Mechanism of Vasopeptidase Inhibitor-Induced Plasma Extravasation: Comparison of Omapatrilat and the Novel Neutral Endopeptidase 24.11/Angiotensin-Converting Enzyme Inhibitor GW796406

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ABSTRACT

We describe N-[(2S)-2-(mercaptomethyl)-3-methylbutanoyl]-4-(1H-pyrazol-1-yl)-L-phenylalanine (GW796406), a vasopeptidase inhibitor (VPI) that possessed ~3-fold selectivity for neutral endopeptidase 24.11 (NEP) versus angiotensin-converting enzyme (ACE) in vitro assays using rat and human enzymes. In the same assays, omapatrilat, the most extensively studied VPI, displayed ~3-fold selectivity for ACE. The in vivo ACE and NEP inhibition profile and the liability of the compounds to increase plasma extravasation were compared at two (low and high) therapeutically equivalent intravenous doses in the rat. At the low dose, both agents inhibited ACE activity by ~85%. Consistent with their in vitro ACE/NEP selectivity, omapatrilat produced 49% inhibition, whereas GW796406 produced >95% inhibition of NEP. Neither compound increased plasma extravasation. When the low dose was administered to rats pretreated with the NEP inhibitor ecadotril to normalize NEP background to ~<5% of control, only omapatrilat significantly increased plasma extravasation. At the high dose, omapatrilat and GW796406 produced profound, nonselective inhibition of ACE (~90%) and NEP (>95%), and they significantly increased plasma extravasation. The activity of the agents as inhibitors of dipeptidylpeptidase IV (DPP IV) and aminopeptidase P (APP) was also investigated. Neither compound inhibited DPP IV. Interestingly, omapatrilat, but not GW796406, was a relatively potent inhibitor of APP (IC_{50} = 260 nM). We investigated whether APP inhibition increased the plasma extravasation liability of GW796406. The low dose of GW796406 administered with apstatin, an APP inhibitor, did not increase plasma extravasation. This finding inferred that APP inhibition is not involved in plasma extravasation in the rat and that APP inhibition does not explain the increased plasma extravasation produced by omapatrilat in NEP-inhibited rats.

Angioedema is a potentially life-threatening adverse effect that occurs in ~0.1% of patients treated with ACEIs. The mechanisms involved in angioedema are not well understood, but increased levels of bradykinin, a preferred substrate for ACE, likely play a key early role. In an attempt to identify more effective antihypertensive drugs, efforts have been directed toward developing vasopeptidase inhibitors (VPIs), agents that are dual inhibitors of ACE and neutral endopeptidase 24.11 (NEP). Inhibition of NEP would be expected to increase levels of the physiologic substrates of the enzyme, the atrial natriuretic peptides that oppose the hypertensive properties of angiotensin II. Consequently, the VPIs may offer greater hemodynamic efficacy than the ACEI. Indeed, clinical trials with omapatrilat have demonstrated a significantly greater decrease in blood pressure than that produced by selective ACEIs (Campese et al., 2001; Azizi et al., 2002; Ferrario et al., 2002). Although NEP does not play a significant role in bradykinin metabolism under normal circumstances, the enzyme does seem to assume a much greater role when ACE is inhibited. Consequently, there has been some concern that the vasopeptidase inhibitors would possess as much, if not greater liability, to cause angioedema as the selective ACEIs. This concern may have some basis since, in

ABBREVIATIONS: ACEI, angiotensin-converting enzyme inhibitor; ACE, angiotensin-converting enzyme; VPI, vasopeptidase inhibitor; NEP, neutral endopeptidase 24.11; GW796406, N-[(2S)-2-(mercaptomethyl)-3-methylbutanoyl]-4-(1H-pyrazol-1-yl)-L-phenylalanine; TCA, trichloroacetic acid; DPP IV, dipeptidylpeptidase IV; APP, aminopeptidase P; DOCA, deoxycorticosterone acetate; MAP, mean arterial pressure; CP99994, (+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine; B2, bradykinin 2; NK1, neurokinin 1.
spite of the apparent greater antihypertensive effect of omapatrilat over ACEIs, results from a large clinical trial in hypertensive patients (OCTAVE) demonstrated that omapatrilat-treated hypertensive subjects had a significantly higher (~3-fold) incidence of angioedema than enalapril-treated subjects (Kostis et al., 2004). The increased liability of omapatrilat to cause angioedema has delayed further development of this agent, and it may have tempered interest in the VPIs as antihypertensive agents. Consequently, in spite of their apparent greater efficacy in reducing blood pressure, the question of whether the VPIs possess true clinical benefit over the selective ACEIs may never be answered.

The relatively low incidence of angioedema, whether in ACEI- or VPI-treated patients, suggests that a variety of other factors, genetic or acquired, predispose a subpopulation of patients to develop the condition. There have been a number of studies conducted to evaluate the mechanisms involved in ACE inhibitor- and VPI-induced angioedema that have demonstrated that affected patients have impaired plasma aminopeptidase P (APP) (Adam et al., 2002; Molinaro et al., 2002a) or dipeptidylpeptidase IV (DPP IV) (Lefebvre et al., 2002) activity. These findings are consistent in that they implicate an impaired metabolism of bradykinin and/or substance P by non-ACE pathways that may assume a greater role in the metabolism of kinins when ACE is inhibited.

Plasma extravasation in the rodent seems to be a reasonable model of the early changes in vascular permeability that occur in ACEI-related angioedema. We have demonstrated that captopril-induced plasma extravasation in this model was enhanced by concomitant inhibition of NEP (Sulpizio et al., 2004). These findings seem consistent with the report that C1 inhibitor deficiency in humans leads to spontaneous angioedema (Markovic et al., 2000). Although C1 inhibitor-deficient mice do not exhibit overt angioedema, they do display increased vascular permeability, a fundamental component of plasma extravasation, which is markedly worsened by acute ACE inhibition (Han et al., 2002). In the present study, we have investigated the effects of omapatrilat and GW796406, a new VPI that displayed 3-fold selectivity for NEP over ACE, for their in vitro and in vivo enzyme inhibition profiles and their liability to increase plasma extravasation in the rat.

**Materials and Methods**

Male Sprague-Dawley strain rats (Charles River Laboratories, Inc., Wilmington, MA) weighing between 250 and 350 g were used in the ex vivo enzyme and plasma extravasation studies. The procedures involving the use of rats in these experiments were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines (NIH Publication 85-23). The animals were housed two per cage with free access to food and water and a 12-h light/dark cycle. Rabbit lung and kidney used in the in vitro enzyme assays were obtained from in-house colony of New Zealand White strain rabbits following euthanasia with intravenous pentobarbital. Human lung and kidney tissues used in the enzyme assays were obtained from the International Institute for the Advancement of Medicine (Exton, PA) and the National Disease Research Interchange (Philadelphia, PA).

**In Vitro ACE and NEP Assays**

**Tissue Preparations.** Human kidney cortex tissue or lung tissue was homogenized in 0.1 M phosphate buffer, pH 7.4, 10 ml/g tissue, using a Tekmar Tissuemizer (Tekmar-Doehrmann, Mason, OH). The homogenate was centrifuged at 10,000g for 20 min at 4°C. The supernatant was then centrifuged at 40,000g for 45 min at 4°C. The pellet from this step was suspended in one-third of the original volume with 5 mM sodium phosphate, pH 7.4, using a motorized Teflon mortar and glass pestle homogenizer. This was centrifuged at 40,000g for 45 min at 4°C. The pellet was suspended in 20 mM Tris, pH 7.5, and stored frozen at −80°C.

**Rabbit kidney and lung crude membrane preparation.** Crude membrane fractions were prepared by homogenizing tissue in 40 volumes of 50 mM Tris, pH 7.5, with 0.25 M sucrose using a Tekmar Tissuemizer. Homogenate was centrifuged at 1000g for 10 min at 4°C. Supernatant was removed and centrifuged at 40,000g for 45 min at 4°C. The pellets were resuspended in a small volume of 20 mM Tris, pH 7.5, and stored frozen at −80°C.

**In Vitro ACE/NEP Inhibition**

For determination of lung ACE activity and kidney NEP activity, approximately 250 mg of tissue was homogenized in 6 volumes of 0.1 M K$_2$HPO$_4$, pH 8.3, 0.3 M NaCl, and 1 μM ZnSO$_4$ using a Teflon glass motor-driven pestle. For lung ACE activity, 40 μl of homogenate was added to conical-bottomed 96-well plates and warmed to 37°C for 5 min. Ten microliters of 7.5 mM hippuryl-His-Leu (1.5 mM final) was added to each sample and incubated for 10 min at 37°C. One hundred microliters of 10% trichloroacetic acid (TCA) was added to each well, and the plates were centrifuged to pellet precipitated proteins. Fifty microliters of supernatant was added to 100 μl of 2 mg/ml o-phthalaldehyde in 10% ethanol and 50 μl of 1 N NaOH in a black fluorometric plate. After 60 min, the plate was read in a fluorometer at 390-nm excitation and 460-nm emission. Standard curves were generated using His-Leu.

Kidney NEP activity was measured by adding 35 μl of homogenate to wells containing 5 μl of buffer or 10 μM phosphoramidon. Plates were warmed to 37°C for 5 min. Ten microliters of 2.5 mM N-dansyl-d-Ala-Gly-p-nitrophe-Gly substrate was added to each sample to yield a 0.5 mM final concentration and incubated for 4 min at 37°C. One hundred microliters of 10% TCA was added, and plates were centrifuged to pellet precipitated proteins. Fifty microliters of supernatant was added to 100 μl of 100% ethanol and 50 μl of 1 N NaOH in a black fluorometric plate. After 10 min, plates were read at 590-emission, 320-nm excitation in a fluorometer. Standard curves were generated using N-dansyl-d-Ala-Gly in buffer. For both the ACE and NEP assays, homogenate protein was measured with a protein assay kit (Bio-Rad, Hercules, CA), and product production was corrected for protein. Increasing concentrations of omapatrilat or GW796406 were tested for inhibition of enzyme activity. IC$_{50}$ values were calculated from the inhibition curves using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

**In Vitro Dipeptidylpeptidase IV and Aminopeptidase P Assays**

**Kidney Brush-Border Preparation.** Kidneys were removed from rats under pentobarbital-induced general anesthesia. Cortical tissue was isolated and homogenized in ice-cold 50 mM mannitol and 2 mM HEPES, pH 7.5. A 1 M solution of MgCl$_2$ was added to the homogenate to produce a final concentration of 10 mM MgCl$_2$. This was stirred on ice for 10 min followed by centrifugation at 2000g for 10 min at 4°C. The supernatant was then centrifuged for 20 min at 35,000g at 4°C. The white fluffy portion of the pellet was removed and centrifuged again as in the previous step. The final pellet was resuspended in 20 mM Tris, pH 7.4.

**Aminopeptidase P.** Aminopeptidase P was measured fluorometrically using 0.2 mM Arg-Pro-Pro as substrate in a 50-μl assay in 50 mM Tris, pH 7.5, buffer. Either 0.5 μg of protein from rat kidney brush-border membrane or human kidney microsome was incubated with the compound for 5 min at 37°C before adding substrate. The
assay was then incubated for 45 min at 37°C. At the end of this period, 50 μl of 1 N NaOH was added to stop the assay followed by 100 μl of 2 mg/ml o-phthalaldehyde in 10% ethanol. Assays were allowed to incubate for 1 h to form a fluorescent product from the reaction of o-phthalaldehyde with free arginine produced by the cleavage of the substrate. Fluorescence was measured using a PerkinElmer Victor 2 fluorometer (PerkinElmer Life and Analytical Sciences, Boston, MA) with 355-nm excitation and 460-nm emission. Standard curves were generated with arginine. Less than 10% of the substrate was used during the assay.

**Dipeptidylpeptidase IV.** DPP IV activity was measured by the cleavage of 0.3 mM Gly-Pro-4-methoxy-2-naphthylamide to Gly-Pro, and a fluorescent product was quantified by the measurement of fluorescence at 355-nm excitation and 430-nm emission. Either 6 ng of rat kidney brush-border membrane or 50 ng of human kidney microsome was incubated with compounds for 5 min before the addition of substrate using 50 mM Tris buffer, pH 8.5. The fluorescence was measured 12 times at 5-min intervals, and the rate of fluorescence production was calculated.

**Determination of ACE Inhibitory Activity in the Anesthetized Rat Angiotensin I Challenge Model.** Male rats (Wistar; 300–450 g; Harlan UK Limited, Bicester, Oxon, UK) were anesthetized with ketamine (40 mg/kg i.m.; Vetar; Pharmacal Upjohn, Crawley, UK), xylazine (8 mg/kg i.m.; Rompun; Bayer AG, Leverkusen, Germany), and sodium pentobarbitone (20 mg/kg i.p.; Sagata; Rhone-Merieux, Dagenham, UK). The trachea was cannulated, and the animals were artificially respired with room air at 3.5 ml/kg, 60 breaths per min, supplemented with O2 at 30 ml/min. Both jugular veins were cannulated, and one vein was used for administration of angiotensin I and supplementary anesthesia (sodium pentobarbitone, i.v. as required), whereas the second venous line was used for administration of drugs by bolus plus infusion. A carotid artery was cannulated to measure blood pressure (SensoNor 820 transducer; SensoNor, Horten, Norway) coupled to a Notocord (Notocord Systems, Paris, France) data acquisition system. Animals were allowed at least 15 min following surgery before administration of angiotensin I.

Intravenous administration of human angiotensin I (Sigma Chemical, Poole, Dorset, UK) induced a short-lived, dose-dependent increase in blood pressure with a dose of 300 ng/kg producing approximately 70% of the maximum pressor response. Angiotensin I (300 ng/kg) was administered repeatedly until a reproducible pressor response was obtained; this usually occurred with three challenges. If stable responses were not obtained within five challenges, the preparation was abandoned. A bolus plus infusion of a test inhibitor compound was then administered, and the angiotensin I challenge was repeated at 15, 30, 45, and 60 min following the start of the infusion. The angiotensin I pressor responses obtained in the presence of the test compound were compared with the last preinhibitor angiotensin I challenge, and the data are expressed as a percentage of inhibition. The bolus/infusion-dosing protocol required to produce approximately 50% inhibition (IC50) of the angiotensin I pressor response was determined. Under these conditions, the IC50 for omapatrilat was a 0.1 mg/kg priming bolus followed by a 0.1 mg/kg/h infusion for 60 min. The IC50 for GW796406 was a 3.0 mg/kg priming bolus followed by a 3 mg/kg/h infusion. Control animals received bolus plus infusion of vehicle.

**Determination of Minimally Effective Antihypertensive Dose in Conscious Instrumented DOCA-Salt Hypertensive Rats.** Male Sprague-Dawley rats (300–400 g; Charles River Laboratories Inc., Raleigh, NC) were uninephrectomized and implanted with DOCA pellet (deoxycorticosterone acetate, 200 mg; Innovative Research of America, Sarasota, FL) and aortic telemetry probes (TA11PA-C40; DSI, St. Paul, MN). Surgical procedures were separated by 7 to 10 days. Rats were allowed free access to drinking water containing 1.6% NaCl and maintained on extruded diet (five pellets/day). Blood pressure was monitored and when mean arterial pressure (MAP) reached approximately 160 mm Hg, animals were placed on study. The following protocol was used to evaluate the effects of i.v.-administered GW796406X in the DOCA-salt hypertensive model. One to 2 days before the experiment, intravenous catheters (polyethylene 50) were implanted in the femoral vein and exteriorized through the skin (dorsal upper thorax). On the day of the experiment, animals received GW796406X (i.v. bolus at 30 mg/kg followed by a 15-min i.v. infusion of 30 mg/kg). Telemetry records of MAP and heart rate were collected for a 3-h period before dosing and a 12-h period following dosing.

**Plasma Extravasation and in Vivo Enzyme Inhibition Studies**

Rats were anesthetized as described above, and the femoral vein was isolated and cannulated with polyethylene 60 tubing to provide a site for drug administration. The animals then received either omapatrilat or GW796406 infused using the same dosing protocol described above to produce a 50% reduction in angiotensin I pressor response (low-dose protocol) or produce a minimally significant antihypertensive effect (high-dose protocol). The dosing regimen used to administer omapatrilat or GW796406X intravenously consisted of a bolus-priming dose followed by a slow infusion. The priming dose was administered intravenously via the femoral vein catheter at a volume of 0.25 ml/100 g body weight given over a 1.5- to 2-min period. Immediately following the bolus injection, the catheter was attached to a syringe infusion pump, and the appropriate dose of GW796406X or omapatrilat was infused at a rate of 40 μl/min for the next 50 min (total volume 2.0 ml). Control rats received a bolus and infusion of vehicle using the same dosing protocol. To determine the effect of NEP inhibition on the low-dose regimen of omapatrilat, rats received an oral dose of 15 mg/kg NEP inhibitor ecallitril 20 min before administration of omapatrilat. To determine the effect of APP inhibition on the low-dose regimen of GW796406, rats received an intravenous injection of the APP inhibitor apstatin at a dose of 0.8 mg/kg 5 min before administration of GW796406.

Approximately 50 min into the infusion, the infusion was stopped, and the rats received a 30 mg/kg dose of Evans blue dye administered at a dose volume of 0.2 ml/100 g body weight via the infusion catheter. Five minutes post-Evans blue injection, the thoracic and peritoneal cavities were opened via a single midline incision. A 0.8- to 1.0-ml blood sample was obtained by cardiac puncture using a heparinized 1-ml syringe and 23-gauge needle and placed on ice. The tip of the right atrium was then cut, and a steel cannula, attached by latex tubing to a peristaltic pump (Harvard Apparatus Inc., Hollisburg, MA), was inserted into the heart at the bottom of the left ventricle and was slid up through ventricle until the tip of the cannula was visible in the aortic arch. The cannula was manually held in place using forceps clamped across the heart. The pump was then started, and the vascular system was perfused with 120 ml of saline delivered at a rate of 40 ml/min, which results in a perfusion pressure pulse of 80 to 100 mm Hg. This procedure is similar to that described by others (Brokaw and White, 1994; Klitzman et al., 1995). The success of the perfusion was evaluated by visually monitoring the liver for the progressive removal of the dark bluish color caused by the presence of Evans blue in the liver tissue. Complete blanching of the liver was achieved in all cases. Following perfusion, a section of the left lung, the entire left kidney, and a 15- to 20-mm segment of the trachea were removed. The lung and kidney were cut into four sections, placed into Falcon tubes (Falcon; BD Biosciences Discovery Labware, Bedford, MA), and flash-frozen in liquid nitrogen. The blood, lung, and kidney samples were submitted for determination of ACE (plasma and lung) and NEP (kidney) activity. The trachea was cleared of any adhering fat and muscle, a cut was made along the entire length to expose the inner surface, and the tissue was blotted dry using a paper hand towel. The trachea was weighed and placed into a 1.5-ml Eppendorf tube containing 0.5 ml of formamide. The tissue remained in the formamide for 24 to 48 h at room temperature to extract Evans blue from the tissue. Duplicate 200-μl aliquots of
each extract were then pipetted into wells of a 96-well clear plastic plate. The concentration of dye in the extracts was determined spectrophotometrically by measuring absorbance at 620 nm. The concentration was interpolated from an Evans blue standard curve run simultaneously and was expressed as nanograms of Evans blue per milligram of trachea.

Ex Vivo ACE and NEP Activity

For evaluation of plasma ex vivo ACE activity, the blood collected by cardiac puncture was spun for 2 min at maximum speed in a microcentrifuge. Plasma was obtained, and 35 μl was added to a conical-bottomed 96-well plate with 5 μl of 1 M KCl, 0.5 M sodium borate, pH 8.3, and 3 μM zinc sulfate. Ten microliters of the supernatant was added in duplicate to a black fluorometric plate containing 100 μl of 0.1% methyl glucamine (catalog no. M2004; Sigma-Aldrich, St. Louis, MO) in 25% 2-hydroxypropyl-β-cyclodextrin (catalog no. 33,260-7; Aldrich Chemical Co., Milwaukee, WI) and 30% polyethylene glycol 200 (catalog no. P-3015; Sigma-Aldrich) vehicle. Apstatin (formerly Sigma-Aldrich catalog no. M2004; Sigma-Aldrich, St. Louis, MO) in 25% 2-hydroxypropyl-β-cyclodextrin (catalog no. 33,260-7; Aldrich Chemical Co., Milwaukee, WI) and 30% polyethylene glycol 200 (catalog no. P-3015; Sigma-Aldrich) vehicle. Apstatin (formerly Sigma-Aldrich catalog no. A-1395) was dissolved for intravenous administration in normal saline.

CP99994 was synthesized at the GlaxoSmithKline facility. Hoe 140 (icatibant) was purchased from Sigma-Aldrich. Both compounds were dissolved in normal (0.9%) saline. Evans blue dye was purchased from Sigma-Aldrich, and was prepared as a solution containing 15 mg/ml in heparinized (33 units/ml) normal saline.

Drugs, Peptides, and Reagents

GW796406, omapatrilat, and ecadotril were synthesized at the GlaxoSmithKline facility (GlaxoSmithKline, Uxbridge, Middlesex, UK) and dissolved for infusion or oral administration (ecadotril) in a vehicle consisting of 70% (100 mg/ml α-methyl glucamine (catalog no. M2004; Sigma-Aldrich, St. Louis, MO) in 25% 2-hydroxypropyl-β-cyclodextrin (catalog no. 33,260-7; Aldrich Chemical Co., Milwaukee, WI) and 30% polyethylene glycol 200 (catalog no. P-3015; Sigma-Aldrich) vehicle. Apstatin (formerly Sigma-Aldrich catalog no. A-1395) was dissolved for intravenous administration in normal saline.

In Vivo ACE and NEP Inhibition and Tracheal Plasma Extravasation. The low-dose protocol of GW796406X (3 mg/kg + 3 mg/kg/h) produced 78, 81, and 99% inhibition of plasma ACE, lung ACE, and kidney NEP, respectively, and it did not affect basal tracheal plasma extravasation in a group of four vehicle-treated rats (Fig. 4). The Evans blue content in the tracheal extracts for these animals was 6.2 ± 1.5 versus 6.9 ± 0.6 mg/mg tissue observed in four vehicle-treated rats. The low-dose protocol of omapatrilat was a bolus dose of 30 mg/kg followed by a 30 mg/kg/h infusion, and that used for GW796406 was a bolus dose of 25 mg/kg followed by a sustained infusion of 25 mg/kg/h (Fig. 3B). These doses were used as the high-dose regimen in the extravasation studies. Similarly, dose-ranging studies were conducted to identify a dose of each agent that produced a sustained 50% inhibition of the increase in blood pressure produced by an intravenous challenge of 300 ng/kg angiotensin I. This was achieved with a bolus dose of 0.1 mg/kg followed by a 0.1 mg/kg/h infusion of omapatrilat or a bolus dose of 3.0 mg/kg/kg followed by a 3.0 mg/kg/h infusion of GW796406 (Fig. 3A). These doses were used as the low-dose regimen in the extravasation studies. Similarly, dose-ranging studies were conducted to identify a dose of each agent that produced a minimally effective antihypertensive effect in conscious instrumented DOCA-salt hypertensive rat. The dose established for omapatrilat was a bolus dose of 30 mg/kg followed by a 30 mg/kg/h infusion, and that used for GW796406 was a bolus dose of 25 mg/kg followed by a sustained infusion of 25 mg/kg/h (Fig. 3B). These doses were used as the high-dose regimen in the extravasation studies.

GW5067X (3 mg/kg/h) produced 78, 81, and 99% inhibition of plasma ACE, lung ACE, and kidney NEP, respectively, and it did not affect basal tracheal plasma extravasation in a group of four vehicle-treated rats (Fig. 4). The Evans blue content in the tracheal extracts for these animals was 6.2 ± 1.5 versus 6.9 ± 0.6 mg/mg tissue observed in four vehicle-treated rats. The low-dose protocol of omapatrilat was a bolus dose of 30 mg/kg followed by a 30 mg/kg/h infusion, and that used for GW796406 was a bolus dose of 25 mg/kg followed by a sustained infusion of 25 mg/kg/h (Fig. 3B). These doses were used as the high-dose regimen in the extravasation studies.

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TABLE 1

In vitro inhibition of rat, rabbit, and human lung ACE and renal NEP by GW796406 and omapatrilat

<table>
<thead>
<tr>
<th>Species</th>
<th>ACE IC₅₀ nM</th>
<th>NEP IC₅₀ nM</th>
<th>ACE/NEP Ratio</th>
<th>ACE IC₅₀ nM</th>
<th>NEP IC₅₀ nM</th>
<th>ACE/NEP Ratio</th>
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</tr>
<tr>
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<td>1.6</td>
<td>2.9</td>
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produced similar inhibitions of both plasma and lung ACE activity; however, omapatrilat was a much less effective inhibitor of renal NEP than was GW796406 (49 versus 99%, respectively).

In view of the disparity in NEP inhibition produced by the two compounds, it was of interest to determine whether omapatrilat would affect tracheal plasma extravasation when imposed upon the same degree of NEP inhibition produced by GW796406. To accomplish this, a second study was performed in which rats were pretreated orally with a dose of 15 mg/kg ecadotril, a selective NEP inhibitor, 30 min before omapatrilat or GW796406 infusion. Under these conditions, NEP was inhibited by >95% in both groups. Infusion of omapatrilat in these rats produced 82 and 81% inhibition of plasma and lung ACE, respectively. Infusion of GW796406 produced 84 and 78% inhibition of plasma and lung ACE, respectively. These levels of ACE inhibition were virtually identical to those produced by these regimens of omapatrilat and GW796406 under ecadotril-free conditions. In ecadotril-treated rats, GW796406 again failed to produce an increase in tracheal Evans blue content (6.9 ± 1.6 ng/mg tissue); however, omapatrilat produced a significant increase in tracheal plasma extravasation, increasing tracheal Evans blue concentration to 20.8 ± 4.6 ng/mg tissue (Fig. 4). The increased plasma extravasation produced by omapatrilat in ecadotril-treated rats was significantly inhibited by CP99994, a neurokinin 1 (NK1) receptor antagonist, and Hoe 140, a bradykinin 2 (B2) receptor antagonist (Fig. 5).

In light of the relatively potent APP inhibition produced by omapatrilat, it was of interest to determine whether APP inhibition might be responsible for the greater extravasation liability of omapatrilat compared with GW796406 in spite of virtually identical inhibition of ACE and NEP. To explore this question, a group of four rats was pretreated with an i.v. dose of 0.8 mg/kg apstatin, an APP inhibitor, 5 min before administering the low-dose protocol of GW796406. Under these conditions, GW796406 once again did not significantly increase tracheal Evans blue concentration (8.7 ± 1.4 ng/mg tissue) (Fig. 6).

When administered at the high-dose protocol, GW796406 and omapatrilat produced virtually identical ACE and NEP inhibition and both agents significantly increased plasma extravasation. GW796406 (25 mg/kg bolus + 25 mg/kg/h) produced 82, 92, and 99% inhibition of plasma ACE, lung ACE, and renal NEP activity, respectively, and significantly increased tracheal Evans blue concentration to 32 ± 14 ng/mg tissue in a group of eight rats. Similarly, the high-dose protocol of omapatrilat (30 mg/kg bolus + 30 mg/kg/h) produced 81, 95, and 97% inhibition of plasma ACE, lung ACE, and renal NEP, respectively, and significantly increased tracheal Evans blue concentration to 31 ± 6 ng/mg tissue in a group of four rats (Fig. 7).
Discussion

Our in vitro enzyme inhibition studies demonstrated that omapatrilat and GW796406 were potent inhibitors of rat, rabbit, and human ACE and NEP. However GW796406 was different in that it displayed ~3-fold selectivity for NEP compared with omapatrilat, which displayed a similar degree of selectivity for ACE. This difference in selectivity, however small, was evident in the ex vivo enzyme inhibition data. Thus, at therapeutically equivalent ACE inhibitory doses, as defined as ability to inhibit angiotensin I to angiotensin II conversion by 50%, both compounds produced similar (~85%) inhibition of ex vivo ACE activity, but GW796406 virtually abolished NEP, whereas omapatrilat only partially inhibited NEP. This was predictable since inhibition of angiotensin I to angiotensin II conversion is likely a specific measure of ACE inhibition, and GW796406 would have greater effect on NEP than omapatrilat at this dose level.

The observation that GW796406 when administered at the low-dose infusion protocol did not increase plasma extravasation in spite of profound inhibition of ACE and NEP was interesting since we previously reported that ecadotril, an NEP inhibitor, markedly worsened the extravasation liability of the selective ACE inhibitors captopril and lisinopril (Sulpizio et al., 2004). To investigate whether, under conditions of NEP inhibition comparable with that produced by GW796406, omapatrilat also remained free of extravasation liability, we tested omapatrilat in rats pretreated with a highly efficacious dose of ecadotril. In these rats, NEP was inhibited by >95%, and omapatrilat now markedly increased plasma extravasation. The increase in extravasation was clearly a consequence NEP inhibition and not due to some
unknown effect of ecadotril, since ecadotril did not increase the extravasation liability of GW796406. In addition, the increased extravasation in the ecadotril/omapatrilat-treated rats was significantly blocked by both B2 receptor and NK1 receptor blockade, confirming a role for both bradykinin and substance P in the response. Thus, it was evident that, for some unexplained reason, GW796406 had inherently less liability to increase extravasation than did omapatrilat. Interestingly, GW796406 also had less liability to increase extravasation than did combinations of ACEIs and ecadotril, which significantly increased plasma extravasation in spite of much lower levels of ACE inhibition than that produced by GW796406 (Sulpizio et al., 2004). This difference between the two agents was not observed at the higher dose regimen. When omapatrilat or GW7967406 was administered at a higher therapeutically equivalent (minimally effective antihypertensive dose in DOCA-salt rat) dose, the compounds produced similar profound (>90%) inhibition of both ACE and NEP, and both compounds significantly increased plasma extravasation.

In an attempt to identify a mechanism that could explain the difference between omapatrilat and GW796406, we examined the activity of these compounds as inhibitors of additional enzymes reported to play a role in plasma extravasation. Two enzymes that have recently been implicated in ACEI-induced angioedema are APP (Adam et al., 2002; Molinaro et al., 2002a) and DPP IV (Lefebvre et al., 2002). Our data indicate that neither GW796406 nor omapatrilat possessed any significant inhibitory activity against DPP IV; however, omapatrilat was found to be a relatively potent inhibitor of APP, having an IC_{50} of 260 nM. In contrast, GW796406 produced essentially no inhibition of APP. Interestingly, the APP inhibitory potency that we found for omapatrilat is greater than that reported for other APP inhibitors (Maggiora et al., 1999), possessing more than 3-fold greater potency than the prototypic APP inhibitor apstatin. We suspected that the APP inhibition could be responsible for the increased extravasation liability of omapatrilat. Unexpectedly, however, we found that pretreatment with the APP inhibitor apstatin did not increase the extravasation liability of GW796406 in the rat model. This finding was interesting and suggested that the increased extravasation liability of omapatrilat does not involve APP inhibition. There is little question that APP has a major role in bradykinin metabolism in the rat when ACE is inhibited. Other investigators have demonstrated that in combination with an ACE inhibitor, APP inhibition produced a further increase in bradykinin-mediated hypotensive responses in the anesthetized rat (Kitamura et al., 1999) and bradykinin-induced vasodilation in isolated rat hearts (Dendorfer et al., 2000). Thus, the reason for the inability of apstatin to enhance GW796406 extravasation liability in our studies was not apparent. However, we were unable to measure APP activity in these rats ex vivo because the high (~40 mg/ml) concentration of albumin in plasma and tissue samples interfered with the APP assay. When we examined the effect of albumin on the assay, we found that a concentration of just 1 mg/ml produced more than 50% inhibition of the assay’s endpoint signal (M. A. Pullen, unpublished observation). Because of this limitation, we were unsure of the degree of APP inhibition achieved by apstatin treatment in our studies. The dose of apstatin that we used (800 μg/kg) was selected based on studies reported by Kitamura et al. (1999). In their studies, an 800 μg/kg intravenous dose of apstatin produced a >2-fold increase in the hypotensive response to bradykinin in rats that persisted from more than 4 h and markedly enhanced the hypotensive effect to bradykinin in lisinopril-treated rats.

In spite of our failure to implicate a role for APP in our rat model, we believe that APP inhibition may indeed have relevance to the increased incidence of angioedema observed for omapatrilat in clinical trials. Adam et al. (2002) reported that low plasma APP activity could be a predisposing factor for the development of angioedema in patients treated with ACE inhibitors. It seems then that, under conditions of ACE inhibition, the metabolism of bradykinin and perhaps substance P is assumed by non-ACE pathways, of which APP has been demonstrated to have the greatest importance. Patients with impaired APP activity may be unable to metabolize bradykinin at a rate sufficient to prevent bradykinin accumulation. Indeed, dramatically elevated bradykinin levels have been reported in patients suffering from hereditary and acquired, including ACEI-associated, angioedema (Nussberger et al., 1998; Molinaro et al., 2002b; Cugno et al., 2003). In light of this, it would seem logical to conclude that APP inhibitory activity is undesirable for a vasopeptidase inhibitor since any inhibition of APP imposed upon a background of ACE and NEP inhibition could render some patients with otherwise “normal” plasma APP levels at risk to develop angioedema.

We recognize that whenever a nonhuman species is used to model human disease caution must be used when predicting the clinical implications of the results. A valid criticism of the rat model of extravasation as a surrogate for angioedema is that only an early component of angioedema—increased vascular permeability—is present. The mechanisms responsible for the progression from increased vascular permeability to overt angioedema in susceptible patients remain speculative and are beyond the context of the current rat model. With that important limitation acknowledged, the mechanism driving the increased vascular permeability in both species clearly involves bradykinin. In most forms of human angioedema, including ACEI-induced and hereditary C1 inhibitor deficiency-mediated, markedly elevated plasma bradykinin levels have been observed (Cugno et al., 2003; Davis, 2005). Similarly, in the rat model, bradykinin is a potent inducer of plasma extravasation and ACEI-induced plasma extravasa-
tion is abolished by B2 receptor blockade. The rat model does allow us to evaluate the liability of test compounds to create a bradykinin-driven vascular environment that is conducive to the development of plasma extravasation. Thus, compounds that possess the greatest liability to cause plasma extravasation in the rat may have greater clinical liability to cause angioedema. However, the predictive value of the model will remain in question until additional clinical experience with the VPIs and related classes of compounds is gained.

In summary, our study demonstrated that GW796406 possessed less liability to increase plasma extravasation than omapatrilat under conditions of total NEP inhibition and submaximal levels of ACE inhibition in the rat. Most of the data generated to date suggest that a number of different enzymes are involved in the angioedema associated with ACE and vasopeptidase inhibitors. Our data in the rat model demonstrating that, under similar ACE and NEP inhibition, omapatrilat causes more plasma extravasation than GW796406 suggest that enzymes in addition to ACE and NEP may be involved.

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