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Pharmacological Characterization of a Novel Sulfonylureid-Pyrazole Derivative, SM-19712, a Potent Nonpeptidic Inhibitor of Endothelin Converting Enzyme

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ABSTRACT—We describe the pharmacological characteristics of SM-19712 {4-chloro-*N*-[[(4-cyano-3-methyl-1-phenyl-1*H*-pyrazol-5-yl)amino]carbonyl]benzenesulfonamide, monosodium salt}. SM-19712 inhibited endothelin converting enzyme (ECE) solubilized from rat lung microsomes with an IC_{50} value of 42 nM and, at 10 – 100 μM, had no effect on other metalloproteases such as neutral endopeptidase 24.11 and angiotensin converting enzyme, showing a high specificity for ECE. In cultured porcine aortic endothelial cells, SM-19712 at 1 – 100 μM concentration-dependently inhibited the endogenous conversion of big endothelin-1 (ET-1) to ET-1 with an IC_{50} value of 31 μM. In anesthetized rats, either intravenous (1 – 30 mg/kg) or oral (10 – 30 mg/kg) administration of SM-19712 dose-dependently suppressed the pressor responses induced by big ET-1. In acute myocardial infarction of rabbits subjected to coronary occlusion and reperfusion, SM-19712 reduced the infarct size, the increase in serum concentration of ET-1 and the serum activity of creatinine phosphokinase. The present study demonstrates that SM-19712 is a structurally novel, nonpeptide, potent and selective inhibitor of ECE, and SM-19712 is a valuable new tool for elucidating the pathophysiological role of ECE.

Keywords: Endothelin, Big endothelin, Endothelin converting enzyme, Phosphoramidon, Acute myocardial infarction

Endothelin-1 (ET-1), a 21-amino acid vasoconstrictor peptide, was initially isolated from the supernatant of cultured porcine aortic endothelial cells (1). Infusion of ET-1 to experimental animals and humans causes an increase in mean arterial pressure (MAP) and reductions in renal blood flow and urinary sodium excretion (2 – 4). Plasma ET-1 levels are elevated in patients with congestive heart failure, myocardial ischemia and acute renal failure (5 – 7). ET-1 may play important roles in cardiovascular physiology and pathophysiology.

ET-1 is biosynthesized from its inactive 38 (human) or 39 (porcine) amino acid precursor, big ET-1, by proteolytic cleavage between Trp^{21} and Val^{22}. The enzyme responsible for this last step of selective processing of big ET-1 is a membrane-bound zinc metalloprotease, endothelin converting enzyme (ECE) (8). The purification, cloning, and functional expression of ECE in rat lung (9), porcine aortic endothelium (10), bovine adrenal cortex (11) and human umbilical vein (12) have been reported.

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It is well known that the physiologically relevant ECE is inhibited by phosphoramidon, a non-selective peptidic metalloprotease inhibitor. Phosphoramidon has been shown to inhibit the pressor and airway contractile responses of big ET-1 in vivo (13, 14), the secretion of ET-1 from cultured endothelial cells (15, 16) and the pathophysiological effects of big ET-1 (17 – 19). Although phosphoramidon is not a selective inhibitor of ECE with approximately 1,000-fold higher affinity to neutral endopeptidase (NEP), ECE inhibitor might be expected to produce therapeutic benefits similar to those proposed for ET receptor antagonists.

Other non-peptidic ECE inhibitors have been reported previously. De Lombaert et al. (20, 21) reported compounds, CGS 26303 and CGS 31447, with IC_{50} values of 1.1 μM and 17 nM for ECE-1, respectively. However both compounds inhibit NEP with approximately 1,000- and 3-fold higher affinity, respectively (IC_{50} values of 0.9 and 4.8 nM, respectively, for NEP). Tsurumi et al. (22) reported FR 901533, isolated from fungal broth, which was shown to have an IC_{50} value of 0.14 μM for ECE-1 with selectivity

against NEP. However, FR 901533 could not inhibit endogenous production of ET-1 by live cells (11). Ahn et al. (23) reported PD 069185 and PD 159790 with IC_{50} values of 0.9 and 2.6 μM, respectively, for human ECE-1. However, these inhibitors had relatively high cytotoxicity (TC_{50} values of 19.6 and 89.0 μM, respectively), and they not only inhibited ET-1 production (IC_{50} values of 11.1 and 28.1 μM, respectively) but also decreased big ET-1 levels (IC_{50} values of 8.7 and 37.5 μM, respectively) in the cell-based assay. Thus, all ECE inhibitors reported up to date are not selective for ECE, do not inhibit the endogenous production of ET-1 or have relatively high cytotoxicity.

In this paper, we report the pharmacological characteristics of SM-19712 {4-chloro-*N*-[[(4-cyano-3-methyl-1-phenyl-1*H*-pyrazol-5-yl) amino] carbonyl] benzenesulfonamide, monosodium salt}, a highly selective, structurally novel and non-peptidic inhibitor of ECE.

MATERIALS AND METHODS

ECE assay

ECE was solubilized from rat lung microsomes as described previously (24). Each test compound (dissolved in DMSO) and rat lung ECE (10 μg protein) were preincubated at 37°C for 15 min in 100 mM Tris-HCl buffer (pH 7.0) containing 1 mM *N*-ethylmaleimide (NEM), 100 μM leupeptin and 20 μM pepstatin A. Then 10 pmol of human big ET-1 (1 – 38) was added, and the mixture was incubated at 37°C for 1 h in a total volume of 200 μl. The reaction was stopped by the addition of EDTA to give a final concentration of 1 mM. Aliquots (100 μl) of the mixture were withdrawn and the production of ET-1 was determined by the ET-1 specific sandwich-type enzyme immunoassay (EIA) method (25), using rabbit anti-ET-1 C-terminal heptapeptide (15 – 21) antibody (30821, see Materials) and horseradish peroxidase (HRP)-labeled rabbit anti-ET-1 N-terminal loop domain antibody (30846, see Materials). The inhibitory activities for ECE of test compounds were indicated by the concentration required to provide 50% inhibition, namely the IC_{50}. The antibody of ET-1 for EIA did not cross-react with ET-3, big ET-1 or big ET-3.

Specificity

The activity of NEP, obtained from porcine kidney, was determined by hydrolysis of the synthetic substrate MOCAc-Pro-Leu-Gly-Leu-A_2p(Dnp)-Ala-Arg-NH_2 as reported previously (26). The activity of angiotensin converting enzyme (ACE), obtained from rabbit lung, was determined by hydrolysis of the synthetic substrate Bz-Gly-His-Leu as reported previously (26).

The specificity of SM-19712 for ECE was examined by measuring the ability of SM-19712 to compete with the radioligand binding or enzyme activities in 13 receptors and 9 enzymes except ECE, NEP and ACE. These assays were performed by Panlabs Taiwan, Ltd. (Taipei, Taiwan).

Cellular assay

Endothelial cells isolated from fresh porcine aortas were cultured as described previously (27). The cells were grown in gelatin-coated 12-well plates at 37°C in a CO_2 incubator. When the cells became confluent (4 – 8 passages), the culture medium was changed to 0.5 ml of serum-free Dulbecco's modified Eagle's medium containing 0.01% heat-inactivated bovine serum albumin. In the absence or presence of test compounds, the cells were incubated at 37°C for 6 h in a CO_2 incubator. After the incubation, the medium was collected, boiled for 10 min, and centrifuged at 8,000 × *g* for 10 min. The resulting supernatant served as sample for the radioimmunoassay (RIA). The RIA for ET-1 was performed as described (15). ET-1 antiserum (a generous gift from Dr. M.R. Brown, Department of Medicine, University of California, San Diego, USA) had no cross-reactivity with big ET-1.

Pressor response in anesthetized rats

Male Sprague-Dawley (SD) rats (300 – 400 g) were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and fixed on a warming surgical bed. A femoral artery catheter was inserted for monitoring MAP (AP-641G; Nihon Kohden, Tokyo) and heart rate (data not shown) (AT-601G, Nihon Kohden), and a femoral vein catheter was used for injection of test compounds, vehicle (saline) or big ET-1. Rats were ganglionic-blocked with pentolinium (10 mg/kg, i.v.) to prevent reflex attenuation of the pressor response to big ET-1 and were stabilized. Test compounds and saline (0.5 ml/kg) were injected 10 min prior to the administration of big ET-1 (1 nmol/kg, i.v.). The ECE inhibitory activity of the test compound in vivo was determined by measuring the change in the peak MAP above base line in response to big ET-1 relative to vehicle-treated rats.

Conscious male SD rats (280 – 400 g) were dosed with test compounds or saline (1 ml/kg) by oral gavage. Rats were anesthetized and prepared as described above. Big ET-1 (1 nmol/kg, i.v.) was injected 1 h after the oral administration of test compounds or saline.

Serum concentration

Male SD rats (7 weeks of age) were administered intravenously or orally a saline solution of SM-19712 (10 or 30 mg/kg, n = 3). Whole blood samples of individual rats were withdrawn through the abdominal aorta at 2, 5, 10, 20, 30 and 60 min after i.v. injection or at 15, 30, 60, 120, 240 and 300 min after p.o. administration.

One mlliliter of each serum sample was added to 2 ml of 150 mM phosphate buffer (pH 2.0) and 50 μl of SM-19659, *N*-[[(4-cyano-3-ethyl-1-phenyl-1*H*-pyrazol-5-yl)amino]car-

bonyl]-4-methylbenzenesulfonamide (10 μg/ml of internal standard solution) and the mixture was extracted with 6 ml of ethyl acetate. Organic layers were separated by centrifugation at 1,000 × g for 15 min and evaporated to dryness. The residue was resolved in 100 μl of HPLC mobile phase and 25 μl of this solution was injected into the HPLC system. HPLC analysis was carried out on a Puresil C18 column (4.6 mm diameter, 150 mm length, 5 μm; Waters, Milford, MA, USA) with a mobile phase of 52% Pic A (Waters) solution and 48% methanol at a flow rate of 1.0 ml/min with a UV detection wavelength of 237 nm.

Plasma concentration time profiles were analyzed using noncompartmental methods. The area under the plasma concentration-time curve (AUC) was estimated by a combination of linear and log trapezoidal methods. Plasma clearance was calculated as dose/AUC. Bioavailability was determined as dose-normalized AUC (p.o.) / dose-normalized AUC (i.v.). The apparent terminal half-life was estimated by least-squares linear regression analysis of the log-transformed concentration-time data.

Acute myocardial infarction in a rabbit model

Male New Zealand White Rabbits (2.6 – 3.5 kg) were anesthetized with sodium pentobarbital (40 mg/kg, i.v.). Rabbits were intubated through a tracheotomy, ventilated using a respirator (SN-480-5; Shinano, Tokyo) and with room air supplemented with 100% O_2 gas. Body temperature was maintained at 36 – 37°C with a heating pad (K-20; Baxter, Irvine, CA, USA). Catheters were placed in the right carotid artery and jugular vein to monitor blood pressure and to administer drugs, respectively. Additional anesthetic was administered through the right femoral vein as needed. Heart rate was measured by a cardiotachometer (AT-600G, Nihon Kohden) triggered by R waves of the ECG. ECG, heart rate and systemic arterial pressure (TP-300T pressure transducer, Nihon Kohden) were continuously monitored during the experiment. A left thoracotomy was performed in the fourth intercostal space, and the heart was suspended in a pericardial cradle. A polyethylene cannula was placed in the left atrium via the atrial appendage. A 4-0 braided-silk suture with a 14-mm taper needle was passed around a large marginal branch of the left coronary artery, approximately 5 mm from its origin. The ends of the silk suture were passed through a small polyethylene tube. The marginal branch was occluded by pulling the snare, which was then fixed by clamping the tube. Myocardial ischemia was confirmed by the regional cyanosis and ST segment elevation in the ECG. Coronary artery occlusion was maintained for 30 min, and reperfusion was allowed for 5 h. Reperfusion was induced by releasing the snare and confirmed by visible hyperemia over the surface. If VF developed, animals were immediately resuscitated by DC countershock (20 W·s) using a defibrillator (MDV-2, Nihon Kohden) with external paddles.

At the end of each experiment, the silk suture under the coronary branch was tightly tied to occlude the artery, and a 1% Evan's blue dye solution was injected into the left atrium. The heart was quickly removed, and the left ventricle was dissected free from other structures and cut into transverse slices 1-mm thick. The slices were incubated in a 1% solution of triphenyltetrazolium chloride for 10 min at 37°C and soaked in 10% formalin to enhance the contrast of the stain. After staining, the slices were sandwiched between two acrylic plates to a uniform 1-mm thickness, and the region of infarct tissue and risk zone were traced through the acrylic plate. The risk and infarct zone were defined as Evans blue-negative and triphenyltetrazolium chloride-negative staining, respectively. The area of infarct and risk zone were determined by planimetry of the tracings (NIH Image). The infarct size was expressed as a percentage of the left ventricle or area at risk zone.

Blood samples were withdrawn at the following 6 time points via the cannula placed in the left atrium: before administration of SM-19712 or vehicle; before occlusion of the coronary artery; just before reperfusion; and 1, 3 and 5 h after reperfusion (time of −40 min, −30 min, 0 h, 1 h, 3 h and 5 h, respectively). The serum concentration of ET-1 was determined using a commercially available ET-1 measuring kit (Kainos Laboratory, Inc., Tokyo) by the luminol chemiluminescent method. The serum activity of creatine phosphokinase (CPK) was determined using a commercially available CPK measuring kit (CPK-II Test Wako; Wako Pure Chemical Industries, Ltd., Osaka).

SM-19712 was administered as a bolus injection (25.9 mg/kg, i.v.) 10 min prior to coronary artery occlusion followed by a continual infusion (1.7 mg/kg/min, i.v.) until the termination of the experiment. The vehicle group received an equivalent amount of 10% PEG 400, which was the vehicle of SM-19712.

Statistical analyses

Williams' test or Student's t-test was performed for statistical analysis. The data were expressed as the mean ± S.E.M. or S.D. with the number of experiments involved.

Materials

ET-1 (1 – 21), human big ET-1 (1 – 38), pepstatin A, leupeptin, phosphoramidon, MOCAc-Pro-Leu-Gly-Leu-A_2p (Dnp)-Ala-Arg-NH_2 and Bz-Gly-His-Leu were purchased from Peptide Institute (Osaka). Antibodies for EIA (30821, 30846) were from International Reagents Corporation (Kobe). PMSF and NEM were from Wako Pure Chemical Industries, Ltd. (Osaka). Pentolinium and BSA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NEP was from Elastin Products Company, Inc. (Owensville, MO, USA). ACE was from Nihon Zohki

Pharmaceuticals Co., Ltd. (Osaka). SM-19712 and SM-19659 were synthesized at Sumitomo Pharmaceuticals Co., Ltd.

RESULTS

In vitro studies

SM-19712 (Fig. 1) is structurally novel benzenesulfonamide derivative modified compound found through the screening of the Sumitomo Pharmaceuticals Library (Patent No. EP 885890). We studied the potency of SM-19712 to inhibit ECE by comparing it with phosphoramidon, a non-selective, peptidic ECE inhibitor. In the enzyme assay, we compared the inhibitory activity for ECE using the solubilized fraction from rat lung membrane. SM-19712 and phosphoramidon concentration-dependently inhibited ECE with an IC_{50} of 42 and 690 nM, respectively, showing that SM-19712 was 16-fold more potent than phosphoramidon.

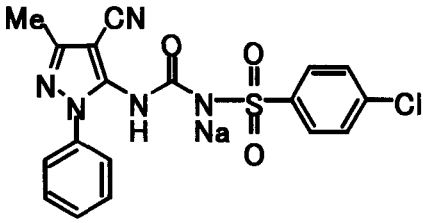

Fig. 1. Structure of SM-19712.

Phosphoramidon inhibited NEP rather more potently than ECE. We tested the selectivity of SM-19712 by examining the inhibitory effects on other metalloproteases such as NEP and ACE. SM-19712 had little effect on these metalloproteases at up to 100 μM (data not shown). In addition, SM-19712 did not affect the radioligand bindings or enzyme activities of any of 13 receptors and 9 enzymes (Table 1). Thus these results show that SM-19712 is a potent and highly selective inhibitor for ECE.

In vivo studies

In anesthetized rats, exogenous big ET-1 (1 nmol/kg, i.v.) increased the MAP, and the maximal pressor response was $\Delta 55.4 \pm 5.4$ mmHg observed at about 10 min in the vehicle group ($n = 47$). When SM-19712 (0.3 – 30 mg/kg, i.v.) and phosphoramidon (0.1 – 10 mg/kg, i.v.) were injected 10 min prior to the administration of big ET-1, they had no effect on the base-line pressure. SM-19712 and phosphoramidon dose-dependently and significantly suppressed the pressor response induced by big ET-1 (Fig. 2), and the estimated ED_{50} values were 5.6 and 1.5 mg/kg, i.v., respectively.

It has been reported that phosphoramidon does not

Table 1. Lack of significant effect of 10 – 300 μM SM-19712 on radioligand binding and enzyme activity

Binding site	Radioligand	% inhibition
Angiotensin AT_1	[^{3}H]Losartan	6
Angiotensin AT_2	[^{125}I]CGP42112A	26
Atrial natriuretic factor	[^{125}I]ANF	−15
Endothelin ET_A	[^{125}I]Endothelin-1	26
Endothelin ET_B	[^{125}I]Endothelin-1	7
Epidermal growth factor	[^{125}I]EGF	−11
GABA uptake	[^{3}H]GABA	15
Inositol trisphosphate	[^{3}H]-1,4,5-IP_3	−6
Insulin	[^{125}I]Insulin	9
Neuropeptide Y_2	[^{3}H]NPY	18
Phorbol ester	[^{3}H]PDBu	10
Tumor necrosis factor-α	[^{125}I]TNF-α	7
Vasoactive intestinal peptide	[^{125}I]VIP	14
Enzyme[a]	**Source**	**% Inhibition**
Calpain (10 μM)	Human erythrocyte	−1
Collagenase IV (100 μM)	Human U937 cells	8
5-Lipoxygenase (30 μM)	RBL-1 cells	21
Nitric oxide synthase, constitutive (10 μM)	Rat cerebellum	−1
Nitric oxide synthase, inducible (10 μM)	RAW 264.7 cells	−2
Phospholipase A_2 (300 μM)	Porcine pancreas	−6
Elastase (30 μM)	Human neutrophil	19
EGF tyrosine kinase (10 μM)	Human recombinant	−6
Protein kinase C (300 μM)	Rat brain	−10

The lack of significant effect at high concentration of SM-19712 is indicative of high specificity for ECE. [a]Concentration of SM-19712 shown in parentheses.

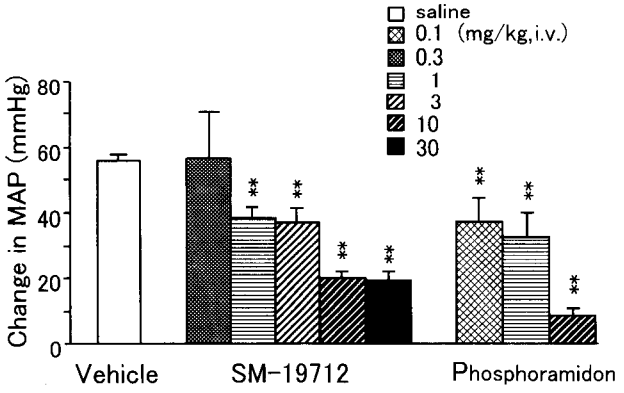

Fig. 2. Effects of intravenously administered SM-19712 (0.3 – 30 mg/kg, n = 3 – 8), phosphoramidon (0.1 – 10 mg/kg, n = 4) and vehicle (saline, n = 47) on the pressor response induced by big ET-1 in anesthetized rats. These compounds were administered 10 min prior to the injection of big ET-1 (1 nmol/kg, i.v.). Data are represented as the mean ± S.E.M. and were evaluated by Williams' test. $^{**}P<0.01$, compared with vehicle.

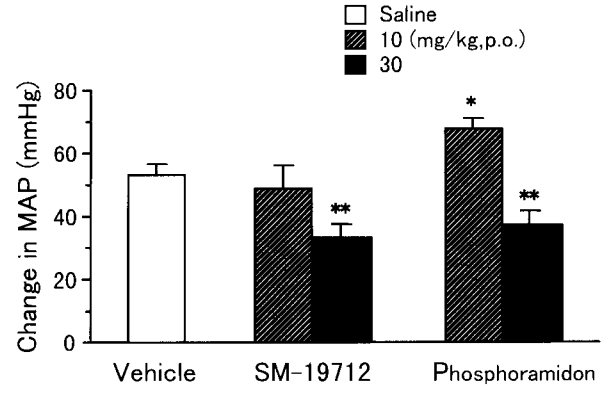

Fig. 3. Effects of orally administered SM-19712 (10, 30 mg/kg, n = 9 – 10), phosphoramidon (10, 30 mg/kg, n = 4 – 7) and vehicle (saline, n = 19) on the pressor response induced by big ET-1 in anesthetized rats. These compounds were administered 1 h prior to the injection of big ET-1 (1 nmol/kg, i.v.). Data are represented as the mean ± S.E.M. and were evaluated by Williams' test. $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle.

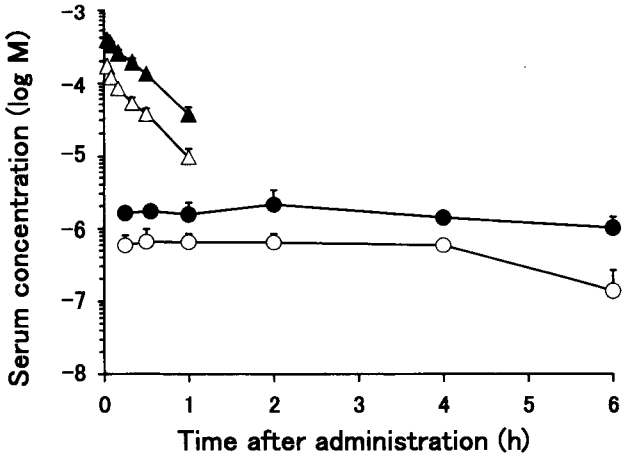

Fig. 4. Serum concentration of SM-19712 after intravenous (△, 10 mg/kg; ▲, 30 mg/kg) and oral (○, 10 mg/kg; ●, 30 mg/kg) administration in SD rats. Each point represents the mean ± S.D. (n = 3).

affect the ET-1-induced hypertensive effect (13). We evaluated the effect of SM-19712 on ET-1-induced pressor response. In anesthetized rats, ET-1 (1 nmol/kg) evoked depressor and pressor responses, and the magnitude of the pressor response was similar to that seen with big ET-1. SM-19712 (10 mg/kg, i.v.) had no effect on the pressor responses induced by ET-1 (data not shown).

We also estimated the efficacies of orally administered SM-19712 and phosphoramidon on the pressor response induced by big ET-1 in anesthetized rats. As shown in Fig. 3, both compounds at a dose of 30 mg/kg, p.o. significantly suppressed the pressor response induced by big ET-1 (SM-19712: 37.2% suppression, phosphoramidon: 29.5% suppression).

Pharmacokinetic parameters

Phosphoramidon is a peptidic compound whose concentration in plasma decreases rapidly after it is administered intravenously to SD rats (22). As shown in Fig. 4 and Table 2, when SM-19712 was administered intravenously to SD rats, the serum concentration decreased with a terminal half-life of 16 min, and the pharmacokinetic parameters were unchanged at the two doses (10 and 30 mg/kg). On

Table 2. Pharmacokinetic parameters for SM-19712 in SD rats

	Dose (mg/kg)	$AUC_{0-\infty}$ (μg·h/ml)	BA[a] (%)	$t_{1/2}$[b] (h)	CL[c] (l/(h·kg))	Vdss[d] (l/kg)	Protein binding (%)
i.v.	30	75	NA	0.27	0.42	0.16	99
	10	23					
p.o.	30	6.5	8.1	NA	NA	NA	
	10	1.8					

Each value indicates the mean (n = 3). NA = not applicable. [a]Bioavailability, [b]Terminal half-life, [c]Plasma clearance, [d]Volume of distribution at steady state.

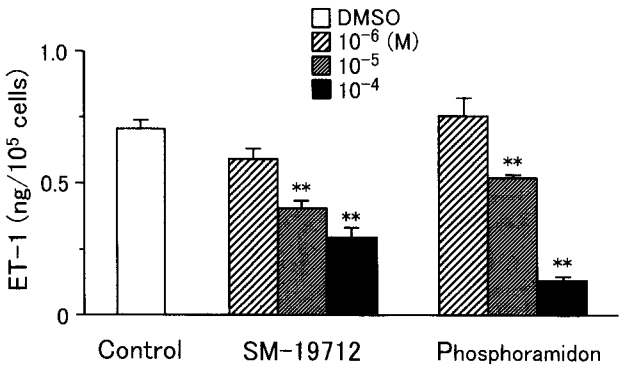

Fig. 5. Effects of SM-19712 and phosphoramidon on the production of ET-1 from endogenous big ET-1 in cultured porcine aortic endothelial cells after incubation for 6 h. Data are represented as the mean ± S.E.M. of 3–9 experiments and were evaluated by Williams' test. $^{**}P<0.01$, compared with the control.

the other hand, the serum concentration of orally administered SM-19712 was sustained from 30 min to 4 h after administration. The bioavailability was 8% and did not differ between the two doses (10 and 30 mg/kg).

The protein binding of SM-19712 in rat serum was determined to be 99%, meaning that only 1% of SM-19712 was free from plasma protein and effective in rat serum. To confirm the effect of protein binding on the inhibitory activity of the compound, we examined the ECE inhibitory activity in vitro in the presence of bovine serum albumin (BSA). In the presence of BSA (2%), the IC_{50} values of SM-19712 and phosphoramidon were 6.3 and 1.0 μM, respectively, so BSA (2%) caused a 150- and 1.4-fold decrease in the inhibitory activity, respectively.

Inhibitory activity for endogenous production of ET-1

Studies were designed to evaluate whether SM-19712 is effective on the endogenous conversion of big ET-1 in intact cells. ECE-1 is expressed in both intracellular (Golgi bodies) and extracellular forms. In pathological states, ECE inhibitor needs to inhibit the endogenous production of ET-1. As shown in Fig. 5, SM-19712 inhibited the endogenous production of ET-1 in cultured porcine aortic endothelial cells with the IC_{50} value of 31 μM, which is as potent as phosphoramidon that has an IC_{50} of 27 μM. In addition, SM-19712 and phosphoramidon increased the production of big ET-1 in cultured porcine endothelial cells (data not shown), reflecting the inhibition of ECE.

Effect of SM-19712 on acute myocardial infarction in a rabbit model

We evaluated the effects of SM-19712 on acute myocardial infarction in a rabbit model. As shown in Fig. 6A, the area at risk expressed as a percentage of the area of the left ventricle did not differ between the SM-19712-treated

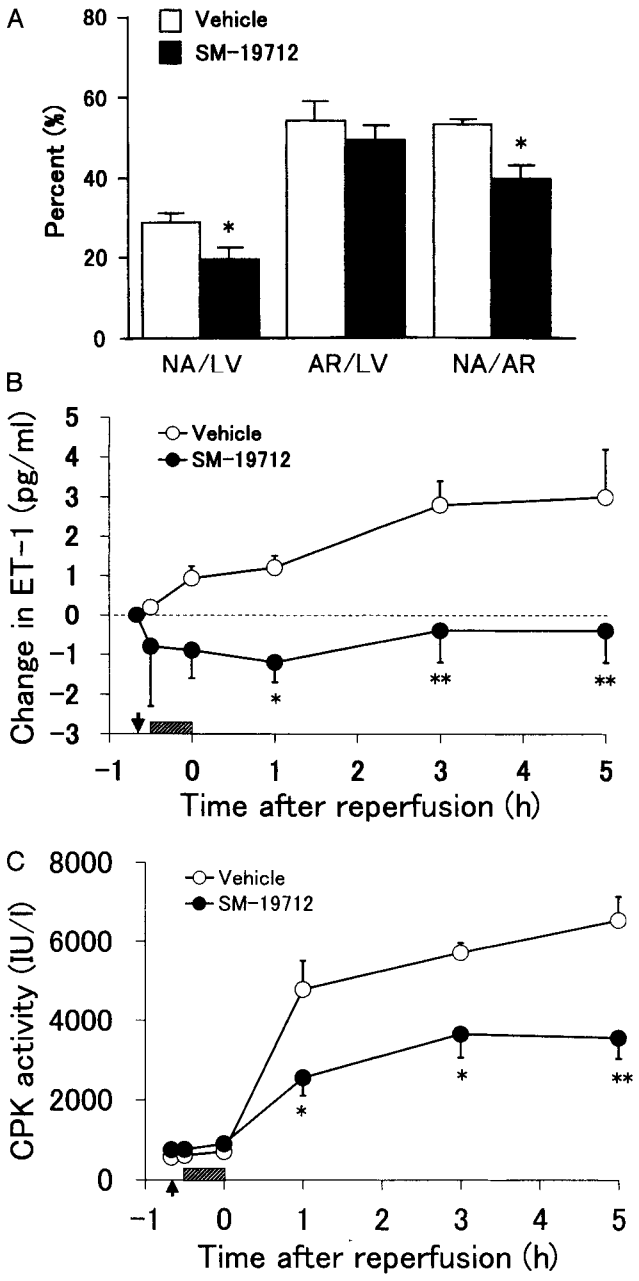

Fig. 6. Effect of SM-19712 on myocardial infarct size (A), serum concentration of ET-1 (B) and serum CPK activity (C) in the rabbits. Rabbits were subjected to 30 min of coronary artery occlusion (CAO) followed by 5 h of reperfusion. SM-19712 was bolus injected 10 min before CAO (25.9 mg/kg, i.v.) and infused throughout the experiment (1.7 mg/(kg·min)). The vehicle group received an equivalent amount of 10% PEG 400. A: AR, LV and NA indicate area at risk, left ventricle area and area at necrosis, respectively. B: Blood samples were collected at the indicated time points, and the serum concentration of ET-1 was measured with a commercially available ET-1 measuring kit by the luminol chemiluminescent method. The hatched bar indicates the CAO period, and the arrow indicates the point at which the bolus was injected and the infusion of SM-19712 started. C: Blood samples were collected at the indicated time points. CPK activity in serum was measured with a CPK measuring kit. Each value represents mean ± S.E.M. of 3–5 experiments. $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle by Student's *t*-test.

group and vehicle-treated group. In the SM-19712-treated group, myocardial infarct size, expressed as a percentage of both the area of the left ventricle and the area at risk, was significantly reduced as compared with that in the vehicle-treated group. SM-19712 also significantly reduced the increase in serum concentration of ET-1 (Fig. 6B) and CPK activity (Fig. 6C) in the vehicle-treated group.

DISCUSSION

In the present study, we demonstrated that SM-19712 was a potent and highly selective ECE inhibitor. The IC_{50} value of SM-19712 for ECE solubilized from rat lung membrane was 42 nM, making it 16-fold more potent than phosphoramidon whose IC_{50} value was 690 nM. In contrast to phosphoramidon, SM-19712 had little effect on other metalloproteases such as NEP and ACE, and it did not affect various radioligand receptor bindings or enzyme activities.

Intravenously administered SM-19712 markedly suppressed the pressor response induced by big ET-1 in a dose-dependent, whereas SM-19712 did not affect the pressor response induced by ET-1. In addition, SM-19712 was an orally active inhibitor for ECE. Therefore, SM-19712 is effective ECE inhibitor both in vitro and in vivo.

The cell compartment where processing of the big ETs by different ECEs occurs is still controversial, with evidence suggesting both the cell surface (13, 28 – 31) and an intracellular location (11, 32 – 34). Only a few compounds such as phosphoramidon and PD 069185 have been reported to inhibit the endogenous conversion of big ET-1 (11, 15, 23), while FR 901533, which efficiently inhibited ECE-1 in vitro with a potency similar to phosphoramidon, is known to be ineffective on the endogenous production of ET-1 (11). In pathological states, an ECE inhibitor needs to inhibit the endogenous production of ET-1. We noted that SM-19712 concentration-dependently inhibited the endogenous conversion of big ET-1 in cultured porcine endothelial cells as potently as phosphoramidon. While PD 069185 decreases the intracellular production of big ET-1 in intact cells, mostly due to cytotoxicity (23), SM-19712 and phosphoramidon do not decrease the production of big ET-1 in cultured porcine endothelial cells.

On the other hand, it is well known that the amino acid sequences of ET-1 and its precursor, big ET-1, involve species differences. Thus, further investigation is necessary to examine whether SM-19712 inhibits the ET-1 production from human big ET-1 in human ECEs and/or human vascular endothelial cells.

Plasma levels of ET-1 are elevated in several cardiovascular disorders such as myocardial infarction, congestive heart failure, acute renal failure, hypertension and atherosclerosis (35). During and following myocardial ischemia and reperfusion, the ET-1 production is stimulated and the coronary constrictor response to ET-1 is enhanced (6, 36, 37). The effects of ET-receptor antagonists, ECE inhibitor and ET monoclonal antibody on myocardial infarction have been examined in animal models, but the findings are controversial. A mixed ET_A/ET_B antagonist, TAK-044, decreased infarct size in ischemia reperfusion-induced myocardial infarction of rats (38, 39), whereas bosentan, a mixed ET_A/ET_B antagonist, did not affect infarct size in rats (40). An ET_A antagonist, PD 156707, also did not reduce infarct size in a porcine model (41), whereas other ET_A antagonists such as FR 139317 and LU 135252 significantly decreased infarct size in rabbits and pigs, respectively, when administered before occlusion of the coronary artery (42, 43). In the present study, we also evaluated the effect of the ECE inhibitor SM-19712 on myocardial infarction of rabbits. SM-19712 significantly reduced infarct size and the serum concentration of ET-1, suggesting that the inhibition of endogenous big ET-1 conversion is effective for the treatment of myocardial infarction. In the present study, the anti-necrotic effect of SM-19712 was incomplete, despite of the fact that this compound completely suppressed the increase in serum ET-1 concentration. It is not easy to explain the reason for this discrepancy, but it may be speculated that ET-1 is likely to be one of many causal factors of myocardial infarction. Most recently, we noted that SM-19712 improved the renal dysfunction and tissue injury induced by renal ischemia/reperfusion (Y. Matsumura et al., unpublished observation). Thus, a specific ECE inhibitor may exert beneficial effects on ischemic tissue damage.

In vivo potencies of SM-19712 did not reflect those in vitro, in comparison with the case of phosphoramidon. The reduced potency of SM-19712 in vivo seemed to be due not to degradation but to serum protein binding. The presence of BSA or rat serum caused a marked decrease in the ECE inhibitory activity of SM-19712 in the enzyme assay, and the percent binding to plasma protein of SM-19712 was determined to be 99%. Hence it seems likely that the discrepancy in inhibitory activities of SM-19712 between in vitro and in vivo mostly results from the protein binding ability.

In conclusion, SM-19712 is a potent and selective ECE inhibitor both in vitro and in vivo. In addition, SM-19712 has beneficial effects on acute myocardial infarction. Therefore, SM-19712 will be a useful tool for elucidating the physiological and pathological roles of ECE. Further studies are required to evaluate whether ECE inhibitors are favorable for the treatment of patients with cardiovascular diseases compared with ET receptor antagonists.

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REFERENCES

1 Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Mitsui T, Yazaki T, Goto K and Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature **332**, 411 - 415 (1988)

2 Mortensen LH, Pawloski CM, Knagy NL and Fink GD: Chronic hypertension produced by infusion of endothelin in rats. Hypertension **15**, 729 - 733 (1990)

3 Leadley RJ, Zhu JL and Goetz KL: Effects of endothelin-1 and sarafotoxin S6b on regional hemodynamics in the conscious dog. Am J Physiol **260**, R1210 - R1217 (1991)

4 Nord EP: Renal actions of endothelin. Kidney Intern **44**, 451 - 463 (1993)

5 Margulies KB, Hildebrand FL, Lerman A, Perrella MA and Burnett JC: Increased endothelin in experimental heart failure. Circulation **82**, 2226 - 2230 (1990)

6 Miyauchi T, Goto K and Masaki T: Increased plasma concentrations of endothelin-1 and big endothelin-1 in acute myocardial infarction. Lancet **2**, 53 - 54 (1989)

7 Tomita Y, Ujiie K, Nakanishi T, Tomura S, Matsuda O, Ando K, Shichiri M, Hirata Y and Marumo F: Plasma endothelin levels in patients with acute renal failure. New Engl J Med **321**, 1127 (1989)

8 Masaki T, Yanagisawa M and Goto K: Physiology and pharmacology of endothelins. Med Res Rev **12**, 391 - 421 (1992)

9 Shimada K, Takahashi M and Tanzawa K: Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. J Biol Chem **269**, 18275 - 18278 (1994)

10 Ohnaka K, Takayanagi R, Nishikawa M, Haji M and Nawata H: Purification and characterization of a phosphoramidon-sensitive endothelin-converting enzyme in porcine endothelium. J Biol Chem **268**, 26759 - 26766 (1993)

11 Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, DeWit D and Yanagisawa M: ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell **78**, 473 - 485 (1994)

12 Shimada K, Matsushita Y, Wakabayashi K, Takahashi M, Matsubara A, Iijima Y and Tanzawa K: Cloning and functional expression of human endothelin-converting enzyme cDNA. Biochem Biophys Res Commun **207**, 807 - 812 (1995)

13 Matsumura Y, Hisaki K, Takaoka M and Morimoto S: Phosphoramidon, a metalloprotinase inhibitor, suppress the hypertensive effect of big endothelin-1. Eur J Pharmacol **185**, 103 - 106 (1990)

14 Fukuroda T, Noguchi K Tsuchida S, Nishikibe M, Ikemoto F, Okada K and Yano M: Inhibition of biological action of big endothelin-1 by phosphoramidon. Biochem Biophys Res Commun **172**, 390 - 395 (1990)

15 Ikegawa R, Matsumura Y, Tsukahara Y, Takaoka M and Morimoto S: Phosphoramidon, a metalloproteinase inhibitor, suppresses the secretion of endothelin-1 from cultured endothelial cells by inhibiting a big endothelin-1 converting enzyme. Biochem Biophys Res Commun **171**, 669 - 675 (1990)

16 Sawamura T, Kasuya Y, Matsushita Y, Suzuki N, Shinmi O, Kishi N, Sugita Y, Yanagisawa M, Goto K, Masaki T and Kimura S: Phosphoramidon inhibits the intracellular conversion of big endothelin-1 to endothelin-1 in cultured endothelial cells. Biochem Biophys Res Commun **174**, 779 - 784 (1991)

17 Matsumura Y, Ikegawa R, Suzuki Y, Takaoka M, Uchida T, Kido H, Shinyama H, Hayashi K, Watanabe M and Morimoto S: Phosphoramidon prevents cerebral vasospasm following subarachnoid hemorrhage in dogs: the relationship to endothelin-1 levels in the cerebrospinal fluid. Life Sci **49**, 841 - 848 (1991)

18 Grover GJ, Sleph PG, Fox M and Trippodo NC: Role of endothelin-1 and big endothelin-1 in modulating coronary vascular tone, contractile function and severity of ischemia in rat hearts. J Pharmacol Exp Ther **263**, 1074 - 1082 (1992)

19 Vemulapalli S, Chiu PJS, Chintala M and Bernardio V: Attenuation of ischemic acute renal failure by phosphoramidon in rats. Pharmacology **47**, 188 - 193 (1993)

20 De Lombaert S, Ghai RD, Jeng AY, Trapani AJ and Webb RL: Pharmacological profile of a nonpeptide dual inhibitor of neutral endopeptidase 24.11 and endothelin-converting enzyme. Biochem Biophys Res Commun **204**, 407 - 412 (1994)

21 De Lombaert S, Stamford LB, Blanchard L, Tan J, Hoyer D, Diefenbacher CG, Wei D, Wallance EM, Moskal MA, Savage P and Jeng AY: Potent non-peptidic dual inhibitors of endothelin-converting enzyme and neutral endopeptidase. Bioorg Med Chem Lett **7**, 1059 - 1064 (1997)

22 Tsurumi Y, Fujie K, Nishikawa M, Kiyoto S and Okuhara M: Biological and pharmacological properties of higher selective new endothelin converting enzyme inhibitor WS79089B isolated from *Streptosporangium roseum* No. 79089. J Antibiot (Tokyo) **48**, 169 - 174 (1995)

23 Ahn K, Sisneros AM, Herman SB, Pan SM, Hupe D, Lee C, Nikam S, Cheng XM, Doherty AM, Schroeder RL, Haleen SJ, Kaw S, Emoto N and Yanagisawa M: Novel selective quinazoline inhibitors of endothelin enzyme-1. Biochem Biophys Res Commun **243**, 184 - 190 (1998)

24 Takahashi M, Matsushita Y, Iijima Y and Tanzawa K: Purification and characterization of endothelin-converting enzyme from rat lung. J Biol Chem **268**, 21394 - 21398 (1993)

25 Suzuki N, Matsumoto H, Kitada C, Masaki T and Fujino M: A sensitive sandwich-enzyme immunoassay for human endothelin. J Immunol Methods **118**, 245 - 250 (1989)

26 Cushman DW and Cheung HS: Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol **20**, 1637 - 1648 (1971)

27 Matsumura Y, Ikegawa R, Takaoka M and Morimoto S: Conversion of porcine big endothelin to endothelin by an extract from the porcine aortic endothelial cells. Biochem Biophys Res Commun **167**, 203 - 210 (1990)

28 Shimada K, Takahashi M, Ikeda M and Tanzawa K: Identification and characterization of two isoforms of an endothelin converting enzyme-1. FEBS Lett **371**, 140 - 144 (1995)

29 Corder R, Khan N and Harrison VJ: A simple method for isolating human endothelin converting enzyme free from contamination by neutral endopeptidase 24.11. Biochem Biophys Res Commun **207**, 355 - 362 (1995)

30 Takahashi M, Fukuda K, Shimada K, Barnes K, Turner AJ, Ikeda M, Koike H, Yamamoto Y and Tanzawa K: Localization of rat endothelin-converting enzyme to vascular endothelial cells and some secretory cells. Biochem J **311**, 657 - 665 (1995)

31 Waxman L, Doshi KP, Gaul SL, Wang S, Bednar RA and Stern AM: Identification and characterization of endothelin converting activity from EAHY 926 cells: evidence for the physiologically relevant human enzyme. Arch Biochem Biophys **308**, 240 - 253 (1994)

32 Emoto N and Yanagisawa M: Endothelin-converting enzyme-2 is a membrane-bound, phosphoramidon-sensitive metalloprotease with acidic pH optimum. J Biol Chem **270**, 15262 - 15268 (1995)

33 Gui G, Xu D, Emoto N and Yanagisawa M: Intracellular localization of membrane-bound endothelin-converting enzyme from rat lung. J Cardiovasc Pharmacol **22**, Suppl **8**, 553 - 556 (1993)

34 Azarani A, Boileau G and Crine P: Recombinant human endothelin-converting enzyme ECE-1b is located in an intracellular compartment when expressed in polarized Madin-Darby canine kidney cells. Biochem J **333**, 439 - 448 (1998)

35 Rubanyi GM and Polokoff MA: Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathology. Pharmacol Rev **46**, 325 - 415 (1994)

36 Watanabe T, Suzuki N, Shimamoto N, Fujino M and Imada A: Endothelin in myocardial infarction. Nature **344**, 114 (1990)

37 Watanabe T, Suzuki N, Shimamoto N, Fujino M and Imada A: Contribution of endogenous endothelin to the extension of myocardial infarct size in rats. Cir Res **69**, 370 - 377 (1991)

38 Kojima M, Kusumoto K, Fujiwara S, Watanebe T and Fujino M: Role of endogenous endothelin in the extension of myocardial infarct size studied with the endothelin receptor antagonist, TAK-044. J Cardiovasc Pharmacol **26**, Suppl **3**, S365 - S368 (1995)

39 Watanabe T, Awane Y, Ikeda S, Fujiwara S, Kubo K, Kikuchi T, Kusumoto K, Wakimasu M and Fujino M: Pharmacology of a non-selective ET_A and ET_B receptor antagonist, TAK-044 and the inhibition of myocardial infarct size in rats. Br J Pharmacol **114**, 949 - 954 (1995)

40 Richard V, Kaeffer N, Hogie M, Tron C, Blanc T and Thuillez C: Role of endogenous endothelin in myocardial and coronary endothelial injury after ischemia and reperfusion in rats: studies with bosentan, a mixed ET_A-ET_B antagonist. Br J Pharmacol **113**, 869 - 874 (1994)

41 Mertz TE, McClanahan TB, Flynn MA, Juneau P, Reynolds EE, Hallak H, Bradford L and Gallagher KP: $Endothelin_A$ receptor antagonism by PD 156707 does not reduce infarct size after coronary artery. J Pharmacol Exp Ther **278**, 42 - 49 (1996)

42 Burke SE and Nelson RA: Endothelin-receptor antagonist FR 139317 reduces infarct size in a rabbit model when given before, but not after, coronary artery occlusion. J Cardiovasc Pharmacol Exp Ther **29**, 87 - 92 (1997)

43 Gonon AT, Wang QD, Shimizu M and Pernow J: The novel non-peptide selective endothelin A receptor antagonist LU 135 252 protects against myocardial ischaemic and reperfusion injury in the pig. Acta Physiol Scand **163**, 131 - 137 (1998)