Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 224-231

THE EFFECTS OF NOVEL CATHEPSIN E INHIBITORS ON THE BIG ENDOTHELIN PRESSOR RESPONSE IN CONSCIOUS RATS

J. Eileen Bird, Thomas L. Waldron, Deborah K. Little, Magdi M. Asaad, Charles R. Dorso, Gerald DiDonato and Jon A. Norman

Departments of Pharmacology, Analytical Research and Cardiovascular Biochemistry
Bristol-Myers Squibb Pharmaceutical Research Institute
Princeton, NJ

Received November 18, 1991

The aspartic protease, cathepsin E, has been shown to specifically cleave big endothelin (big ET-1) at the Trp^{21}-Val^{22} bond to produce endothelin (ET-1) and the corresponding C-terminal fragment. To determine whether cathepsin E is a physiologically relevant endothelin converting enzyme (ECE), three novel and potent inhibitors of cathepsin E were administered to conscious rats prior to a pressor challenge with big ET-1. One of the inhibitors of cathepsin E, SQ 32,056 (3 mg/kg i.v.), blocked the big ET-1 response. However, this dose of SQ 32,056 also blocked the pressor response to ET-1. Phosphoramidon specifically inhibited the Big ET-1 pressor response. These results suggest that ECE is not cathepsin E.

Endothelin (ET-1) is a 21 amino acid vasoconstrictor peptide first described by Yanagasawa *et al.* (1). From the cDNA sequence encoding the 203 amino acid precursor of ET-1, Yanagasawa *et al.* predicted that a 39 amino acid intermediate peptide must exist that would be further processed by cleavage at a Trp^{21}-Val^{22} bond by an endothelin converting enzyme (ECE) to yield ET-1. The 39 amino acid intermediate has been designated big ET and has only 0.5% to 1.0% of the *in vitro* activity of ET (2,3). However, the *in vivo* activity of big ET-1 is similar to ET-1 suggesting that cleavage of big ET-1 by ECE occurs under physiological conditions.

Matsumura *et al.* (4) first described the purification of an aspartic protease with cathepsin D-like activity that cleaved big ET-1 to produce immunoreactive ET-1. Subsequently, this aspartic protease activity was identified as cathepsin E and shown to cleave big ET-1 selectively at the Trp^{21}-Val^{22} bond (5). Ikegawa et al. (6) demonstrated that pepstatin, a general inhibitor of aspartic proteases, blocked the conversion of big ET-1 to ET-1 in endothelial cell extracts. Their results support the concept of cathepsin E functioning as ECE. However, Shields et al. (7) reported that intact endothelial cells secrete ET-1 in the presence of 0.1 μM pepstatin, suggesting that ECE is not an aspartic protease. Recently, a metalloprotease activity was described by McMahon et al. (8) that cleaved big ET-1 at the Trp^{21}-Val^{22} bond.

0006-291X/92 $1.50

Additionally, they showed that the nonspecific metalloprotease inhibitor, phosphoramidon, blocked a big ET-1 pressor response in anesthetized rats suggesting that ECE is a metalloprotease.

In the current study, three novel and potent inhibitors of cathepsin E are described which were used to further investigate the identity of ECE *in vivo*. Phosphoramidon, and SQ 28,603, a specific inhibitor of neutral endopeptidase (NEP) 24.11 (9), were also evaluated as inhibitors of ECE in conscious, normotensive rats.

Methods

Purified human cathepsin E was generously provided by Dr. M. Samloff, Univ. Calif., Los Angeles. The cleavage of big ET-1 by cathepsin E was demonstrated by incubating big ET-1 (60 μM) with 0.06 ml of cathepsin E (50 μg/ml) in 0.05 M sodium acetate, pH 5.0, containing 0.05 M NaCl for increasing time periods. The resulting peptide products were separated by reverse phase HPLC using a C18 Vydac column. The gradient was composed of 0.1% TFA (A) and acetonitrile containing 0.1% TFA (B) was run for 25 minutes from 10%B to 50% B. Absorbance at 215 nm was monitored and selected peaks were collected for analysis by ion spray mass spectrometry as previously described (10). Cathepsin E activity was also assayed in the above buffer using Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu as a substrate at a final concentration of 25 μM at 37 °C. Hydrolysis of the Phe-Nph bond was monitored by the decrease in absorbance at OD_{300} as previously described (11). IC_{50} values were determined by incubating increasing concentrations of the indicated inhibitor with the assay mix.

Male Sprague-Dawley rats (12 weeks) were prepared surgically according to the method of Weeks and Jones (12). Catheters (PE-10) were placed in the descending aorta and vena cava of anesthetized rats and extruded through the skin on the back of the neck. Two weeks later, rats were injected with a bolus of Big ET-1 (1.0 nmol/kg) to obtain a control pressor response (n=4/treatment group). When blood pressure returned to baseline, three consecutive i.v. doses of inhibitor or vehicle (45% ethanol plus equimolar HCl or 5% $NaHCO_3$) were given immediately preceding a challenge with Big ET-1. Phosphoramidon was given in 2.5% $NaHCO_3$ (1.0, 3.0, 10.0 mg/kg); and SQ 28,603 were given in 5% $NaHCO_3$ (3.0, 10.0, 30.0 mg/kg); and SQ 32,056, SQ 32,602, and SQ 32,285 (0.3, 1.0, 3.0 mg/kg) were given in 45% ethanol plus equimolar HCl.

The effects of SQ 32,056 and SQ 32,602 (3.0 mg/kg), phosphoramidon (10.0 mg/kg) and vehicles were examined on the pressor response induced by ET-1 (0.3 nmol/kg i.v.) (Peptides International), following a similar injection protocol as described above. The effect of SQ 32,056 (3.0 mg/kg) on the pressor response induced by norepinephrine (NE) (1.0 μg/kg) and angiotensin I (310 ng/kg i.v.) was also measured.

For the evaluation of NEP activity of SQ 28,603 *ex vivo*, male Sprague-Dawley rats (250-300 g) were dosed i.v. with 10, 30 or 100 μmol/kg SQ 28,603. The animals were killed at the indicated time intervals, by CO_2 asphyxia and cervical dislocation; four rats were used for each time point. The kidneys were isolated, decapsulated and placed in 20 ml of chilled 0.0625 M Tris-HCl buffer (pH 7.6) containing 0.1% Triton X-100, then homogenized. The kidney homogenates were diluted 200- to 250-fold with the assay buffer for the NEP assay, and 50 μl of the diluted homogenates was assayed. NEP activity was determined spectrophotometrically using the substrate Glutaryl-Ala-Ala-Phe-b-Naphthylamide according to the procedure described by Almenoff and Orlowski (13).

Results are expressed as mean ± SEM. The difference between the initial pressor response to Big ET-1 or ET-1 and the response after inhibitor was compared with the difference in vehicle-treated rats by analysis of variance with specific mean differences detected by contrasts. The difference between the initial NE-induced response and

the response after SQ 32,056 was compared to the difference in vehicle-treated rats by the unpaired t-test. The effect of each vehicle dose on the pressor response was compared to the initial response by paired t-test.

Results

Purified cathepsin E cleaved human big ET-1 at the Trp^{21}-Val^{22} bond to yield only endothelin and the corresponding C-terminal fragment. As shown in Figure 1, only 2 peptide peaks were evident after 4 hours of digestion by cathepsin E, labeled peak A and peak B. These peaks were identified by ion spray mass spectrometry. The experimental ion for peak A was 1807.49 which was in excellent agreement with the calculated ion of 1807.92. The assignment for this peptide was VNTPEHVVPYGLGSPRS, the C-terminal fragment. The experimental ion for peak B was 2495.90 which is 4 Daltons higher than the calculated ion of 2491.83 for endothelin 1. This was due to the reducing conditions used during sample preparation and analysis which reduced the 2 disulfide bonds and added 4 protons to endothelin.

The spectrophotometric substrate Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu was used to evaluate several tripeptidic statine-containing compounds as inhibitors of cathepsin E. The IC_{50} values for the 3 potent and novel compounds shown in Table 1 are SQ 32,056 5 nM; SQ 32,285 95 nM; and SQ 32,602 88 nM.

The inhibitor of Cathepsin E, SQ 32,056 significantly inhibited the pressor response induced by Big ET-1 (Control: 142±6% baseline MAP; 1.0 mg/kg: 111±5%; 3.0 mg/kg: 120±3%). These results are summarized in Figure 2. This compound was

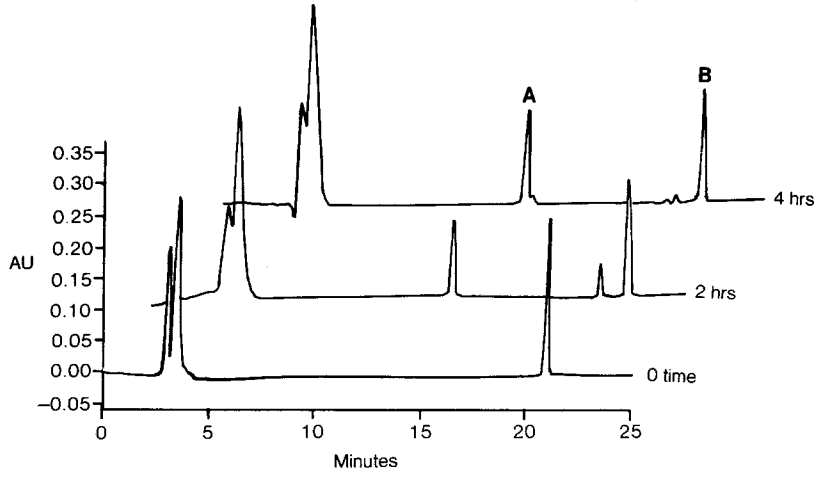
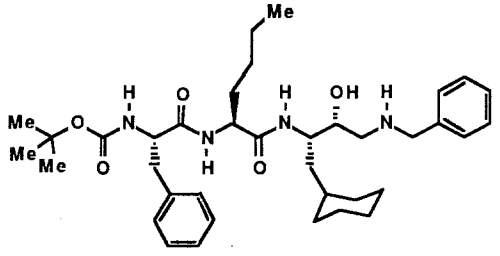
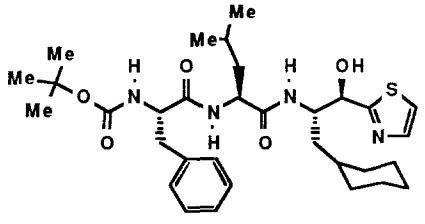
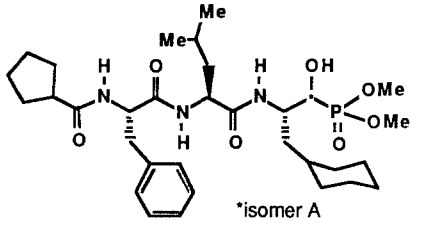


Figure 1. Digestion of big endothelin by cathepsin E. Big ET-1 was incubated with cathepsin E for the indicated times as described in methods. The two peptide products, labeled as peak A and peak B, were separated by reverse phase HPLC as described in methods. Absorbance at 215 nm was monitored and the resulting peptide peaks were recovered and analyzed by ion spray mass spectrometry.

Table 1. Structure and potency of 3 novel inhibitors of cathepsin E. Cathepsin E was assayed using Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu as a substrate as described in Methods. IC_{50} values represent the concentration of indicated inhibitor required to inhibit the decrease in OD_{300} by 50%.

	IC_{50} (nM)
SQ 32,056 	5
SQ 32,285 	95
SQ 32,602  *isomer A	88

more potent than phosphoramidon as an inhibitor of the Big ET-1-induced pressor response (ED_{50}=0.68 mg/kg i.v.). The response to Big ET-1 was potentiated after the lowest dose of SQ 32,056 (0.3 mg/kg: 158±13% baseline MAP) compared with vehicle-treated rats ($p<0.05$). The lowest dose of the Cathepsin E inhibitor, SQ 32,602 (0.3 mg/kg) inhibited the pressor response to Big ET-1 ($p<0.05$) when compared to the change in vehicle-treated rats, but the two higher doses (1.0, 3.0 mg/kg) had no significant effect. The pressor response to Big ET-1 was 124±3%, 143±8%, and 143±5% baseline MAP after the 3 doses of this compound. The third inhibitor of

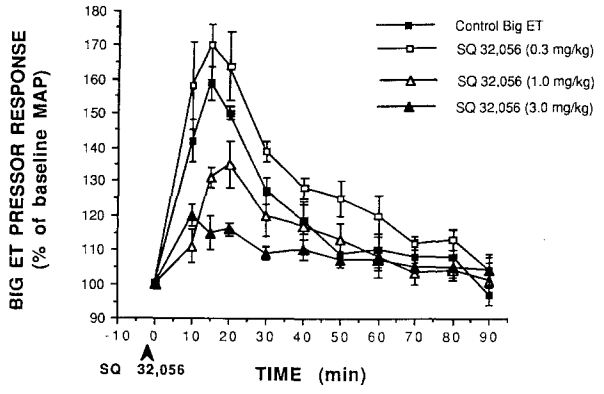
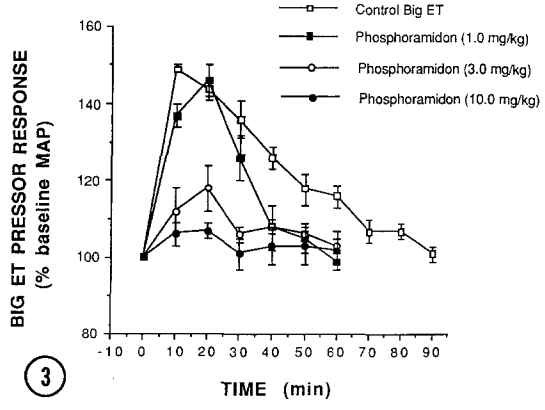


Figure 2. The effects of i.v. administration of the Cathepsin E inhibitor, SQ 32,056 on the pressor response induced by Big ET-1 (1.0 nmol/kg) were examined in conscious, normotensive rats (n=4). The control response was evaluated prior to dosing with SQ 32,056 at 0.3, 1.0, and 3.0 mg/kg. The pressor response is given as % of baseline mean arterial pressure.

Cathepsin E tested, SQ 32,285 did not significantly change the pressor response to Big ET.

Phosphoramidon inhibited the pressor response induced by Big ET-1 in a dose-dependent manner (3.0 mg/kg: 112±6% baseline MAP; $p<0.01$) when compared to vehicle-treated animals (Figure 3). After the 10.0 mg/kg dose of phosphoramidon,



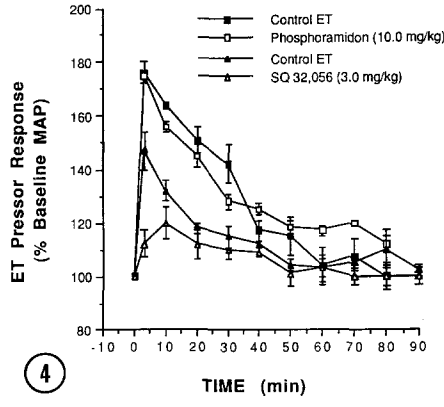


Figure 3. The effects of i.v. administration of phosphoramidon on the pressor response induced by Big ET-1 (1.0 nmol/kg) were examined in conscious, normotensive rats (n=4). The control response was evaluated prior to dosing with phosphoramidon at 1.0, 3.0 and 10.0 mg/kg. The pressor response is given as % of baseline mean arterial pressure.

Figure 4. The effects of i.v. administration of the Cathepsin E inhibitor, SQ 32,056 and of phosphoramidon on the pressor response induced by ET-1 (0.3 nmol/kg) were examined in 2 groups of conscious, normotensive rats (n=4/group). The control response to ET-1 is presented for each experimental group. The pressor response is given as % of baseline mean arterial pressure.

there was a 6% increase in baseline MAP in response to Big ET-1 which was significantly different from the change in vehicle-treated animals (ED_{50}=1.7 mg/kg). There was partial suppression of the pressor response to Big ET-1 after the 30 mg/kg dose of the NEP inhibitor, SQ 28,603, but the difference between the initial response to Big ET-1 and the response after SQ 28,603 (Δ=27.3±9.7% baseline MAP) and the difference in vehicle-treated rats (Δ=13.5±6.5%) did not reach statistical significance. SQ 28,603 inhibited renal NEP activity in a dose-related fashion *ex vivo*. Inhibition was maximal at 15 minutes after each dose (10 μmol/kg i.v. = 38.5 ± 0.6%; 30 μmol/kg = 69.4 ± 1.5%; 100 μmol/kg = 86.4 ± 1.8%). One hour after administration of all doses of SQ 28,603, NEP was inhibited by approximately 30-40% and remained constant for three hours. Thus the dose of SQ 28,603 used in our *in vivo* studies was sufficient to inhibit renal NEP *ex vivo* by 86%.

The Cathepsin E inhibitor, SQ 32,056 (3.0 mg/kg) also inhibited the peak pressor response to ET-1 (112±5% baseline MAP) when compared to vehicle-treated rats (Figure 4). Phosphoramidon (10 mg/kg) had no significant effect on the ET-1-induced pressor response. There was no hypotension noted during the first 90 min after phosphoramidon (10 mg/kg) or SQ 32,056 (3.0 mg/kg) in control, normotensive rats. The pressor response to norepinephrine (149±6% baseline MAP), but not angiotensin I, was inhibited by SQ 32,056 (115±1%) when compared to the change in vehicle-treated rats.

Discussion

The preparation of cathepsin E used in the current studies cleaved big ET-1 only at the Trp^{21}-Val^{22} bond as previously demonstrated by Lees, et al. (5). Mass spectrometry was used to identify the two peptide products generated after extensive proteolysis in this study and gave the same result as previously reported using protein sequencing techniques (8). The pH optimum was for this preparation of cathepsin E was 3.5 and the enzyme had no detectable activity above pH 6.0 regardless of the presence of ATP at a concentration of 6.5 mM. Consequently, all of the inhibitors were identified and their IC_{50} determination done at pH 5.0.

The 3 inhibitors identified under these assay conditions had several common features. All of the compounds were tripeptide analogs with different functionalities at the N-terminus and C-terminus. These inhibitors were Phenylalanyl-Leucyl-Cyclohexylphenylalanyl derivatives with the exception of SQ 32,056, the most potent of the series, which had a norleucine replacement for leucine. The N-terminus was acylated with tertiary butyloxycarbonyl (t-boc) as in SQ 32,056 or SQ 32,285 or cyclopentanecarbonyl in the case of SQ 32,602. The C-terminal functionality is varied with each compound: SQ 32,056, an amino alcohol (14); SQ 32,285, a hydroxythiazole (15) and SQ 32,602 a hydroxyphosphonate (16).

The three novel and potent inhibitors of cathepsin E identified in this study were used to investigate the *in vivo* role of cathepsin E in the conversion of big ET-1 to ET-1. SQ 32,056 inhibited the pressor response to Big ET-1 in conscious rats which is consistent with the hypothesis that ECE is a Cathepsin E-like enzyme. However, SQ 32,056 also inhibited the pressor response induced by ET-1 and NE indicating either a lack of specificity for ECE, or a lack of ECE inhibitory activity. The mechanism of inhibition of response to the different pressor agents is unclear. SQ 32,056 did not exert hypotensive effects in control rats suggesting that it is not a non-specific vasodilator. These results coupled with the lack of consistent inhibition of the Big ET-1-induced pressor response by the other Cathepsin E inhibitors suggest that Cathepsin E is not the physiologically relevant endothelin-converting enzyme in spite of its ability to specifically cleave big ET-1 at the Trp^{21}-Val^{22} bond *in vitro*.

Phosphoramidon significantly inhibited the peak pressor response induced by Big ET-1 but not ET-1 confirming reports by other investigators that phosphoramidon inhibited ECE in anesthetized rats (8,17) and in cultured cells (18). SQ 28,603, the specific inhibitor of NEP 24.11, had no significant effect on the Big ET-1 pressor response. Pollock has reported that an infusion of the NEP inhibitor, thiorphan did not inhibit the cardiovascular or renal effects of Big ET-1 in anesthetized rats (19). McMahon has reported an ED_{50} for thiorphan for inhibition of the Big ET-1-induced pressor response of approximately 60 mg/kg, i.v. in ganglion-blocked anesthetized rats (8). It is possible that a higher dose of SQ 28,603 might have a significant inhibitory effect on ECE in our conscious rat model, but our *ex vivo* results suggest that the highest dose used in this study was effective for inhibition of renal NEP 24.11. These results suggest that ECE may be a metalloprotease different from NEP 24.11.

In summary, we have shown for the first time that novel, potent inhibitors of Cathepsin E *in vitro* are not specific, potent inhibitors of the Big ET-1-induced pressor response *in vivo*. These results in conscious rats confirm previous reports that ECE is a phosphoramidon-sensitive metalloprotease, which appears to be different from NEP 24.11 and suggest that ECE is not the aspartic protease, Cathepsin E.

References

1. Yanigasawa M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki. (1988) Nature 332, 411-415.
2. Kimura S., Y. Kasuya, T. Sawamura, O. Shinmi, Y. Sugita, M. Yanigasawa, K. Goto, and T. Masaki. (1989) J. Cardiovascular Pharmacol. 13 (Suppl 5), S5.
3. Fukuroda T., K. Noguchi, S. Tsuchida, M. Nishikibe, F. Ikemoto, K. Okada, and M. Yano. (1990) Biochem. Biophys. Res. Comm. 172, 390-395.
4. Matsumura Y., R. Ikegawa, M. Takaoka and S. Morimoto. (1990) Biochem Biophys Res Commun. 167, 203-210.
5. Lees W.E., S. Kalinka, J. Meech, SJ Capper, ND Cook and J. Kay. (1991) FEBS Lett. 273, 99-102.
6. Ikegawa R., Y. Matsumura, M. Takaoka and S. Morimoto. (1990) Biochem. Biophys. Res. Commun. 167, 860-866.

7. Shields P.P., T.A. Gonzales, D. Charles, J.P. Gilligan and W. Stern (1991) Biochem Biophys. Res. Commun. 177, 1006-1012.
8. McMahon E.G., M.A. Palomo, W.M. Moore, J.F. McDonald, and M.K. Stern. (1991) Proc. Natl. Acad. Sci. 88, 703-707.
9. Seymour A.A., J.A. Norman, M.M. Asaad, S.A. Fennell, B. Abboa-Offei, D.K. Little V.J. Kratunis, N.G. Delaney, J.T. Hunt, G. DiDonato. (1991) J. Pharmacol. Exp. Ther. 256, 1002-1009.
10. Norman J.A., D.K. Little, M. Bolgar and G.. DiDonato (1991) Biochem. Biophys. Res. Comm. 175, 22-30.
11. Norman J.A., D.K. Little, C.A. Free, T. Dejneka, H. Weber and D.E. Ryono. (1989) Biochem. Biophys. Res. Comm. 161, 1-7.
12. Weeks J.R. and J.A. Jones. (1960) Proc. Soc. Exptl. Biol. Med. 104, 646-649.
13. Almenoff J. and M. Orlowski. (1983) Biochemistry 22, 590-599.
14. Natarajan S., C.A. Free, F.F. Sabo, J. Lim, E.R. Spitzmiller, S.G. Samaniego, S.A. Smith and L.M. Zanoni. (1988) Proc. 10th Am. peptide symposium, peptides, chemistry and biology, p131.
15. Ryono D.E., H.N. Weller, C.A. Free, J.M. DeForrest, E.R. Spitzmiller, T. Dejneka, E.F. Sabo, M.B. Rom, S.G. Samaniego, S. Smith, R.S. Oehl and R.J Scalese. (1988) National medicinal chemistry symposium. poster presentation.
16. Patel D.V., K. Rielly-Gauvin and D.E. Ryono. (1990) Tetrahedron Lett. 31, 5587-5590.
17. Matsumura Y., K. Hisaki, M. Takaoka, and S. Morimoto. (1990) Eur. J. Pharmacol. 185, 103-106.
18. Takada J., K. Okada, T. Ikenaga, K. Matsuyama, and M. Yano. (1991) Biochem. Biophys. Res. Commun. 176 (2), 860-865.
19. Pollock D.M., and T.J. Opgenorth. (1991) Am. J. Physiol. 261, R257-R263.