-2 and cFP. JA-2 inhibited EP24.15 in a concentration-dependent manner, similarly to cFP. The K_i values obtained for JA-2 and cFP were 23 and 19 nM respectively. Like cFP, JA-2 acts as a competitive inhibitor, as confirmed by Lineweaver–Burk analysis (Figure 3). Furthermore, preincubation studies also confirmed (as with cFP) rapid binding (results not shown). Thus we conclude that JA-2 is equipotent to cFP in its inhibition of EP24.15. We have isolated the two diastereoisomers of JA-2 by HPLC; although their absolute

Figure 3 Inhibition curve of EP 24.15 by JA-2 and cFP

Upper panel: recombinant EP24.15 (0.5 μ g) was incubated with 0.1 μ g of QFS for 30 min at 37 $^{\circ}$ C in 2.5 ml Tris-buffered saline containing 0.1 mM DTT in the presence of increasing concentrations of inhibitor. Each point is the mean $\pm$ S.D. for duplicate measurements taken from four separate experiments. Symbols: $\bullet$, JA-2; $\circ$, cFP. Inset: Lineweaver–Burk plot (v , nmol of product/h per μ g of enzyme; S , μ M QFS) of JA-2 incubated with EP 24.15 with various concentrations of QFS, confirming the competitive nature of the inhibitor.

stereochemistry has not yet been determined, the calculated potencies (K_i values 22 and 65 nM) were of the same order of magnitude.

To determine the selectivity of JA-2 we examined its ability to inhibit the other thermolysin-like zinc metallopeptidases, ACE, ECE, NEP and EP24.15. Neither ACE nor ECE was inhibited by JA-2 at an inhibitor concentration of 500 or 100 μ M respectively; only slight (17%) inhibition of NEP was observed with 100 μ M JA-2. The closely related EP24.16 was inhibited by JA-2, although the inhibitory potency of JA-2 for EP24.15 was 20–30-fold greater than for EP24.16 (Figure 4). The inhibition of

Figure 4 Inhibition curves of EP24.15 (●) and EP24.16 (○) by JA-2

Recombinant EP24.15 or EP24.16 (0.5 μg) was incubated with 0.1 μg of QFS for 30 min at 37 °C in 2.5 ml Tris-buffered saline (including 0.1 mM DTT for EP24.15) in the presence of increasing concentrations of inhibitor. Each point is the mean for duplicate measurements in a single experiment. The calculated K_i for EP24.16 was 610 nM.

EP24.16 by JA-2 was similar to that of the enzyme by cFP- α AY-pAB (results not shown) and was in accordance with the reported inhibition of EP24.16 by cFP-AAF-pAB [19,20]. This result was not unexpected, given the very similar substrate specificities of these two enzymes.

In further attempts to improve the inhibitory potency of JA-2, several modifications were made to the compound. Compounds JA-3 and JA-7 were identical to JA-2 with the exception of the replacement of the carboxy group with a hydroxamic acid and a thiol group respectively. These groups have been reported to display a higher binding affinity for zinc metalloproteases [21]; however, JA-7 inhibited EP24.15 poorly and JA-3 was approx. 10% as potent as JA-2. The removal of the pAB group from the JA-2 compound also resulted in a 90% decrease in potency. Further characterization of these compounds was therefore not pursued.

DISCUSSION

JA-2 is the first inhibitor that we have been able to test and report that possesses the desirable characteristics of potency, selectivity and biological stability. The replacement of the second alanine residue of cFP with Aib, to give JA-2, imparted proteolytic stability without any loss of potency and specificity. By analogy with cFP, JA-2 is expected to bind to EP24.15 with the α -aminobutyric acid residue occupying the S_2' site and being the P_2' residue according to the Schechter–Berger nomenclature [22]. The active-site Zn^{2+} ion presumably co-ordinates the N-terminal carboxy group, as with cFP.

The achievement of biological stability without the loss of potency or selectivity is encouraging in the light of several previous attempts to produce a desirable inhibitor of EP24.15. The reduced amide derivative of cFP (JA-1), although completely stable under the conditions described here for JA-2, was found to be 1/1000 as potent as cFP. The loss of potency is most probably attributable to a pronounced conformational change in the molecule caused by a loss of regional planarity. In a previous attempt to develop more stable inhibitors of EP24.15, we synthesized a series of analogues of cFP in which the second alanine residue was replaced by D-Ala, Asp, Val, Ser or Leu, in an effort to decrease the susceptibility at the Ala-Tyr bond. The D-Ala and Asp substitutions abolished inhibitory activity, whereas activity remained with the Ser, Val and Leu substitutions,

although at decreased potency. Biological stability was enhanced 20-fold (Ser) and 10-fold (Val, Leu) in comparison with cFP. However, the Val-substituted analogue was cleared from the circulation of conscious rabbits at the same rate as cFP and, like cFP, blunted the pressor response to angiotensin I, again indicative of ACE inhibition [13].

So far, the most potent and selective inhibitor of EP24.15 reported is the tetrapeptide Z-(L,D)Phe(PO₂CH₂)(L,D)Ala-Arg-Phe [9]. This compound, which incorporates a phosphinic acid, has a K_i for EP24.15 of 0.16 nM and is more than three orders of magnitude less potent towards EP24.16 (K_i 530 nM). Furthermore, at 1 mM this inhibitor does not affect the activity of other zinc peptidases (ACE and NEP). This compound is reportedly stable after 15 hours of incubation with rat brain homogenate (F. Checler, personal communication); however, given the well-characterized tryptic and Phe-specific carboxypeptidase activities in blood and tissues [23], the utility of this compound *in vivo* is likely to be limited.

Other available inhibitors include *N*-[(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolinecarbonyl]-L-phenylalanine (SA898), which incorporates a thiol group, a better zinc-chelating group than the carboxy group. The result is a potent (K_i 9.1 nM) inhibitor; however, this compound is not selective, also significantly inhibiting ACE with a K_i of 170 nM [24]. In addition, there are no results on the stability of this compound *in vitro* or *in vivo*. The phosphonamide peptide *N*-(phenylethylphosphonyl)-Gly-L-Pro-L-aminohexanoic acid is again a potent inhibitor (K_i 7.5 nM), although equipotent in its inhibition of EP24.15 and EP24.16 [25]. Again, this compound is probably hydrolysed in biological fluid, limiting its use in studies *in vivo*.

The JA-2 inhibitor does not inhibit ACE and ECE and does not significantly inhibit NEP at concentrations up to 100 μM; it is approx. 1/30 as potent in its inhibition of EP24.16 in comparison with EP24.15. The inhibition of EP24.16 by JA-2 was similar to that by cFP- α AY-pAB and in agreement with the inhibition of EP24.16 by cFP-AAF-pAB [19,20]. Hence the stabilization of cFP's scissile bond via the replacement of Ala with Aib has not altered the potency or specificity of the parent compound.

In summary, we have described the development of a potent, stable and EP24.15-preferential inhibitor that provides a valuable tool for further defining the precise physiological role of EP24.15. The use of JA-2 *in vivo* might help to assess the involvement of EP24.15 in bradykinin degradation and hence in the regulation of blood pressure, in addition to the identification of the role of EP24.15 in the regulation of gonadotrophin-releasing hormone [26] and neurotensin [27] within the neuroendocrine system. We are currently examining the stability and hypotensive utility of this inhibitor *in vivo*. Preliminary whole-animal studies confirm that JA-2 is indeed stable *in vivo*; interestingly, they suggest a role for EP24.15 and EP24.16 in the regulation of circulating bradykinin levels [28].

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