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European Journal of Pharmacology 478 (2003) 61–71
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Pharmacological characterization of YM598, an orally active and highly potent selective endothelin ET_A receptor antagonist

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Received 1 May 2003; received in revised form 13 August 2003; accepted 19 August 2003

Abstract

We describe here the pharmacology of (*E*)-*N*-[6-methoxy-5-(2-methoxyphenoxy)[2,2′-bipyrimidin]-4-yl]-2-phenylethenesulfonamide monopotassium salt (YM598), a novel selective endothelin ET_A receptor antagonist synthesized through the modification of the ET_A/ET_B non-selective antagonist, bosentan. YM598 inhibited [^{125}I]endothelin-1 binding to cloned human endothelin ET_A and ET_B receptor, with K_i of 0.697 and 569 nM, and inhibited endothelin-1-induced increases in intracellular Ca^{2+} concentration in human and rat endothelin ET_A receptor. YM598 also inhibited endothelin-1-induced vasoconstriction in isolated rat aorta with a pA_2 value of 7.6. In vivo, YM598 inhibited the pressor response to big endothelin-1, a precursor peptide of endothelin-1. DR_2 values of YM598 in pithed rats were 0.53 mg/kg, i.v. and 0.77 mg/kg, p.o., and its antagonism in conscious rats was maintained for more than 6.5 h at 1 mg/kg, p.o. In contrast, YM598 had no effect on the sarafotoxin S6c-induced depressor or pressor responses. YM598 showed not only superior antagonistic activity and higher-selectivity for endothelin ET_A receptor in vitro, but at least a 30-fold higher potency in vivo than bosentan. In conclusion, YM598 is a potent and orally active selective endothelin ET_A receptor antagonist.

Keywords: YM598; Antagonist; Endothelin ET_A receptor

1. Introduction

Endothelin, a potent vasoconstrictive 21-amino acid peptide, was originally isolated from the conditioned medium of cultured porcine vascular endothelial cells in 1988 (Yanagisawa et al., 1988). Since that time, the family of endothelin peptides (endothelin-1, endothelin-2, endothelin-3) has been detected in humans (Inoue et al., 1989). In addition, sarafotoxin, a cardiotoxic peptide isolated from snake venom, has been shown to have a high degree of homology with endothelin (Kloog et al., 1988). The physiological actions of endothelin are mediated by binding of these peptide ligands to specific cell surface endothelin receptors, two subtypes of which have been cloned and stably expressed in mammals (Arai et al., 1990; Sakurai et al., 1990). Endothelin ET_A receptors exhibit selectivity for endothelin-1 and endothelin-2 over endothelin-3 and sarafotoxin S6c, whereas endothelin ET_B receptors have nearly equipotent affinity for all known members of the endothelin family including sarafotoxin S6c (Arai et al., 1990; Sakurai et al., 1990; Williams et al., 1991b). In the cardiovascular system, endothelin ET_A receptors are expressed abundantly on smooth muscle cells and cardiac myocytes, and mediate contraction and mitogenic actions of endothelin (Sakurai et al., 1992; Ohlstein and Douglas, 1993). Endothelin ET_B receptors are expressed predominantly on vascular endothelial cells and to a much lesser extent on vascular smooth muscle cells, and cause relaxation of constricted smooth muscle via release of intracellular mediators such as nitric oxide and prostacyclin (Warner et al., 1989). Largely because of its ability to constrict vascular and nonvascular smooth muscle, endothelin has been implicated in the pathogenesis of myocardial infarction, hypertension, heart failure, atherosclerosis, cerebral and coronary vasospasm, renal failure and asthma. In addition, it has been reported that plasma endothelin-1 levels are increased in these diseases (for review, see Rubanyi and

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doi:10.1016/j.ejphar.2003.08.031

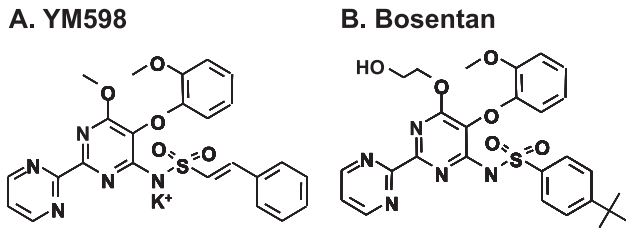

Fig. 1. Chemical structure of YM598 (A) and bosentan (B).

Polokoff, 1994). In some animal models, endothelin receptor antagonists have proven to be efficacious, suggesting that these agents will be useful for the treatment of cardiovascular diseases (for review, see in Miyauchi and Masaki, 1999).

It has been reported that replacement of the benzenesulfonamide group of the endothelin ET_A/ET_B non-selective antagonist bosentan with 2-phenylethenylsulfonamide led to the discovery of a new class of endothelin ET_A selective antagonists (Clozel et al., 1994; Harada et al., 2001a). (*E*)-*N*-[6-methoxy-5-(2-methoxyphenoxy)[2,2′-bipyrimidin]-4-yl]-2-phenylethenesulfonamide monopotassium salt (YM598, Fig. 1A) is a novel, potent and orally active selective endothelin ET_A receptor antagonist synthesized at Yamanouchi Pharmaceutical, through the modification of bosentan (Fig. 1B) (Harada et al., 2001b). This is the first report on the pharmacological profile of YM598 as investigated in vitro and in vivo.

2. Materials and methods

2.1. Materials

YM598, bosentan and 3-{[6-{[(4-*tert*-butylphenyl)sulfonyl]amino}-5-(2-methoxyphenoxy)-2,2′-bipyrimidin-4-yl]oxy}-*N*-(2,6-dimethylphenyl)propanamide (K-8794) were synthesized at Yamanouchi Pharmaceutical (Ibaraki, Japan). [^{125}I]endothelin-1 (specific activity, 2200 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA, USA). Endothelin-1, big endothelin-1 and sarafotoxin S6c were from Peptide Institute (Osaka, Japan). Fura-2-acetoxymethylester (Fura 2-AM) was from Dohjin Chemicals (Kumamoto, Japan). Chinese hamster ovary (CHO) cells and rat vascular smooth muscle (A10) cells were from the American Tissue Culture Collection (Rockville, MD, USA). Minimum essential medium (MEM)-α, Dulbecco's modified Eagle's medium (DMEM), LipofectAMINE, fetal bovine serum and trypsin-EDTA were from Gibco (Grand Island, NY, USA). Bovine serum albumin was from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade purchased from Wako (Osaka, Japan).

2.2. Cell culture

CHO cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in MEM-α supplemented with nucleosides and 10% fetal bovine serum. CHO cells were stably transfected with the human endothelin ET_A and ET_B receptor expression constructs by the LipofectAMINE method and stable transfectants were selected with 1 μM amethopterin. A10 cells were cultured at 37 °C under a humidified 95% air/5% CO_2 atmosphere in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin.

2.3. Membrane preparation

Plasma membranes were prepared from CHO cells, rat A10 cells (expressing rat endothelin ET_A receptors, Lin et al., 1991) and rat cerebellum (expressing rat endothelin ET_B receptors, Williams et al., 1991a). Confluent CHO cells and A10 cells were washed with phosphate-buffered saline (PBS) and harvested into ice-cold 10 mM Tris–HCl, pH 7.4, containing 5 mM EDTA followed by homogenization. Male Wistar rats were sacrificed by decapitation and their cerebellum was removed into ice-cold 10 mM Tris–HCl, pH 7.4, containing 5 mM EDTA followed by homogenization. Cells and tissue lysate were centrifuged at $800 \times g$ for 10 min to remove unbroken cells and nuclei. The supernatant was centrifuged at $100{,}000 \times g$ for 30 min at 4 °C, and the pellet was resuspended in 50 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$ and stored in small aliquots which included sufficient protein for about 100 assays (20 μg for recombinant human endothelin ET_A and ET_B receptors, 2.4 mg for rat A10, 240 μg for rat cerebellum) at −80 °C until use. Protein was determined by the method of Bradford using bovine serum albumin as a standard.

2.4. Binding assay

Endothelin receptor binding assays were performed according to the method of Webb et al. (1993) as described previously with modifications. Briefly, competition studies were performed in a total volume of 250 μl containing 25 μl [^{125}I]endothelin-1 (200 pM for recombinant human endothelin ET_A and ET_B receptors, 500 pM for rat A10 and cerebellum), 25 μl competing compounds or 100 nM endothelin-1 to define nonspecific binding, and incubation buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 0.01% bovine serum albumin). Reaction was initiated by the addition of 200 μl of plasma membrane suspension reconstructed by incubation buffer, which contained 0.2 μg (recombinant human endothelin ET_A and ET_B receptor), 24 μg (rat A10), or 2.4 μg (rat cerebellum) of membrane protein. After the incubation period (3 h for recombinant human endothelin ET_A and ET_B receptor, 2 h for rat A10 and cerebellum, room temperature), the reaction was terminated by the addition of 3 ml of ice-cold incubation buffer followed by rapid filtration through Whatman GF/C filters. The filters were rinsed twice and the radioactivity retained on the filters was counted using a gamma counter at 60%

efficiency. Each assay was performed 4–6 times in duplicate. For saturation binding studies, each plasma membrane preparation was incubated with various concentrations of [^{125}I]endothelin-1 (1.5–800 pM) in the absence or presence of different concentrations of YM598. Assay conditions were the same as those described for competition binding. Maximal specific binding was calculated as total binding minus nonspecific binding. The concentration of test compound that caused 50% inhibition (IC_{50}) of the specific binding of [^{125}I]endothelin-1 was determined by regression analysis of displacement curves. Inhibitory dissociation constant (K_i) was calculated from the following formula: $K_i = IC_{50}/(1+[C]/K_d)$, where $[C]$ is the concentration of radioligand present in the tubes and K_d is the dissociation constant of radioligand obtained from the Scatchard plot (Cheng and Prusoff, 1973).

2.5. Receptor specificity

The specificity of YM598 for endothelin receptors was examined by measuring the ability of YM598 to compete with receptor-specific ligands in 59 different ligand binding assays. YM598 was tested at 10 μM. These assays were performed by NovaScreen (Hanover, MD, USA).

2.6. In vitro functional inhibitory potency: measurement of intracellular Ca^{2+} concentration

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed according to the method described previously (Grynkiewicz et al., 1985; Tahara et al., 1998) with minor modification. CHO cells expressing human endothelin ET_A receptor and A10 cells were plated on cover glasses (13.5 mm in diameter) and serum-starved for 12 h. Cell monolayers were loaded in Hank's balanced salt solution (HBSS: 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.4) with Fura 2-AM (4 μM/cover glass) for 1 h at 37 °C. They were then washed, transferred to Fura 2-free HBSS and incubated for an additional 30 min at 37 °C. The cover glass was placed into a quartz cuvette containing 2 ml HBSS buffer and maintained at 37 °C with continuous stirring. When thermal equilibrium was reached, the fluorescence signal was recorded with a CAF-110 spectrofluorometer (Japan Spectrometer, Tokyo, Japan) with 340/380 nm excitation and 500 nm emission wavelengths. After recording the baseline signal for a short while, vehicle or test compound was added to the cuvette. Two minutes after the test compounds were added, endothelin-1 was added to the cuvette to stimulate the mobilization of $[Ca^{2+}]_i$ in the presence or absence of the test compounds. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (R_{max}) with the nonfluorescent Ca^{2+} ionophore, ionomycin (25 μM), after which minimal fluorescence (R_{min}) was obtained by adding 3 mM EGTA. From the ratio of fluorescence at 340 and 380 nm, $[Ca^{2+}]_i$ was calculated using the following equation: $[Ca^{2+}]_i\ (nM) = K_d \times [(R - R_{min})/(R_{max} - R)] \times b$. The term b is the ratio of fluorescence of Fura 2 at 380 nm in zero and saturation Ca^{2+}. R_{max}, R_{min}, and the value of b were yielded twice, at the beginning and end of the experiment, and the mean values were used in calculation. K_d is the dissociation constant of Fura 2 for Ca^{2+}, assumed to be 224 nM. The activity of the test compound was evaluated by expressing the increase in $[Ca^{2+}]_i$ as the percentage of that to treatment with endothelin-1 (10 nM), determined in each experiment using the same preparation of cells. IC_{50} values of test compound were determined by regression analysis.

2.7. In vitro functional inhibitory potency: rat aortic ring contraction

Antagonism of endothelin-1-induced vasoconstriction was evaluated with isolated rat aortic rings because this response is mediated by endothelin ET_A receptors in this tissue (Panek et al., 1992). Male Wistar rats (320–370 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and the thoracic aorta was quickly removed and placed in a Krebs–Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 11.1 mM glucose) with 95:5 O_2/CO_2 to maintain pH 7.4. The endothelium was removed by gentle rubbing of the intimal surface using a small cotton ball and each ring was suspended in a 10-ml isolated siliconized organ chamber containing gassed (95:5 O_2/CO_2) and warmed (37 °C) Krebs–Henseleit solution. Vessel segments were attached to an isometric force transducer (TB-611T, Nihon Kohden, Tokyo, Japan) linked to a physiographic recorder for monitoring tension change. Baseline tension was set at 1.0 g and the tissues were allowed to equilibrate for 1 h. The tissues were maximally contracted with phenylephrine (1 μM) followed by challenge with acetylcholine (1 μM). A negative relaxant response to acetylcholine confirmed the absence of endothelium. After washing out these agents, the rings were stimulated to contract with 60 mM KCl repeatedly until the contractile response to KCl became stable before starting experiments. Cumulative concentration–response curves to endothelin-1 were performed in the presence or absence of YM598 after a 30-min pretreatment period. Contractile responses were expressed as a percentage of the response elicited by 60 mM KCl. The effective concentration of endothelin-1 causing 50% maximum response (EC_{50}) in the presence or absence of YM598 was determined by regression analysis. The negative logarithm of the molar concentration of antagonist required to produce a 2-fold rightward shift of concentration–response curves to agonist (pA_2) value was determined as an index of potency by the equation: $pA_2 = \log(\text{concentration ratio} - 1) - \log[B]$, where concentration ratio is the ratio of EC_{50} values with and without antagonist and

[*B*] is the concentration of antagonist. Regression analysis of the plot log (concentration ratio − 1) against the log [*B*] (Schild plot) allowed us to confirm the competitive nature of the antagonist by assessing its slope (Arunlakshana and Schild, 1956).

2.8. In vivo functional inhibitory potency: inhibition of pressor response to big endothelin-1 in pithed rats

In vivo antagonistic activity in pithed rats was evaluated according to the method of Clozel et al. (1994) as described previously. Briefly, after tracheal intubation, male Wistar rats (280–320 g) were pithed with a steel rod under sodium pentobarbital anesthesia (60 mg/kg, i.p.) and artificially ventilated with room air. The right common carotid artery and the left femoral vein were cannulated for blood pressure measurements and i.v. administration of drugs, respectively. After stabilization of blood pressure, various doses of (1 ml/kg) YM598 or vehicle (distilled water) were injected. Five minutes later, the first dose of big endothelin-1 was injected intravenously in a volume of 0.5 ml/kg. Increasing doses were injected in a cumulative manner (0.1–3.2 nmol/kg, i.v.), with each dose being given after stabilization of the effect of the previous dose on blood pressure. In another series of experiments, the oral activity of YM598 was tested. Various doses of (5 ml/kg) YM598 or vehicle (0.5% methyl cellulose) were orally administered by gastric gavage with a cannula. About 20 min later, the rats were anesthetized with sodium pentobarbital, and 30 min after dosing were pithed and ventilated. About 1 h after oral administration of YM598, waiting for stabilization of blood pressure, the first dose of big endothelin-1 was injected intravenously. In this study, DR_2 value was defined as the dose of YM598 required to produce a 2-fold rightward shift of dose–response curves to big endothelin-1 in diastolic blood pressure.

2.9. In vivo functional inhibitory potency: effect on big endothelin-1- or sarafotoxin S6c-induced changes in blood pressure in conscious normotensive rats

Male Wistar rats (270–360 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.). The right common carotid artery and the left jugular vein were cannulated with a polyethylene tube for determination of blood pressure and heart rate, and for i.v. administration of big endothelin-1. The animals were allowed to recover for 2 days after the operation, during which time they were housed in individual cages with free access to rat chow and water. Rats were then placed in individual cages, and big endothelin-1 (0.5 nmol/kg) was intravenously administered three times at intervals of 1 h. Thirty minutes after the third administration of big endothelin-1, various doses of YM598, bosentan or vehicle (0.5% methyl cellulose) (5 ml/kg) were orally administered by gastric gavage with a cannula. Repeated doses of big endothelin-1 were administered 30 min later, followed every 60 min over a 6-h period and finally again 24 h after administration with YM598, bosentan, or vehicle. The activity of the test compound was evaluated by expressing the pressor response in mean blood pressure as the percentage of that to the third administration of big endothelin-1. In another set of experiments, the effects on sarafotoxin S6c-induced depressor and pressor responses were also tested. Instead of big endothelin-1, sarafotoxin S6c (0.3 nmol/kg) was intravenously administered and various doses of YM598, K-8794, a selective endothelin ET_B receptor antagonist (Sonoki et al., 1997), and vehicle were orally administered. The activity of the test compound was evaluated by expressing the depressor and pressor response as the percentage of those to the third administration of sarafotoxin S6c.

2.10. Expression of results

Values are expressed as the mean ± S.E.M. or mean value with 95% confidence interval. *N* represents the number of animals or separate experiments in each group unless otherwise noted. Data were analyzed using the SAS software (SAS Institute, NC, USA). The difference between control and drug treatment groups was analyzed by a two-way repeated measures of analysis of variance (ANOVA). When significant differences were identified by two-way repeated measures ANOVA, Dunnett's multiple range test was used. A *P* value less than 0.05 was considered to be significant.

2.11. Ethical considerations

The protocol for this study was approved by the Animal Ethical Committee of Yamanouchi Pharmaceutical.

3. Results

3.1. Binding assays

The potency of YM598 in inhibiting [^{125}I]endothelin-1 binding to endothelin ET_A and ET_B receptors was examined using membranes from CHO cells expressing human endothelin ET_A and ET_B receptors, and rat cells and tissues. YM598 inhibited the specific binding of [^{125}I]endothelin-1 to endothelin ET_A and ET_B receptors in a concentration-dependent manner (Fig. 2A and B). K_i values of YM598 were 0.697 ± 0.132 nM ($n = 6$) and 1.53 ± 0.16 nM ($n = 4$) for human and rat endothelin ET_A receptors, respectively. In contrast, YM598 exhibited low affinities for human and rat endothelin ET_B receptors, with K_i values of 569 ± 90 nM ($n = 6$) and 155 ± 11 nM ($n = 4$), respectively. Binding experiments were also performed for bosentan (Fig. 2A and B). K_i values of bosentan were 2.28 ± 0.26 nM ($n = 6$) and 7.99 ± 1.68 nM ($n = 4$) for human and rat endothelin

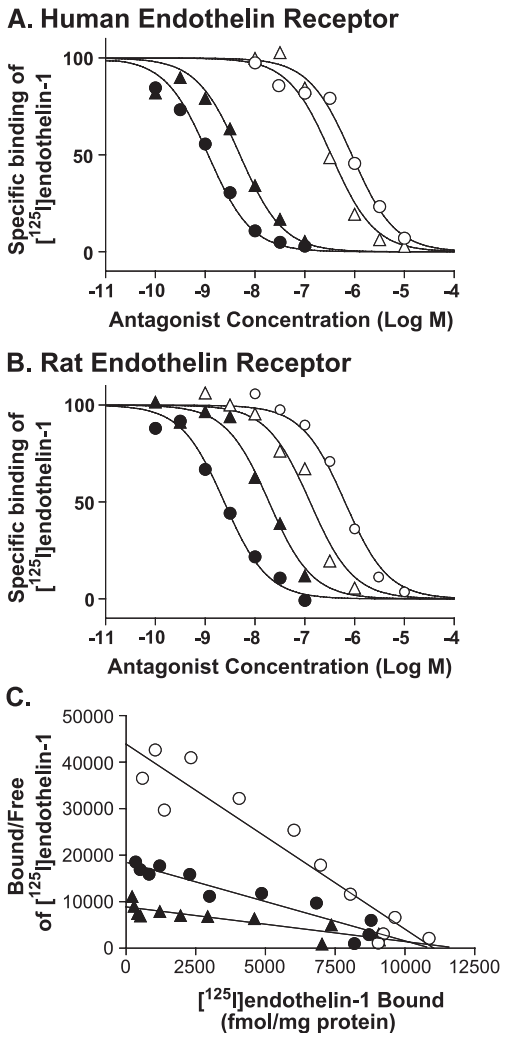

Fig. 2. Binding properties of YM598 to human and rat endothelin ET receptors. (A) Inhibition of specific binding of [^{125}I]endothelin-1 human endothelin ET_A and ET_B receptors by YM598 (●, ET_A; ○, ET_B) and bosentan (▲, ET_A; △, ET_B). Results are representative data from six experiments performed in duplicate. Results are expressed as a percentage of specific binding in the absence of unlabeled agents. (B) Inhibition of specific binding of [^{125}I]endothelin-1 to rat endothelin ET_A and ET_B receptors by YM598 (●, ET_A; ○, ET_B) and bosentan (▲, ET_A; △, ET_B). Results are representative data from four experiments performed in duplicate. Results are expressed as a percentage of specific binding in the absence of test compounds. (C) Scatchard plots of [^{125}I]endothelin-1 binding to human endothelin ET_A receptors in the absence (○) or the presence of 1 (●) and 3 nM (▲) YM598. Results are representative data from three experiments performed in duplicate.

ET_A receptors, and those for human and rat endothelin ET_B receptors were 174 ± 19 nM ($n = 6$) and 34.9 ± 3.0 nM ($n = 4$), respectively.

To further evaluate the properties of the interaction between YM598 and the endothelin ET_A receptor, [^{125}I]endothelin-1 saturation binding studies were performed using membranes from CHO cells expressing human endothelin ET_A receptor. It was evident that increasing the concentration of YM598 caused successive decreases in the slope of the lines (Fig. 2C), indicating a kind of change in the K_d value of [^{125}I]endothelin-1 binding. YM598 did not have a significant effect on the B_{max} (maximal [^{125}I]endothelin-1 binding).

3.2. Receptor specificity

The specificity of YM598 was determined in 59 radioligand binding assays. Table 1 shows that YM598 had no significant inhibiting activity at 10 μM, except at the diltiazem binding site. However, in a functional assay (KCl-induced contraction in rabbit aorta), YM598 (up to 10 μM) did not inhibit L-type Ca^{2+}-channel-mediated (KCl-induced) contraction, whereas diltiazem did (data not shown).

3.3. In vitro functional inhibitory potency: measurement of intracellular Ca^{2+} concentration

Addition of endothelin-1 to Fura 2-loaded CHO cells expressing human endothelin ET_A receptor and rat A10 cells resulted in a concentration-dependent increase in $[Ca^{2+}]_i$, whereas sarafotoxin S6c did not exert any effect at concentrations up to 100 nM. EC_{50} values of endothelin-1 were 10.5 ± 4.3 nM ($n = 6$) and 6.1 ± 3.8 nM ($n = 6$) in CHO cells expressing human endothelin ET_A receptor and rat A10 cells, respectively. The maximal effects were attained at 100 nM endothelin-1 (CHO cells: 386 ± 103 nM, rat A10 cells: 207 ± 32 nM, $n = 6$ in each cell) and their quantities were considered to be almost the same as those induced by other agonists (Roullet et al., 1997). YM598 concentration-dependently inhibited the increase in $[Ca^{2+}]_i$ induced by 10 nM endothelin-1 in both CHO cells and A10 cells, with IC_{50} values of 26.2 ± 3.6 nM for CHO cells ($n = 6$) and 26.7 ± 8.2 nM for A10 cells ($n = 6$), respectively (Fig. 3). Bosentan also inhibited the $[Ca^{2+}]_i$ increases in both cells with IC_{50} values of 53.5 ± 9.2 nM for CHO cells ($n = 6$) and 39.4 ± 5.5 nM for A10 cells ($n = 6$), respectively (Fig. 3). YM598 and bosentan did not show any agonistic or antagonistic effects on the basal $[Ca^{2+}]_i$.

3.4. In vitro functional inhibitory potency: aortic ring contraction

Endothelin-1 induced contraction of rings prepared from rat thoracic aorta in a concentration-dependent manner (Fig. 4). The contractile response induced by endothelin-1 in this tissue is mediated by the endothelin ET_A receptor (Panel et al., 1992). YM598 (10–1000 nM) antagonized this endothelin-1-induced vasoconstriction without reducing the maximum response (Fig. 4), but had no direct effect on basal tone even at 1000 nM. The pA_2 value of YM598 analyzed by the Schild plot was 7.6 (95% confidence interval: 6.5–7.8), with a slope of 0.84 ($n = 6$–8).

Table 1
Effect of 10 μM YM598 on radioligand binding in various ligand-specific bindings

Receptor	Radioligand	% inhibition
Adenosine	[^{3}H]NECA	− 1.81
Alpha-1	[^{3}H]Prazosin	13.35
Alpha-2	[^{3}H]RX821002	5.36
Beta	[^{3}H]Dihydroalprenolol	− 34.13
Benzodiazepine (peripheral)	[^{3}H]PK11195	− 0.37
Dopamine (ns)	[^{3}H]Spiperone	− 5.16
$GABA_A$	[^{3}H]GABA	12.78
Glutamate (AMPA)	[^{3}H]AMPA	− 2.39
Glutamate (Kainate)	[^{3}H]Kainic Acid	− 1.46
Glutamate (NMDA)	[^{3}H]CGP39653Tiotidine	7.69
Glycine	[^{3}H]Strychnine	− 12.40
Histamine H_1	[^{3}H]Pyrilamine	− 45.40
Histamine H_2	[^{3}H]Tiotidine	14.37
Imidazoline 2 (central)	[^{3}H]2-BFI	− 5.09
Melatonin	[^{125}I]2-Iodomelatonin	− 3.32
Muscarinic (ns central)	[^{3}H]QNB	− 1.18
Muscarinic (ns peripheral)	[^{3}H]QNBStrychnine	4.44
Nicotinic	[^{3}H]Epibatidine	− 1.97
Purinergic P_{2Y}	[^{35}S]ADPβS	21.74
Serotonin (ns)	[^{3}H]LSD	− 153.0
Sigma (ns)	[^{3}H]DTG	− 10.90
Opiate (ns)	[^{3}H]Naloxone	16.76
Calcium (L-type)	[^{3}H]Diltiazem	106.92
Calcium (L-type)	[^{3}H]Nitrendipine	− 21.08
Calcium (N-type)	[^{125}I]Conotoxin GVIA	17.62
$GABA_A$ (chloride)	[^{3}H]TBOB	5.03
Glutamate (MK-801)	[^{3}H]MK-801	− 0.93
Potassium (ATP)	[^{3}H]Glibenclamide	11.27
Potassium (Ca^{2+} Activated)	[^{125}I]Charybdotoxin	3.78
Sodium	[^{3}H]Saxitoxin	− 10.85
Norepinephrine uptake	[^{3}H]Nisoxetine	− 22.75
Serotonin uptake	[^{3}H]Citalopram	9.93
Estrogen	[^{125}I]3,17β-Estradiol	− 34.95
Glucocorticoid	[6,7-^{3}H]Triamcinolone acetonid	30.14
Progesterone	[^{3}H]Promogestone	9.78
Testosterone	[^{3}H]Methyltrienolone	15.27
Angiotensin AT_1	[^{125}I]Sar^1,Ile^8-angiotensin II	5.20
Angiotensin AT_2	[^{125}I]Tyr^4-angiotensin II	45.34
Bradykinin 2	[^{3}H]Bradykinin	− 16.69
CGRP (central)	[^{125}I]CGRP	16.52
CGRP (peripheral)	[^{125}I]CGRP	35.38
Cholecystokinin A	[^{125}I]Cholecystokinin-8	14.36
Cholecystokinin B	[^{125}I]Cholecystokinin-8	19.67
Neurokinin NK_1	[^{3}H]Substance P	− 3.22
Neurokinin NK_2	[^{125}I]Neurokinin A	37.55
Neuropeptide Y (ns)	[^{125}I]Neuropeptide Y	2.91
Neurotensin	[^{3}H]Neurotensin	− 11.88
Somatostatin (ns)	[^{125}I]Somatostain-14	13.07
Vasoactive intestinal peptide	[^{125}I]Vasoactive intestinal peptide	13.83
Vasopressin V_1	[^{3}H]Vasopressin-1 antagonist	24.37
Leukotriene B_4	[^{3}H]Leukotriene B_4	7.34
Leukotriene D_4	[^{3}H]Leukotriene D_4	5.33
Thromboxane A_2	[^{3}H]SQ29,548	22.26
Atrial natriuretic peptide A	[^{125}I]Atrial natriuretic peptide	2.27
Corticotropin releasing factor	[^{125}I]Tyr^0-oCorticotropin releasing factor	− 5.07
Epidermal growth factor	[^{125}I]Epidermal growth factor	− 10.14
Oxytocin	[^{3}H]Oxytocin	− 4.98
Platelet activating factor	[^{3}H]Hexadecyl Platelet activating factor	2.56
Thyrotropin releasing hormone	[^{3}H]Thyrotropin releasing hormone	− 2.76

ns = non-selective. GABA = γ-aminobutylic acid. AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid. NMDA = *N*-methyl-D-aspartate. ATP = adenosine 5′-triphosphate. CGRP = calcitonin gene-related peptide. NECA = 5′-(*N*-ethylcarboxamido)adenosine. BFI=(2-benzofuranyl)-2-imidazoline. QNB = quinuclidinyl benzilate. ADPβS = adenosine 5′-*O*-2-thiodiphosphate. LSD = lysergic acid diethylamide. DTG = 1,3-di(2-tolyl)-guanidine. TBOB = *tert*-butyl-bicyclo-orthobenzoate.

3.5. In vivo functional inhibitory potency: inhibition of pressor response to big endothelin-1 in pithed rats

The i.v. administration of big endothelin-1 (0.1–3.2 nmol/kg), precursor peptide of endothelin-1, induced dose-dependent pressor responses and elicited a maximum in-

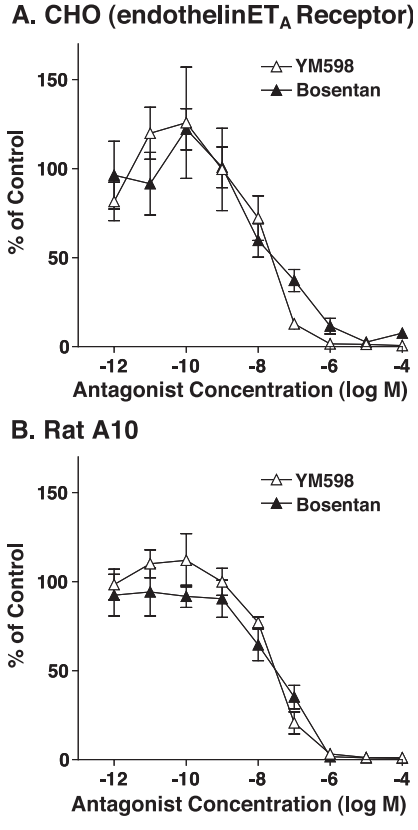

Fig. 3. Inhibitory effect of YM598 (△) and bosentan (▲) on the increase in $[Ca^{2+}]_i$ induced by endothelin-1 in CHO cells expressing human endothelin ET_A receptor (A) and in rat A10 cells (B). Results are expressed as a percentage of the $[Ca^{2+}]_i$ increase induced by 10 nM ET-1. Each data point with vertical bar represents the mean ± S.E.M. of six experiments. CHO = Chinese hamster ovary cells.

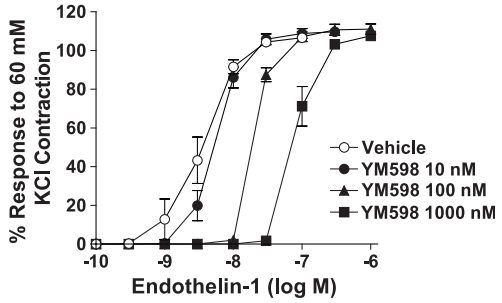

Fig. 4. Effect of YM598 (○, vehicle; ●, 10; ▲, 100; ■, 1000 nM) on endothelin-1-induced contraction of isolated rat aorta. Endothelin-1 concentration–response curves are shown in the presence of increasing concentrations of YM598. Results as expressed as a percentage of the maximal response by 60 mM KCl. Each data point with vertical bar represents the mean ± S.E.M. of six to eight experiments.

crease in diastolic blood pressure of about 100 mm Hg in pithed male Wistar rats (Fig. 5). YM598 (0.1, 0.3, 1 mg/kg) dose-dependently inhibited this big endothelin-1-induced pressor response and produced a parallel rightward shift of it following both i.v. and p.o. administration, with DR_2 values of 0.53 mg/kg, i.v. and 0.77 mg/kg, p.o., respectively (Fig. 5). The calculated i.v./p.o. ratio from these DR_2 values was 0.69. On the other hand, DR_2 values of bosentan were

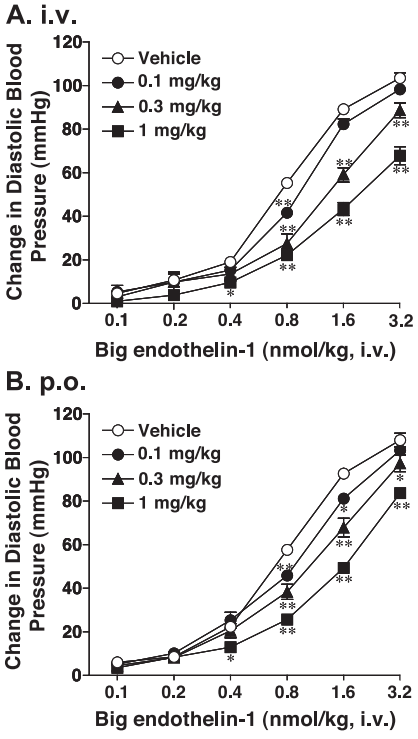

Fig. 5. Effects of i.v. (A) and p.o. (B) administration of YM598 (○, vehicle; ●, 0.1; ▲, 0.3; ■, 1 mg/kg) on the increase in diastolic blood pressure induced by increasing doses of big endothelin-1 in pithed rats. Each data point with vertical bar represents the mean ± S.E.M. of six experiments. Statistical analysis was performed by two-way repeated measures ANOVA followed by Dunnett's multiple range test. *$P<0.05$, **$P<0.01$ compared with corresponding vehicle values.

5.1 mg/kg, i.v. and 25.2 mg/kg, p.o., and the calculated i.v./p.o. ratio of bosentan was 0.20.

3.6. In vivo functional inhibitory potency: effects on big endothelin-1- or sarafotoxin S6c-induced changes in blood pressure in conscious normotensive rats

Effects of YM598 and bosentan on big endothelin-1-induced pressor response were investigated in conscious normotensive rats. Intravenous administration of big endothelin-1 (0.5 nmol/kg) elicited an increase in mean blood pressure (113.1 ± 3.3 to 169.5 ± 3.7 mm Hg) that reached a peak 10 to 15 min after injection and returned to the baseline within 1 h in conscious rats. Mean blood pressure was not altered 30 min after the oral administration of YM598 (0.1, 0.3, 1 mg/kg). Oral administration of YM598 (0.1, 0.3, 1 mg/kg) dose-dependently inhibited this big endothelin-1-induced pressor response, with maximum inhibitory effect observed 30 min after oral administration at every dose (Fig. 6A). At a dose of 1 mg/kg, p.o., YM598 produced approx-

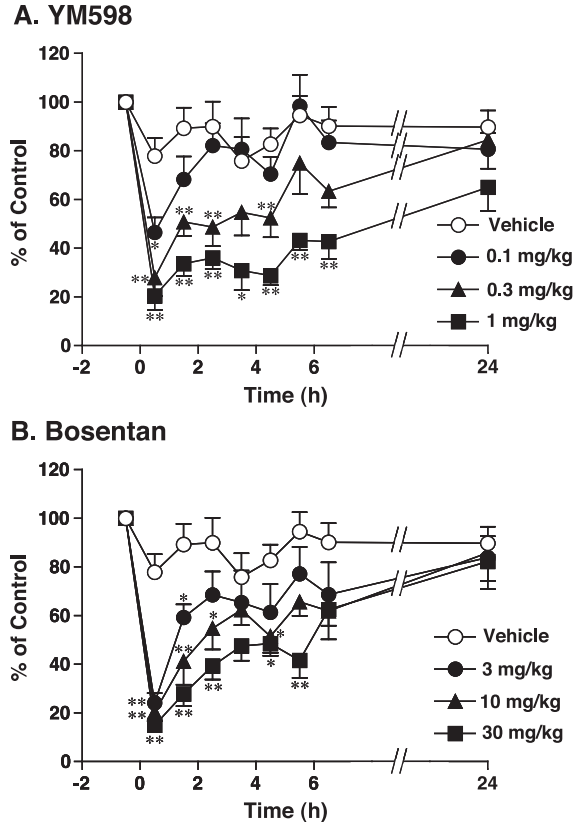

Fig. 6. Effects of YM598 (A) and bosentan (B) on big endothelin-1-induced (0.5 nmol/kg, i.v.) increase in mean blood pressure in conscious rats after oral dosing. Results are expressed as a percentage of the increase in mean blood pressure induced by 0.5 nmol/kg big endothelin-1 before administration of test compounds. Each data point with vertical bar represents the mean ± S.E.M. of six experiments. Statistical analysis was performed by two-way repeated measures ANOVA followed by Dunnett's multiple range test. *$P<0.05$, **$P<0.01$ compared with vehicle value at the corresponding time.

imately 80% inhibition of the big endothelin-1-induced pressor response, and approximately 60% inhibition was sustained for at least 6.5 h. Moreover, there was approximately 25% inhibition even at 24 h after oral administration at a dose of 1 mg/kg (vehicle: 89.7 ± 6.8%, YM598: 65.0 ± 9.7%, Fig. 6A). Oral administration of bosentan (3, 10, 30 mg/kg) also dose-dependently inhibited the pressor response induced by big endothelin-1 (0.5 nmol/kg), with maximum inhibitory effect observed 30 min after oral dosing (Fig. 6B). However, at least a 30-fold higher oral dose (30 mg/kg) of bosentan, compared to YM598, was required to produce a similar extent and duration of inhibition of the pressor response to big endothelin-1 (Fig. 6B).

Effects of YM598 and K-8794, a selective ET_B receptor antagonist (Sonoki et al., 1997), on depressor and pressor responses induced by the selective ET_B receptor agonist sarafotoxin S6c were also investigated in conscious normotensive rats. Intravenous administration of sarafotoxin S6c (0.3 nmol/kg) produced a transient depressor response (− 23.9 ± 1.7 mm Hg) followed by a sustained pressor response (30.6 ± 2.1 mm Hg). Oral administration of YM598 (1, 3, 10 mg/kg, p.o.) did not significantly inhibit the depressor and pressor responses induced by sarafotoxin S6c at all doses (two-way ANOVA repeated measures) (Fig. 7A,B). On the other hand, oral administration of K-8794 (0.3 to 30 mg/kg) dose-dependently inhibited the initial depressor and pressor responses induced by sarafotoxin S6c (Fig. 7C,D). Bosentan at 30 mg/kg p.o. also inhibited the depressor response to sarafotoxin S6c but not the pressor response to sarafotoxin S6c (data not shown).

4. Discussion

The development of high-affinity and subtype-selective endothelin receptor antagonists provides a tool by which we

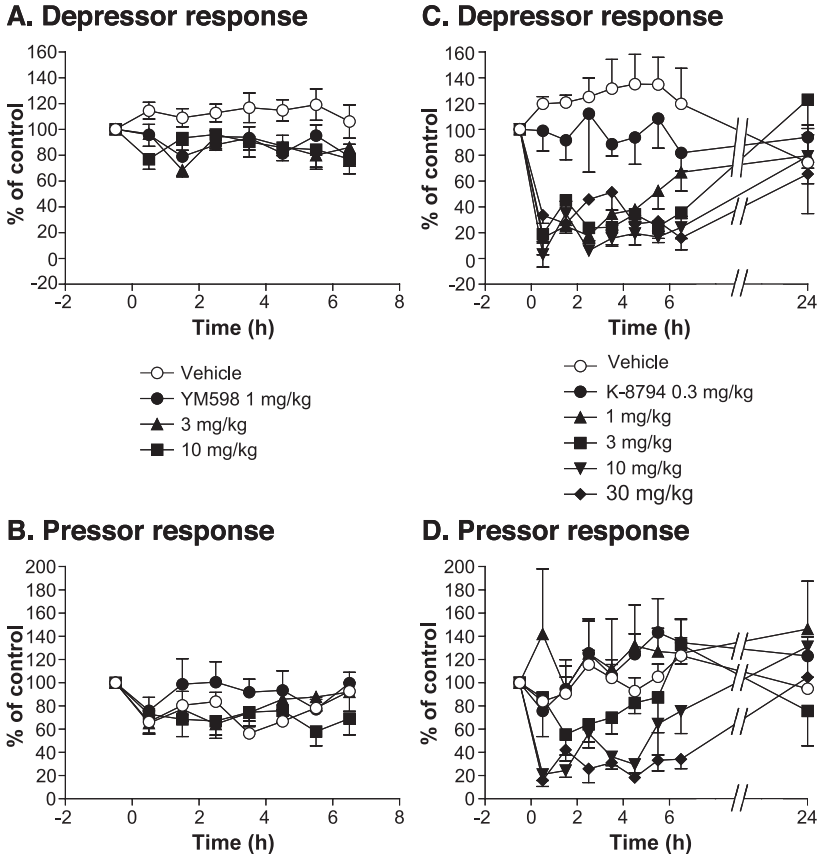

Fig. 7. Effects of YM598 (A, B) and K-8794 (C, D) on sarafotoxin S6c-induced (0.3 nmol/kg, i.v.) changes in mean blood pressure in conscious rats after oral dosing. Results are expressed as a percentage of the decrease (A, C) or increase in mean blood pressure induced by 0.3 nmol/kg sarafotoxin S6c before administration of test compounds. Each data point with vertical bar represents the mean ± S.E.M. of six to eight (A, B) or three to nine (C, D) experiments. (A) Depressor response to sarafotoxin S6c in the presence of YM598. Statistical analysis was performed by two-way repeated measures ANOVA (not significant). (B) Pressor response to sarafotoxin S6c in the presence of YM598. Statistical analysis was performed by two-way repeated measures ANOVA (not significant). (C) Depressor response to sarafotoxin S6c in the presence of K-8794. Statistical analysis was performed by two-way repeated measures ANOVA followed by Dunnett's multiple range test compared with vehicle value at the corresponding time. K-8794 (1 to 30 mg/kg, p.o.) significantly inhibited sarafotoxin S6c-induced decrease in mean blood pressure for 0.5 to 6.5 h ($P < 0.05$). (D) Pressor response to sarafotoxin S6c in the presence of K-8794. Statistical analysis was performed by two-way repeated measures ANOVA followed by Dunnett's multiple range test compared with vehicle value at the corresponding time. K-8794 (10, 30 mg/kg, p.o.) significantly inhibited sarafotoxin S6c-induced increase in mean blood pressure (10 mg/kg: 0.5 h and 3.5 to 4.5 h; 30 mg/kg: 2.5 to 6.5 h, $P < 0.05$).

are able to clarify the role of endothelin in pathophysiology. To date, many pharmaceutical companies have reported the discovery of a number of non-peptide endothelin receptor antagonists, which show endothelin ET_A receptor selectivity or non-selectivity (Roux et al., 1997; Ohlstein et al., 1996, 1998; Opgenorth et al., 1996; Clozel et al., 1994). However, it is still unclear what kind of antagonists against which subtype of endothelin receptor may be more clinically useful. Some evidence suggests that selective endothelin ET_A receptor antagonists may be better. Firstly, the endothelin ET_B receptor is known to mediate the release of nitric oxide, which results in vasodilation, and endothelin ET_B receptor blockade abolishes this beneficial effect (Rubanyi and Polokoff, 1994). Secondly, the endothelin ET_B receptor is involved in the clearance of endothelin-1 from circulation, and endothelin ET_B receptor blockade causes an increase in plasma endothelin-1 concentration (Fukuroda et al., 1994; Willette et al., 1998). Thirdly, the administration of selective endothelin ET_B receptor antagonists in humans produced a decrease in forearm blood flow, which suggests that endothelin ET_B receptor antagonism causes vasoconstriction (Verhaar et al., 1998).

Thus, on the basis of the principle that endothelin ET_A receptor antagonists may be preferable as therapeutic drugs for diseases associated with endothelins, we tried to find a new selective endothelin ET_A receptor antagonist through the modification of bosentan, known as a non-peptide endothelin ET_A/ET_B mixed antagonist (Clozel et al., 1994; Harada et al., 2001b). The present studies describe the characterization of YM598, which is an orally active endothelin receptor antagonist and shows a good endothelin ET_A receptor selectivity over endothelin ET_B receptor in vitro and in vivo. In radioligand-binding assay, YM598 retained high affinities for the human and rat endothelin ET_A receptor with about 800- and 100-fold greater affinities for human and rat endothelin ET_A receptors than for their endothelin ET_B receptors (0.697 ± 0.132 and 569 ± 90 nM for human endothelin ET_A and ET_B receptor; 1.53 ± 0.16 and 155 ± 11 nM for rat endothelin ET_A and ET_B receptor). The affinities of bosentan for human endothelin ET receptors were reported to be 6.5 ± 0.4 and 343 ± 3.5 nM (Clozel et al., 1994), and these values were almost the same as those yielded in the present study (2.28 ± 0.26 and 174 ± 19 nM for human endothelin ET_A and endothelin ET_B receptors). In the present study, Bosentan showed approximately 80-fold and 4-fold greater selectivity for the endothelin ET_A receptor in humans and rats. So YM598 showed higher affinity and selectivity for the endothelin ET_A receptor than bosentan, and had no species difference with regard to antagonistic activity for endothelin-1 binding to endothelin receptors. To examine whether YM598 interacts reversibly or irreversibly with the endothelin ET_A receptor, we investigated [^{125}I]endothelin-1 saturation binding with human cloned endothelin ET_A receptor in the presence or absence of YM598. Pre-incubation of the endothelin ET_A receptor with YM598 reduced the affinity of [^{125}I]endothelin-1 binding without changing the B_{max} value, which indicated that YM598 interacts reversibly and competitively with the endothelin ET_A receptor.

To assess the specificity of YM598 for the endothelin receptor, YM598 was tested at 10 μM in a variety of radioligand competition assays using 59 receptors. YM598 did not affect radioligand binding except at the diltiazem binding site. In a functional assay (KCl-induced contraction in rabbit aorta), however, YM598 did not inhibit KCl-induced contraction. These data indicate that YM598 might not provide functional inhibition of the effects of L-type Ca^{2+}-channel blockers, including diltiazem. As a point of reference, a 10-μM concentration is approximately 10,000-fold higher than the K_i of YM598 for binding at the endothelin ET_A receptor. These data show the specificity of YM598 for the endothelin receptor.

The ability of YM598 to antagonize endothelin-1-induced functional responses in vitro was investigated by measuring the inhibitory effects of endothelin-1 on the increases in $[Ca^{2+}]_i$ in both CHO cells expressing human endothelin ET_A receptor and rat A10 cells. YM598 concentration-dependently antagonized the increase in $[Ca^{2+}]_i$ stimulated by 10 nM endothelin-1. Almost identical IC_{50} values in both cells indicate that the antagonistic activity of YM598 in humans is equal to that in rats. We also evaluated the effect of YM598 on the contractile response of isolated rat aorta for endothelin ET_A receptor-mediated contraction, because endothelin-1 is a potent constrictor of smooth muscle and the endothelin ET_A receptor is the predominant mediator of endothelin-1 activity in rat aorta (Panek et al., 1992). YM598 produced a parallel and rightward shift of the endothelin-1 concentration–response curve without affecting the maximal force to yield a pA_2 value of 7.6. These results indicate that YM598 is a functional endothelin ET_A receptor antagonist.

To evaluate the antagonistic effect of YM598 in vivo, we examined the effects of both intravenous and oral administration of YM598 in rats. The pressor response induced by exogenous big endothelin-1 is considered to be mediated by the endothelin ET_A receptor in rats (Haleen et al., 1993). In pithed rats, intravenous and oral administration of YM598 produced a parallel rightward shift of big endothelin-1-induced dose–response curves, with DR_2 values of 0.53 mg/kg, i.v. and 0.77 mg/kg, p.o. The calculated i.v./p.o. ratio from these DR_2 values was 0.69. In contrast, DR_2 values of i.v. and p.o. administration and calculated i.v./p.o. ratio of bosentan were 5.1 mg/kg, 25.2 mg/kg, and 0.20, which are almost equal to previously reported data (Clozel et al., 1994). The relative potencies of YM598 to bosentan in inhibiting exogenous big endothelin-1 were approximately 10-fold on i.v. and 30-fold on p.o. administration. These results indicate that YM598 has potent antagonistic activity in vivo as well as in vitro and may be easily absorbed by oral administration with a higher oral bioavailability than bosentan. We also evaluated the inhibitory effect of YM598 on the pressor response to exogenous big endothelin-1 in conscious normotensive rats at the same doses used in

pithed rats. The magnitude of maximum inhibition was about 55%, 70%, and 80% at the doses of 0.1, 0.3, and 1 mg/kg of YM598, 30 min after oral administration. At the same doses, the duration of action was 2.5 h, over 6.5 h, and over 24 h, while a 30-fold higher oral dose of bosentan was needed to bring about a response equal to that of YM598. These results suggest that YM598 has a long-lasting effect and that its oral antagonistic activity to endothelin response via the endothelin ET_A receptor is 30-fold more potent than that of bosentan in vivo.

Finally, to ascertain whether YM598 retains endothelin ET_A receptor selectivity in vivo, we investigated the effects of YM598 on sarafotoxin S6c-induced responses in conscious normotensive rats, and compared the results with those of K-8794, a selective endothelin ET_B receptor antagonist. Oral administration of YM598 at up to 10 mg/kg affected neither the depressor nor pressor responses to sarafotoxin S6c. However, K-8794 potently suppressed depressor and pressor responses to sarafotoxin S6c at 1 to 10 mg/kg oral administration. This result is consistent with those previously reported (Sonoki et al., 1997, Sawaki et al., 2000), which showed K-8794 to be ineffective on the pressor response to big endothelin-1 at 30 mg/kg in rats. These results indicate that YM598 is a selective endothelin ET_A receptor antagonist in vivo, at least up to 10 mg/kg.

In conclusion, we have developed a new selective endothelin ET_A receptor antagonist, YM598, through the modification of bosentan. YM598 is a potent, orally active endothelin ET_A receptor antagonist with a long duration of action. This compound blocks endothelin ET_A receptors but not endothelin ET_B receptors both in vitro and in vivo, and its endothelin ET_A receptor antagonistic activities were more potent than those of bosentan. Although it has not been clarified whether blockade of endothelin ET_A receptors only or of both endothelin ET_A and ET_B receptors is more beneficial in the long-term treatment of diseases associated with endothelins, the potency and selectivity of YM598 provide a new tool in analyzing the pathophysiological role of the endothelin ET_A receptor in various disorders in which endothelins are implicated.

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