Pheophorbide a, a Potent Endothelin Receptor Antagonist for Both ET_A and ET_B Subtypes

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Many crude drugs were screened for their capacity to inhibit the binding of endothelin-1 (ET-1) to ET receptors ; several crude drugs showed significant binding inhibitory activity. Pheophorbide a (1), a potent non-peptide ET receptor antagonist, was isolated from Altemisiae capillaris Flos ("Inchinko" in Japanese), which has been utilized as a remedy for hepatitis in Oriental medicine. In receptor binding experiments, compound 1 inhibited ET-1 binding specifically to both the ET_A receptor (ET_AR) and ET_B receptor (ET_BR), with IC_{50} values of 8.0×10^{-8} and 2.1×10^{-7} M, respectively. Thus, compound 1 is an ET-1 binding inhibitor; however, it exhibited no affinity for the other receptors of angiotensin II and atrial natriuretic peptide. We also evaluated the inhibitory activity of porphyrin compounds, and found that some exhibited moderate activity.

Keywords pheophorbide a; endothelin receptor antagonist; Inchinko; endothelin-1; porphyrin; crude drug

Endothelin-1 (ET-1), a potent vasoconstrictor peptide consisting of 21 amino acid residues, was originally isolated from the supernatant of cultured vascular endothelial cells.[1] Two isopeptides of ET-1, termed ET-2 and ET-3, were subsequently discovered.[2] The actions of ET appear to be mediated by at least two distinct receptor subtypes, designated ET_A (selective for ET-1 and ET-2) and ET_B (equally sensitive to all three isopeptides).[3] The ET_A receptor (ET_AR) mediates the vasoconstrictor and mitogenic responses of ET,[4] while the ET_B receptor (ET_BR) is associated with vasodilator activity through the release of endothelium-derived relaxing factor (EDRF) from the endothelium.[5] However, there is now increasing evidence that ET_BR mediates not only vasodilator activity but also vasoconstrictor responses.[6] For the development of therapeutic drugs for diseases in which ET is involved in the pathogenesis, it may be necessary to block both receptor subtypes for ET receptor antagonists. Many ET receptor antagonists have recently been reported[7]; for example, BQ-123 and FR-139317 for ET_AR, IRL1038 for ET_BR, and PD142893 for both ET_AR and ET_BR; most of these are peptides. Two representative non-peptide antagonists have been reported, 27-*O*-caffeoyl myricerone and Ro46-2005, the former, obtained from a natural source, is highly potent but selective for ET_AR,[8] while the latter is the first synthetic orally active antagonist for both ET_AR and ET_BR; however, its inhibitory activity is week.[9] Our screening program has been directed to search for potent non-peptide ET-1 antagonists for both ET_AR and ET_BR among the many crude drugs which are used in Chinese medicines. We found a highly potent receptor antagonist, compound **1**, obtained from the extract of Artemisiae capillaris Flos (Japanese name, "Inchinko," the ears of *Artemisia capillaris* THUNB., Compositae). This paper describes the isolation and identification of compound **1**, an active component of "Inchinko," and its receptor binding inhibitory activity, as well as that of its related compounds.

Results and Discussion

We examined many important crude drugs with various activities for the ET-1 receptor binding assay. As shown in Table I, six crude drugs exhibited significant binding inhibitory activity. The most potent extract, derived from a chloroform extract of "Inchinko," was

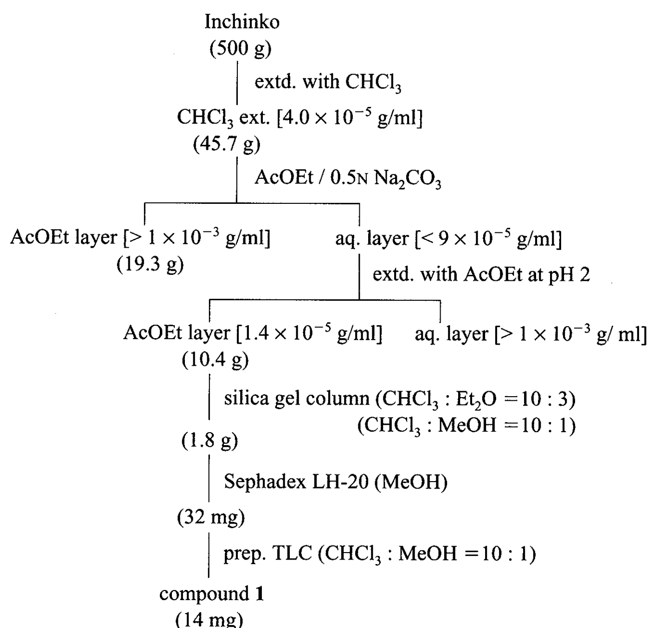

Chart 1. Isolation Procedure for "Inchinko"

[ ] indicates receptor binding inhibitory activity (IC_{50}).

TABLE I. Inhibitory Activity of Crude Drugs on [^{125}I]ET-1 Binding to ET_AR

Crude drug	Extract[a]	IC_{50} (mg/ml)
Artemisiae capillaris Flos (Inchinko)	$CHCl_3$	0.0037
Selini Fructus (Zyasyooshi)	$CHCl_3$	0.025
Pogostemi Herba (Kakko)	AcOEt	0.039
Alismatis Rhizoma (Takusya)	AcOEt	0.11
Eucommiae Cortex (Totyuu)	AcOEt	0.13
Evodiae Fructus (Gosyuyu)	AcOEt	0.17

a) Each crude drug was first extracted with EtOH, and the extracts were then extracted successively with *n*-hexane, $CHCl_3$, and AcOEt.

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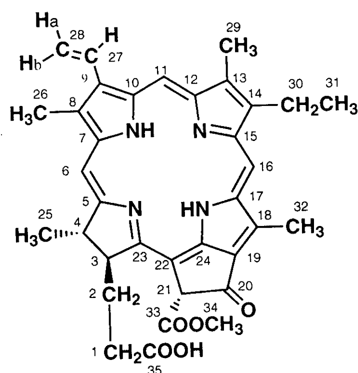

Fig. 1. Pheophorbide a (**1**)

selected for purification and was isolated by monitoring the inhibition of ET-1 binding (Chart 1). The active acid ingredient was subjected to silica gel chromatography on Sephadex LH-20, followed by preparative TLC, to give compound **1** (Fig. 1). Compound **1**, a deep-green amorphous solid, exhibited a molecular ion peak at m/z 592 in the FAB-MS, and two UV absorptions at λ_{max} 408 and 666 nm, these being characteristic absorptions of porphyrin compounds. In the ^{1}H-NMR spectrum, an unusual downfield shift of three methine protons (δ, 9.40, 9.25 and 8.52) and most substituents of compound **1** were observed. These spectral data suggested that compound **1** was pheophorbide a, possessing a chlorin macrocycle; it was identified as pheophorbide a by direct comparison with an authentic sample. Pheophorbide a, which has photodynamic action,[10] is known to be a significant degradation product of chlorophyll a. It is, however, an original component of "Inchinko," not an artifact, because the conversion of chlorophyll a to pheophorbide a did not occur during this isolation procedure.

Compound **1** inhibited the binding of ET-1 to the ET_AR (porcine aorta membranes) and ET_BR (rat brain membranes) with almost the same IC_{50} values, 8.0×10^{-8} and 2.1×10^{-7} M, respectively. The flavone and chromone compounds, arcapillin and capillarisin,[11] the major choleretic and liver-protective principles of "Inchinko," which were obtained in this separation procedure, showed no inhibitory activity for either ET_AR or ET_BR at 10^{-4} M.

Saturation binding experiments were performed with both ET_AR and ET_BR using [^{125}I]ET-1 and varying concentrations of compound **1**. On the Scatchard plots, the apparent K_D values for ET-1 increased in the presence of increasing concentrations of compound **1**; however, the B_{max} values were unchanged in comparison with that in the absence of compound **1** on both receptor subtypes (data not shown). Thus, the inhibition of ET-1 binding to both ET_AR and ET_BR by compound **1** was considered to be competitive. The specificity of compound **1** was demonstrated using other radioligand binding assays and it was shown to have no affinity for the receptors of angiotensin II (Ang II) and atrial natriuretic peptide (ANP) at 10^{-5} M (data not shown).

To study the structure–activity relationships, compound **1**-related compounds, which were easily prepared or commercially available, were evaluated for their capacity to inhibit ET-1 binding to ET_AR and ET_BR (Table II).

Table II. Receptor Binding Inhibitory Activity for Pheophorbide a-Related Compounds

No.	Compound	IC_{50} (μM)	
		ET_AR[a]	ET_BR[b]
1	Pheophorbide a	0.080 ± 0.0093	0.21 ± 0.034
2	Methyl pheophorbide a	5.1 ± 0.48	8.4 ± 0.84
3	Chlorophyll a	> 100	> 100
4	Hematoporphyrin IX	1.3 ± 0.29	3.5 ± 0.27
5	Hematin	> 100	> 100
6	Coproporphyrin I	> 100	> 100
7	Methylpyrroporphyrin XXI	0.58 ± 0.097	0.78 ± 0.089
8	Methylpyrroporphyrin XXI ethyl ester	> 100	> 100
9	29*H*,31*H*-Phthalocyanine	> 100	> 100
10	2,3,7,8,12,13,17,18-Octaethyl-21*H*,23*H*-porphine	> 100	> 100

Each value represents the mean ± S.E. of five experiments. *a*) Porcine aortic smooth muscle membranes. *b*) Rat brain membranes.

None of them displayed potency greater than that of compound **1** on receptor binding. In particular, compounds **8**, **9** and **10**, which bear no carboxyl group in the molecules, and the chelated compounds, **3** and **5**, showed no binding inhibitory activity. The active compounds, **1** and **7**, which bear one carboxyl substituent, markedly decreased the inhibitory activity by esterification of the carboxyl group. The inhibitory activity was decreased by increasing the number of carboxyl groups. These results indicate that only one carboxyl group may play a very important role, such as being involved in electrostatic interaction at the binding site, and that the porphyrin structure itself may contribute to providing the appropriate hydrophobicity and to retaining the rigid conformation of the ligand.

Compound **1**, which is a potent non-peptide receptor antagonist for both ET_AR and ET_BR, should be a useful tool to clarify the physiological and/or pathophysiological role of endothelin and its receptor subtypes.

Experimental

UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and IR spectra were taken with a JASCO VALOR-III spectrometer. The ^{1}H-NMR spectra were recorded on a JEOL GSX-270 spectrometer, using tetramethylsilane as an internal standard. FAB-MS were measured with a JEOL JMS D-300 mass spectrometer. Column chromatography was carried out on Kieselgel 60 (230—400 mesh) and Sephadex LH-20. Preparative TLC were run on Kieselgel $60F_{254}$ plates (Merck). Radioactivity was determined with an Aloka autowell gamma system, ARC-251.

Materials Crude drugs were purchased from Uchida Wakanyaku Co., Ltd. [^{125}I]ET-1 and ET-1 were obtained from Amersham and Peptide Institute, Inc., respectively. Pheophorbide a was purchased from Wako Pure Chemical Industries Co., Ltd. Chlorophyll a, hematoporphyrin IX, hematin, coproporphyrin I, methylpyrroporphyrin XXI ethyl ester, 29*H*,31*H*-phthalocyanine, and 2,3,7,8,12,13,17,18-octaethyl-21*H*,23*H*-porphine were purchased from Aldrich Chemical Co., Ltd. Methyl pheophorbide a was synthesized by the methylation of pheophorbide a with diazomethane, and methylpyrroporphyrin XXI was prepared by the hydrolysis of methylpyrroporphyrin XXI ethyl ester with aqueous sodium hydroxide in our laboratory.

Extraction Each crude drug (100 g) was extracted with EtOH (350 ml) under reflux for 2 h. After the EtOH extracts were concentrated *in vacuo*, the residues were extracted successively with *n*-hexane, $CHCl_3$ and AcOEt (100 ml each).

Isolation of Compound 1 "Inchinko" (500 g) was extracted with $CHCl_3$ (2.5 l) under reflux for 2 h. After the $CHCl_3$ extract was concentrated *in vacuo*, the residue (45.6 g) was dissolved in AcOEt (200 ml) and ex-

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tracted with 0.5 N Na_2CO_3 (70 ml × 3). The aqueous layer was adjusted to pH 2 with 1 N HCl and was reextracted with AcOEt (150 ml × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and evaporated *in vacuo* to give a residue (10.4 g). This residue was subjected to silica gel chromatography, and eluted with $CHCl_3$–Et_2O (10 : 3) followed by $CHCl_3$–MeOH (10 : 1) to afford the active fraction (1.8 g). The fraction was further separated on a Sephadex LH-20 column, using MeOH as an eluent. The active fraction (32 mg) was finally purified by preparative TLC with $CHCl_3$–MeOH (10 : 1), to give compound **1** (14 mg).

Compound **1**: Deep-green amorphous solid. IR (KBr): 2925, 1740, 1700, 1615, 1260, 1150 cm^{-1}. UV λ_{max}^{MeOH} nm (ε): 408 (84500), 666 (38000). FAB-MS *m/z*: 592 (M^+). ^{1}H-NMR ($CDCl_3$) δ: 1.63 (3H, t, J= 7.5 Hz, 31-H), 1.80 (3H, d, J=7.3 Hz, 25-H), 2.28 (2H, m, 2-H), 2.59 (2H, m, 1-H), 3.13 (3H, s, 29-H), 3.34 (3H, s, 26-H), 3.57 (2H, q, J=7.3 Hz, 30-H), 3.62 (3H, s, 32-H), 3.85 (3H, s, 34-H), 4.18 (1H, m, 3-H), 4.43 (1H, dq, J=7.3, 2.0 Hz, 4-H), 6.11 (1H, dd, J=11.5, 1.5 Hz, 28-H_a), 6.21 (1H, dd, J=17.8, 1.5 Hz, 28-H_b), 6.23 (1H, s, 21-H), 7.89 (1H, dd, J=17.8, 11.5 Hz, 27-H), 8.52 (1H, s, 6-H), 9.25 (1H, s, 11-H), 9.40 (1H, s, 16-H).

ET-1 Receptor Binding Assay Receptor membranes were prepared from the smooth muscle layer of porcine aorta (ET_A site) and from rat brain (ET_B site). The porcine aorta was dissected free from fatty tissue, minced with scissors, and then homogenized in twice the volume of 50 mM Tris–HCl, pH 7.4, containing 0.25 M sucrose, 3 mM ethylenediamine tetraacetic acid, 5 U/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 μM (*p*-amidinophenyl) methanesulfonyl fluoride (buffer A). After centrifugation at 1000 × ***g*** for 30 min at 4 °C, the resultant supernatant was further centrifuged at 100000 × ***g*** for 30 min at 4 °C. The pellet was suspended in buffer A and recentrifuged at 100000 × ***g*** for 30 min, and the final pellet was then resuspended in buffer A. The aorta membrane fractions were stored at −80 °C until use. Rat brain membrane fractions were prepared by the same procedure. Aliquots of aorta and brain membrane homogenates were incubated at 25 °C for 2 h with 2.0×10^{-11} M [^{125}I]ET-1 and various concentrations of the compounds in 50 mM Tris–HCl, pH 7.4, containing 0.9% sodium chloride and 0.5% bovine serum albumin (buffer B), in a 96-well filtration plate assembly (Multiscreen Filtration System, Millipore) (total volume 250 μl). Reactions were terminated by filtration *in vacuo* through HVPP filters (pore size 0.45 μm, Millipore). The filters were washed 4 times with a total volume of 550 μl of buffer B, and the radioactivity was determined with a gamma counter. Non-specific binding was determined in the presence of 4×10^{-7} M non-radioactive ET-1.

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