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EFFECTS OF METALLOPROTEASE INHIBITORS ON SMOOTH MUSCLE ENDOTHELIN-CONVERTING ENZYME ACTIVITY

JOSEPH L. BALWIERCZAK, PAIVI J. KUKKOLA, PAULA SAVAGE and ARCO Y. JENG*

Research Department, Pharmaceuticals Division, Ciba-Geigy Corporation, Summit, NJ 07901, U.S.A.

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Abstract—The enzyme responsible for the conversion of exogenous big endothelin-1 to endothelin-1 by porcine coronary arterial smooth muscle has been shown to be a metalloprotease. The potencies of eight metalloprotease inhibitors for this endothelin-converting enzyme were determined. CGS 25015, CGS 26129, and thiorphan inhibited the enzyme activity monophasically with IC_{50} values of 2.6, 2.4, and 190 μM, respectively. In contrast, the data obtained using phosphoramidon as an inhibitor were best fit by a two-site model. The biphasic concentration–response curve had IC_{50} values of 4.6 μM and 2.2 mM. Three analogs of phosphoramidon were also tested for enzyme inhibition. Removal of the rhamnose moiety of phosphoramidon reduced the potency (IC_{50} = 15 μM), whereas substitution of the rhamnose by *N*-[2-(2-naphthyl)ethyl] improved the potency (IC_{50} = 2.0 μM). These results identify a thiol and a phosphonyl series of compounds as smooth muscle endothelin-converting enzyme inhibitors. The structure–activity relationships revealed that an aromatic or aliphatic group in the P_2' position or an aromatic group in the P_1 position of the inhibitor significantly increased the potency.

Key words: endothelin-converting enzyme; metalloprotease inhibitors; phosphoramidon; vascular smooth muscle

ET-1† is a potent, peptidic vasoconstrictor originally isolated from the conditioned medium of porcine aortic endothelial cells [1]. It has been shown that the conversion of a precursor form of ET-1, big ET-1, to ET-1 by a putative ECE is essential for expression of full biological activity of the mature peptide [2]. Therefore, extensive research has been focused on the identification of this enzyme, and three classes of proteolytic enzyme have been demonstrated to possess ECE activity, viz. aspartyl [3–5], thiol [6], and metallo [7–9] proteases.

Subsequent studies have demonstrated that a metalloprotease may be responsible for the conversion of big ET-1 to ET-1 *in vivo*. Fractionation of cultured vascular endothelial cells reveals two types of metallo ECE activities. These include a membrane-bound, phosphoramidon-sensitive enzyme and a cytosolic enzyme that is insensitive to phosphoramidon [9]. Since phosphoramidon has been shown to suppress the production of ET-1 in cultured endothelial cells [8] and to block the pressor response induced by intravenously administered big ET-1 in the rat [10], the membrane-bound metalloprotease has been suggested to be the physiologically relevant ECE. The location of this enzyme in the cell membrane has led investigators to hypothesize that ECE converts big ET-1 extracellularly [11], although evidence for an intracellular membrane-bound ECE has also appeared [12].

The secretion of ET-1 by endothelial cells is a polarized event. Wagner *et al.* [13] examined the directionality of ET-1 release by human umbilical vein endothelial cells cultured on amniotic membranes and demonstrated that about 80% of ET-1 is found in the basolateral compartment. These results imply that big ET-1 may be processed by a membrane-bound ECE in the extracellular space between endothelial and smooth muscle cells.

In addition to endothelial cells, vascular smooth muscle cells also have been shown to convert exogenous big ET-1 to ET-1 [14], and a phosphoramidon-sensitive, membrane-bound protease is responsible for this conversion [15]. Using smooth muscle preparations of denuded porcine coronary arterial strips, we have developed a simple assay for the measurement of conversion of big ET-1 to ET-1 [16]. The K_m value for this conversion is similar to that reported using human umbilical vein endothelial cells or bovine aortic endothelial cells [17]. Thus, smooth muscle ECE may be as important as endothelial ECE in processing big ET-1 released by endothelial cells. In this paper we report the effects of various structurally dissimilar metalloprotease inhibitors on the conversion of big ET-1 to ET-1 by smooth muscle ECE.

* Corresponding author: Dr. Arco Y. Jeng, Ciba-Geigy Corp. (LSB-3129), 556 Morris Avenue, Summit, NJ 07901. Tel. (908) 277–5924; FAX (908) 277–4739.

† Abbreviations: ET-1, endothelin-1; and ECE, endothelin-converting enzyme.

MATERIALS AND METHODS

Materials. Porcine big ET-1 and [^{125}I]ET-1 were

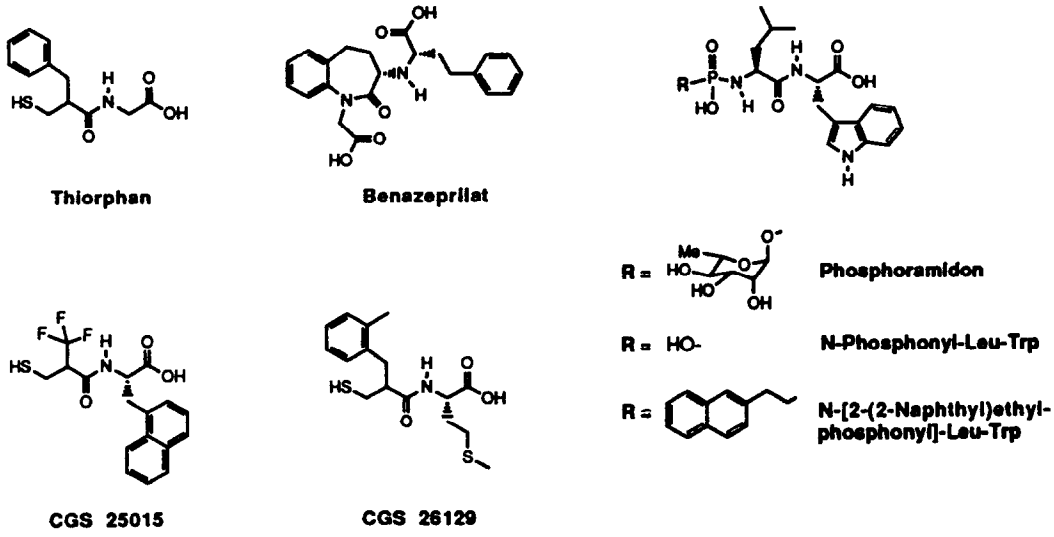

Fig. 1. Structures of metalloprotease inhibitors used in this study.

purchased from American Peptide (Santa Clara, CA) and New England Nuclear (Boston, MA), respectively. Phosphoramidon was a product of Sigma (St. Louis, MO). Leu-Trp was obtained from Research Plus (Bayonne, NJ). Thiorphan, CGS 25015 (α-[*N*-[1-oxo-3-thio-2-(trifluoromethyl)-propyl]-amino]-1-naphthalenepropanoic acid), and benazeprilat were synthesized at the Ciba-Geigy Corp. (Summit, NJ). Goat anti-rabbit antibodies coupled to magnetic beads were obtained from Ciba-Corning (East Walpole, MA).

Chemical synthesis. *N*-Phosphonyl-Leu-Trp was synthesized as the Tris sodium salt. Briefly, 87 mg (0.65 mmol) of *N*-chlorosuccinimide in 2 mL of toluene was treated with 144 μL (0.65 mmol) of dibenzylphosphite at room temperature under N_2 atmosphere. The reaction mixture was warmed occasionally to 50°, and after 3 hr the supernatant was cannulated to an ice-cold solution containing 204 mg (0.5 mmol) of Leu-Trp-OBzl and 91 μL (0.65 mmol) of tricthylaminc in 5 mL of dichloro-methane. After stirring at 0° for 1 hr and then at room temperature for 16 hr, the reaction mixture was partitioned between ethyl acetate and saturated $NaHCO_3$. The organic phase was washed with brine, dried over anhydrous $MgSO_4$, and concentrated. Chromatography on 10 g silica gel (eluant, 1:2 EtOAc:hexane) gave 200 mg of *N*-(*O*-dibenzyl-phosphonyl)-Leu-Trp-OBzl as a colorless oil (60% yield). To synthesize *N*-phosphonyl-Leu-Trp, 80 mg (0.12 mmol) of the above compound was mixed with 30 mg (0.36 mmol) of anhydrous $NaHCO_3$ and 20 mg of 10% palladium on carbon in 10 mL of 90% methanol saturated with H_2 for 3 hr at room temperature. The catalyst was removed by filtration through celite. The filtrate was concentrated, redissolved in a small volume of methanol, and precipitated by addition of ether. About 40 mg of *N*-phosphonyl-Leu-Trp, Tris sodium salt, was obtained (71% yield).

CGS 26129 (*N*-[3-mercapto-2(*R*)-(2-methyl-phenyl)methyl-1-oxopropyl]-L-methionine) and *N*-[2-(2-naphthyl)ethyl-phosphonyl]-Leu-Trp were synthesized according to published procedures [18, 19].

Smooth muscle ECE assay. Enzyme assay was performed according to previously published procedures [16]. Porcine hearts were obtained from a local abattoir. Coronary arteries were isolated and placed in a petri dish containing oxygenated Krebs buffer. Excess fat and connective tissue were removed, and the coronary arteries were cut into 4-mm ring segments and denuded by rubbing with a wooden applicator stick. The ring segments were then cut open to form strips.

Tissue strips were incubated at 37° in 200 μL of PBS containing 5 μM big ET-1, and 20-μL aliquots of the reaction mixture were withdrawn every 15 min up to 1 hr for the measurement of ET-1 production. After the 1-hr incubation period, the tissues were rinsed with PBS and blotted. To determine the effects of inhibitors on the conversion of big ET-1 to ET-1, the tissues were preincubated subsequently with desired concentrations of inhibitors in PBS at 37° for 20 min prior to the addition of big ET-1 to the incubation medium. The final concentration of big ET-1 was 5 μM. Aliquots of the reaction mixture were withdrawn as described above for the measurement of ET-1 production in the presence of a metalloprotease inhibitor. In some experiments, the tissues were washed again and incubated for a third time with 5 μM big ET-1 in the absence of inhibitors to determine if the inhibitory effects were reversible.

Measurement of ET-1. ET-1 was quantitated by a radioimmunoassay (RIA) using rabbit antibodies that recognize specifically the carboxyl terminal tryptophan of ET-1. Samples obtained from the smooth muscle ECE assay were diluted to 200 μL with an RIA buffer containing 0.1% Triton X-100, 0.2% bovine serum albumin, and 0.02% NaN_3 in

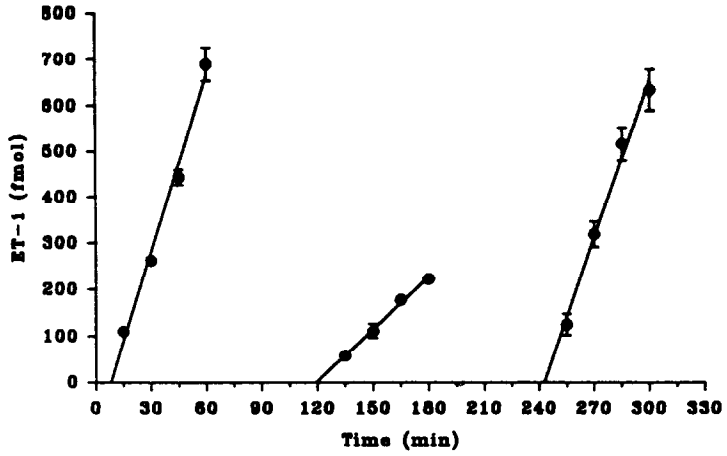

Fig. 2. Reversible inhibition of smooth muscle ECE activity by CGS 26129. Each tissue strip was incubated with 5 μM big ET-1, and the rate of ET-1 production was determined during the first hour to serve as the control. The tissues were then rinsed with PBS, blotted, and treated with 100 μM CGS 26129 for 20 min prior to the addition of 5 μM big ET-1 for the measurement of the rate of ET-1 production. Subsequently, these tissues were washed again, and the rate of ET-1 production was again measured in the absence of the inhibitor. Each data point represents the mean ± SEM (N = 6), and the lines were generated by linear regression. The rates of ET-1 production, measured in chronological order, were 12.8, 3.7, and 11.5 fmol/min.

PBS and incubated at 4° overnight with 25 μL each of [^{125}I]ET-1 (10,000 cpm/tube) and 1:20,000-fold diluted antibodies. Goat anti-rabbit antibodies coupled to magnetic beads (70 μg) were then added to each tube, and the reaction mixture was incubated further for 30 min at room temperature. The beads were pelleted using a magnetic rack. The supernatant was decanted, and the radioactivity in the pellet was counted in a gamma counter. Total and nonspecific binding were measured in the absence of nonradioactive ET-1 and anti-ET antibodies, respectively. Under these conditions, ET-1 and big ET-1 displaced [^{125}I]ET-1 binding to the antibodies with IC_{50} values of 21 ± 2 and 260,000 ± 66,000 fmol (mean ± SEM, N = 3–5), respectively [20].

Data analysis. The time-dependent conversion of big ET-1 to ET-1 was analyzed by linear regression. Inhibition of smooth muscle ECE activity was calculated by comparing the rates of ET-1 production in the presence and absence of the metalloprotease inhibitors using the same coronary arterial strip. At least three tissue segments, each from different hearts, were used for every inhibitor concentration in each experiment, and the full concentration–response curve of each inhibitor was repeated 2–3 times.

An IBM-compatible version of ALLFIT was used to fit data to a one-site model [21], and an iterative nonlinear least-squares fit was used to analyze data for a two-site model [22]. Statistical significance of a two-site fit was determined from an F value calculated using the variance of the data [23].

RESULTS

The rates of conversion of big ET-1 to ET-1 by different strips of denuded porcine coronary artery varied significantly. However, repeated incubations of the same tissue with an identical concentration of big ET-1 yielded similar rates of conversion over a period of 4 hr [16]. This characteristic allowed the determination of a control rate of ET-1 production by a single smooth muscle strip followed by measurement of the rate under various conditions. The effects of metalloprotease inhibitors on the conversion of big ET-1 were investigated using this protocol. These inhibitors included thiorphan, CGS 25015, CGS 26129, benazeprilat, and phosphoramidon analogs (Fig. 1). As an example of our experimental procedure, the effect of CGS 26129 on the conversion of big ET-1 is shown in Fig. 2. The initial control rate of ET-1 production, measured

Table 1. Summary of the potencies of the metalloprotease inhibitors in the smooth muscle ECE assay

Inhibitor	IC_{50} (μM)	N
Benazeprilat	>1000	6
CGS 25015	2.6 ± 0.4*	6
CGS 26129	2.4 ± 1.5	6
Thiorphan	190 ± 83	6
Leu-Trp	>3000	6
NEP-Leu-Trp	2.0 ± 0.4	6
N-Phosphonyl-Leu-Trp	15 ± 4.4	4–6
Phosphoramidon (site 1)	4.6 ± 1.1	5–9
(site 2)	2200 ± 960	

* Mean ± SEM (N).

The IC_{50} values for phosphoramidon were obtained from a two-site fit, while those of other inhibitors were obtained from a one-site fit. NEP-Leu-Trp, *N*-[2-(2-naphthyl)ethylphosphonyl]-Leu-Trp.

between 0 and 60 min, was 12.8 fmol/min. In the presence of 100 μM CGS 26129, the rate of ET-1 production decreased to 3.7 fmol/min (measured between 120 and 180 min in Fig. 2), a 71% inhibition of the smooth muscle ECE activity. After the tissue was rinsed, the production of ET-1 was measured again in the absence of the inhibitor during the 240- to 300-min period. A rate of 11.5 fmol/min was obtained, similar to the initial control value. Thus, this protocol not only enabled the evaluation of the inhibitory activity of a compound at a specific concentration, but also could determine whether or not the inhibition was reversible. Under these conditions, the inhibition of smooth muscle ECE by all metalloprotease inhibitors tested was readily reversible (results not shown).

The concentration–response curves for the metalloprotease inhibitors in the smooth muscle ECE assay are shown in Fig. 3. Benazeprilat did not display any inhibitory activity even at a concentration as high as 1 mM. CGS 25015, CGS 26129, and thiorphan inhibited ECE activity monophasically with IC_{50} values of 2.6, 2.4, and 190 μM, respectively (Table 1). In contrast, the data obtained using phosphoramidon as an inhibitor were best fit by a two-site model. The biphasic concentration–response relationship had IC_{50} values of 4.6 μM and 2.2 mM. Removal of the rhamnose moiety of phosphoramidon reduced its potency to 15 μM, whereas substitution of the rhamnose by *N*-[2-(2-naphthyl)ethyl] improved the potency to 2.0 μM. Interestingly, these two analogs of phosphoramidon inhibited ECE in a monophasic manner (Fig. 3B). The phosphonyl group was found to be essential for inhibitory activity, since the dipeptide Leu-Trp only inhibited ECE activity by 38% at a concentration of 3 mM (Fig. 3B).

DISCUSSION

ET-1 is a potent vasoconstrictor and has been implicated in the pathogenesis of various disease states such as acute myocardial infarction, pulmonary hypertension, essential hypertension, congestive heart failure, and acute renal failure [24]. The posttranslational processing of this peptide has been elucidated; preproendothelin-1 is first cleaved by dibasic-pair-specific endopeptidases to generate big ET-1, which is subsequently cleaved by ECE to produce ET-1 [1]. Therefore, inhibition of ECE is expected to decrease the production of ET-1 and could be beneficial for diseases where ET-1 plays a pathogenic role.

We previously developed a simple assay for the measurement of conversion of big ET-1 to ET-1 by smooth muscle [16]. The K_m value for this conversion was found to be 32 μM, a concentration that is substantially higher than the 2 pM big ET-1 determined in normal human plasma [25]. At a glance, these results seem to suggest that the concentration of circulating big ET-1 is too low for an effective processing by ECE. However, since big ET-1 may be packaged in secretory vesicles and then converted by ECE, which is located either in the membrane of these vesicles or at the plasma membrane surface of endothelial cells [11], the local concentration of big ET-1 in the vicinity of ECE may be significantly greater than the K_m value for big ET-1 conversion. As a comparison, high concentrations of neuropeptides in secretory vesicles have been observed; they range from about 1 mM for cholecystokinin and dynorphin peptides to about 100 mM for oxytocin [26]. Further research on the immunolocalization of ECE and big ET-1 will help clarify this matter.

In this study, the effects of two chemical series of metalloprotease inhibitors on vascular smooth muscle ECE activity were examined. These are phosphoramidon analogs and thiol-containing compounds, the latter being represented by thiorphan, CGS 25015, and CGS 26129. The inhibition of ECE activity exerted by these two classes of inhibitors was reversible. An unexpected finding was that the data obtained using phosphoramidon as an inhibitor of ECE were best fit with a two-site model, while the thiol-containing compounds and phosphoramidon analogs inhibited ECE activity monophasically. The reason for this biphasic inhibition by phosphoramidon is not clear at present. This compound has been shown to inhibit the partially purified ECE from porcine aortic endothelial cells in a monophasic manner [27]. Thus, ECE from smooth muscle may have different characteristics when compared with the endothelial enzyme.

Another difference between smooth muscle and endothelial ECE is revealed by the structure–activity relationships of the inhibitors. It has been shown that phosphoramidon, CGS 25015, and CGS 26129 inhibit endothelial ECE activity with IC_{50} values of 4, 18, and 58 μM, respectively [27]. However, these three compounds inhibit smooth muscle ECE activity almost equipotently with IC_{50} values between 2.4 and 4.6 μM (Table 1). These results show that the smooth muscle enzyme is less selective toward the thiol- and phosphonyl-containing inhibitors than the endothelial enzyme. Nevertheless, both enzymes are clearly distinguished from other well characterized metalloproteases such as angiotensin converting enzyme and neutral endopeptidase 24.11, because potent inhibitors for the latter two enzymes, benazeprilat and thiorphan [28, 29], had little or no effect on smooth muscle and endothelial ECE activities (Table 1 and Ref. 27).

The conversion of big ET-1 to ET-1 by smooth muscle seems to be carried out by more than one type of enzyme. Figure 3 shows that CGS 25015, CGS 26129, thiorphan, or analogs of phosphoramidon could inhibit at most 80% of big ET-1 conversion by smooth muscle, whereas Z-Phe-AlaCHN_2, a specific thiol protease inhibitor, has been shown to inhibit this conversion slightly [16]. When the concentration–response curves for metalloprotease inhibitors were determined in the presence of 0.5 mM Z-Phe-AlaCHN_2, a complete inhibition of big ET-1 conversion was observed (data not shown). Therefore, about 20% of the conversion of big ET-1 may be mediated by a thiol protease in smooth muscle preparations. In contrast, endothelial ECE activity is entirely inhibitable by metalloprotease inhibitors [27]. The physiological significance of this thiol ECE remains to be investigated.

A comparison of the structure–activity relation-

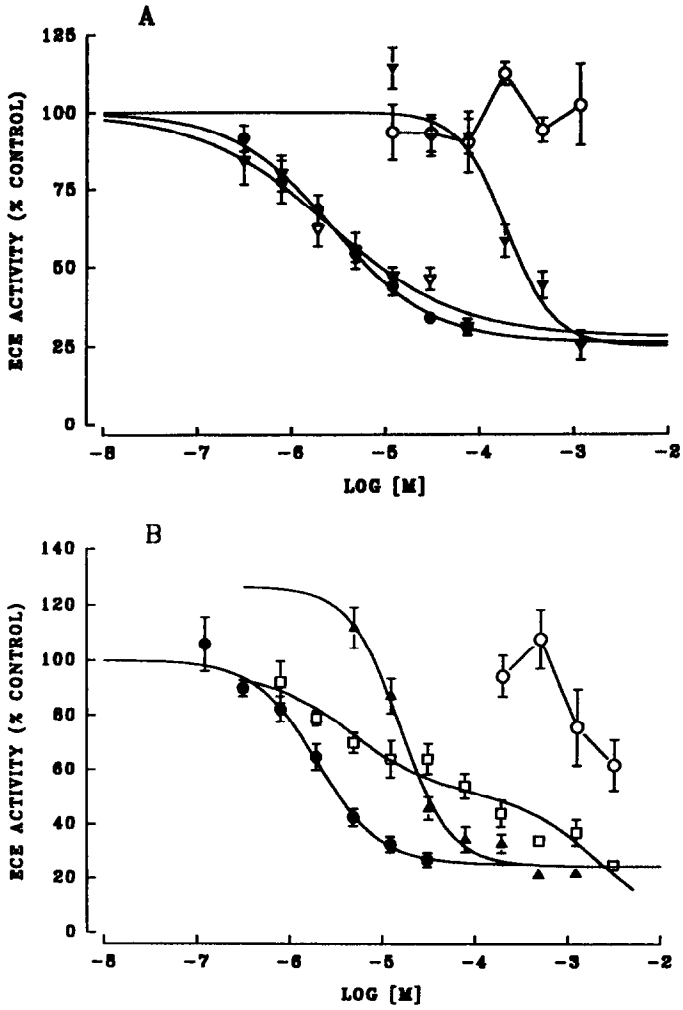

Fig. 3. Concentration-dependent inhibition of smooth muscle ECE activity by metalloprotease inhibitors. Control ECE activity was 14.3 ± 0.5 fmol/min (mean ± SEM, N = 21). Each data point represents the mean ± SEM. The symbols are: (A) o, benazeprilat (N = 6); ●, CGS 25015 (N = 6); ▽, CGS 26129 (N = 6); and ▼, thiorphan (N = 6); and (B) ●, *N*-[2-(2-naphtyl)ethyl-phosphonyl]-Leu-Trp (N = 6); ▲, *N*-phosphonyl-Leu-Trp (N = 4–6); □, phosphoramidon (N = 5–9); and o, Leu-Trp (N = 6). The data obtained using benazeprilat and Leu-Trp were simply connected by line segments. A one-site model was used to fit data generated by CGS 25015, CGS 26129, thiorphan, *N*-[2-(2-naphthyl)ethyl-phosphonyl]-Leu-Trp, and *N*-phosphonyl-Leu-Trp, whereas results obtained using phosphoramidon were fit by a two-site model.

ships of the thiol-containing compounds revealed that an aromatic group (in the case of CGS 25015) or an aliphatic group (in the case of CGS 26129) in the P_2' position significantly increased the potency for inhibition of smooth muscle ECE activity. Likewise, phosphoramidon, which contains an indole group in the P_2' position, showed similar potency when compared with that of CGS 25015 and CGS 26129. These results suggest that smooth muscle ECE may have a hydrophobic pocket at the S_2' subsite. Removal of the rhamnose moiety of phosphoramidon decreased the potency of the parent compound only slightly (3-fold) (Table 1). This is consistent with the finding that the rhamnose moiety of phosphoramidon is not required for inhibition of the big ET-1-induced pressor response in anesthetized rats [30]. Table 1 also shows that an aromatic substitution of the rhamnose group of phosphoramidon improved the inhibitory activity, indicating that there may be an additional hydrophobic pocket at the S_1 subsite of the enzyme. The information generated from this study will aid in the design of specific inhibitors for smooth muscle ECE.

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