Notes

HALOEMODINS, A NEW CLASS OF ENDOTHELIN-1 TYPE B (ET_B) RECEPTOR BINDING INHIBITORS

YUE-ZHONG SHU,* MAREDITH ARCURI, MICHAEL R. KOZLOWSKI, RICHARD R. WANG, KIN SING LAM, LI-PING CHANG and DOLORES M. PIRNIK

Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 5100, Wallingford, CT 06492, U.S.A.

PATRICIA M. ROSE

Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543, U.S.A.

(Received for publication May 25, 1994)

Since the discovery of endothelin (ET, later ET -1), multiple receptors for the peptide have been described. Thus far, two of these, termed ET_A and ET_B, have been pharmacologically characterized and cloned (for review see 1). The ET_A receptor appears to be the predominant vascular smooth muscle receptor, and selectively binds to ET-1 over ET-3, resulting in vasoconstriction. The isopeptide non-selective ET_B receptor is widely expressed (*e.g.* in liver and uterus) and is probably the predominant receptor in CNS and kidney, but its function is less well understood. It is known that the circulating blood levels of endothelins in patients are usually increased in a variety of cardiovascular, renal and inflammatory disease states. Several characteristics of ET-1-induced vasoconstriction are consistent with it being involved in the production of hypertension in man. An ET receptor antagonist with appropriate affinity and selectivity may be useful in the treatment of hypertension, ischaemic or cyclosporin-induced renal damage, ulcerogenesis and cancer[2~3]. To date, many structurally diverse ET_A receptor selective and ET_A/ET_B receptor non-selective antagonists have been reported, only two peptidic ET_B receptor selective antagonist, IRL-1038[4] and RES-701-1[5], have been identified. Clearly, the discovery of a non-peptidic ET_B receptor selective antagonist would be very useful to elucidate the function of this receptor subtype in physiological regulation. A high-throughput screen for identifying ET_B receptor binding inhibitors was therefore developed. The screen was based on the measurement of binding of ^{125}I-ET-1 to the human ET_B receptor, which was cloned and expressed in Chinese hamster ovary cells (CHO-ET_B) by our laboratories[6].

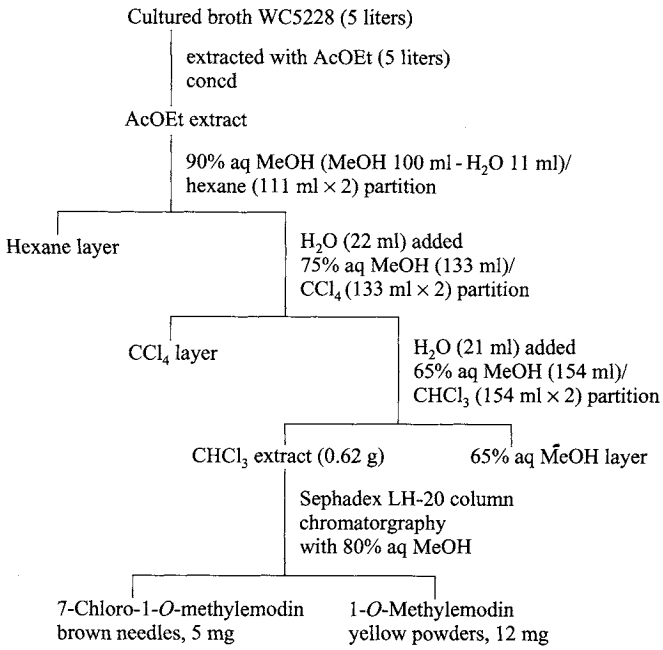
During the screening of microbial fermentation extracts for their ability to inhibit the binding of ^{125}I-ET-1 to the human ET_B receptor, an extract of *Fusarium aquaeductuum* WC-5228, was found to be active. When subjected to an activity-guided fractionation, one active compound, 7-chloro-1-*O*-methylemodin, was isolated. This report describes the isolation and characterization of this compound, the preparation and testing of its analogs, and their inhibitory effects on ET_B receptor binding.

Results and Discussion

Binding of ^{125}I-ET-1 to CHO-ET_B cells was inhibited by ET-1 in a concentration-dependent fashion with an IC_{50} of 0.16 nM (mean of 2 experiments which produced similar values). ET-2 and ET-3 also inhibited ^{125}I-ET-1 binding with potencies similar to that of ET-1 (IC_{50} values = 0.55 nM and 0.35 nM, respectively). Thus, the binding site on CHO-ET_B cells appears to be typical of the non-isopeptide selective ET_B subtype[6~10]. Binding was not inhibited by a number of unrelated peptides such as angiotensin II, bradykinin, and gp120 (data not shown).

During the testing of a large number of natural products in a high-throughput screen for ET_B binding inhibitors, an extract of fungal strain WC-5228 was found to inhibit ^{125}I-ET-1 binding to CHO-ET_B cells. Fungal strain WC-5228 was isolated from a moist dark brown loam soil sample collected in St. Louis, Missouri. Taxonomic studies on this strain was carried out from growth on Potato-Dextrose Agar (PDA). Colonies on PDA are cream color to lightly yellow and slow growing reaching 4.5~5.0 cm in diameter in two weeks at room temperature. The reverse pigment is absent and becoming dark yellow brown with age. The colony surface is wrinkled with aerial mycelium scarce to absent. Microconidia and chlamydospores are absent. Conidia are born directly from phialides arising from hyphae in groups resembling sporodochia. They are multi-septate (3~4 septa), crescent to strongly curved with potential lateral

Fig. 1. Isolation procedures of 7-chloro-1-*O*-methylemodin and 1-*O*-methylemodin.

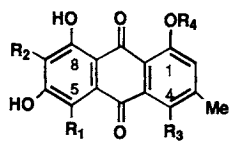


cells without hooks or notches. The conidia are $40 \sim 60\,\mu m \times 5\,\mu m$ in size. The characteristics described above are in general agreement with descriptions of *Fusarium aquaeductuum* given by NELSON *et al.*[11] Therefore fungal strain WC-5228 was identified as *Fusarium aquaeductuum*.

The compound responsible for the inhibitory activity, 7-chloro-1-*O*-methylemodin was isolated from the fungal extract using activity-guided fractionation (Fig. 1). The IC_{50} for 7-chloro-1-*O*-methylemodin was 50 μM. Its non-chlorinated analog, 1-*O*-methylemodin, also isolated from the extract, but was not active at the maximum concentration of 7.0 mM. 7-Chloro-1-*O*-methylemodin did not inhibit ^{125}I-ET-1 binding to the ETA receptor expressed in A10 cells[12] at 6.3 mM.

Four chlorinated emodins (5-chloroemodin, 7-chloroemodin, 5,7-dichloroemodin, and 4,5,7-trichloroemodin) and two brominated emodins (7-bromoemodin and 5,7-dibromoemodin) were prepared from emodin. Their structures are shown in Fig. 2; Table 1 shows the IC_{50} values of these compounds in the ET_B receptor binding assay. Monochlorinated, dichlorinated, and monobrominated emodins were active inhibitors of ET_B binding with potencies in the micromolar range (IC_{50}: $100 \sim 158\,\mu M$). The 5,7-dibromoemodin was, however, much less active ($IC_{50} = 500\,\mu M$) and 4,5,7-trichloroemodin showed no activity.

Fig. 2. Structures of emodin derivatives.



$R_1 = R_2 = R_3 = R_4 = H$
emodin

$R_1 = R_2 = R_3 = H$, $R_4 = Me$
1-*O*-methylemodin

$R_1 = R_3 = R_4 = H$, $R_2 = Cl$
7-chloroemodin

$R_1 = R_2 = R_3 = Cl$, $R_4 = H$
4,5,7-trichloroemodin

$R_1 = R_2 = Br$, $R_3 = R_4 = H$
5,7-dibromoemodin

$R_1 = R_3 = H$, $R_2 = Cl$, $R_4 = Me$
7-chloro-1-*O*-methylemodin

$R_1 = Cl$, $R_2 = R_3 = R_4 = H$
5-chloroemodin

$R_1 = R_2 = Cl$, $R_3 = R_4 = H$
5,7-dichloroemodin

$R_1 = R_3 = R_4 = H$, $R_2 = Br$
7-bromoemodin

Based on preliminary studies of structural requirements for activity, it appears that halogen substitution on the anthraquinone nucleus is essential for activity. Thus, the non-halogenated analog, 1-*O*-methylemodin, as well as emodin itself, were inactive. Of the six chlorinated and brominated derivatives prepared, most showed activity similar to 7-chloro-1-*O*-methylemodin, although somewhat weaker. Dibromo and trichloro substitutions resulted in significantly diminished activity.

Several non-peptidic compounds possessing an-

Table 1. ET_B receptor binding inhibition by emodin and haloemodin derivatives.

Compound	IC_{50} (μM)
Emodin	*
1-*O*-Methylemodin	*
7-Chloro-1-*O*-methylemodin	50
5-Chloroemodin	158
7-Chloroemodin	100
5,7-Dichloroemodin	120
4,5,7-Trichloroemodin	*
7-Bromoemodin	100
5,7-Dibromoemodin	500

* Inactive.

thraquinone ring system such as WS009[13] have previously been reported as ET_A-selective or ET_A/ET_B-nonselective inhibitors. To the best of our knowledge, the haloemodin type anthraqunones represent the first reported class of non-peptidic ET_B-selective inhibitors, to clarify whether the haloemodin derivatives act as endothelin receptor antagonists further functional testing in a relevant assay will be necessary. The function of the ET_A receptor in producing vasoconstriction is well established, but less well understood is the function of the ET_B receptor. Furthermore, the importance of the interaction between ET_A and ET_B receptor stimulation in tissues containing both subtypes of receptor is also unclear. Having both ET_A- and ET_B-selective compounds as tools, it should now prove possible to more accurately define the functions of each of these receptor subtypes.

Experimental

Binding Assay

The cloning of the human ET_B receptor and its expression in CHO cells has been previously described[6]. The ET_B receptor binding assay was carried out by culturing CHO-ET_B cells in Hams F12 medium supplemented with 10% bovine calf serum, 300 μg/ml geneticin (antibiotic G418) plus 2 mM L-glutamine in T-175 flasks. The cells from one confluent flask were detached with trypsin and used to seed a 1,050-cm^2 roller bottle. Medium was removed and replaced with 200 ml of fresh medium every 3 days, as well as 24 hours prior to use. Cells were used when confluency was reached. On the day of the assay, the CHO-ET_B cells were removed from the roller bottle by scraping, collected by centrifugation, and resuspended in PBG (PBS supplemented with 0.1% glucose and 0.1% BSA). A suspension of 3.5 mg of cells in 80 μl was placed into each well of a microtiter plate and 10 μl of ^{125}I-ET-1 (2,200 Ci/mmol, NEN) at 0.4 nM was added. In addition, each well contained 10 μl of sample to be tested or vehicle. The cells were incubated for 3 hours at 4°C, washed twice with ice-cold PBG, and collected onto a glass fiber filtermat using a cell harvester (Tomtec; Orange, CT). The filtermat was washed twice with ice-cold PBS supplemented with 0.1% BSA to remove unbound ligand. Radioactivity bound to the cells was quantified by scintillation counting (Betaplate, LKB). Non-specific binding of a control well, defined as that occurring in the presence of 1 μM unlabeled ET-1 (10 μl), was subtracted from the total binding to yield specific binding. The ET_A receptor binding was carried out with rat aortic smooth muscle A10 cells, which expressed the rat ET_A receptor, using modifications of a previously described procedure[12].

Fermentation

The producing organism used in this study was *F. aquaeductuum* WC-5228. Frozen vegetative preparations were maintained in 10% glycerol - 5% sucrose solution stored at −80°C for use as working stocks. A seed culture of *F. aquaeductuum* WC-5228 was prepared by transferring 4 ml of the frozen vegetative stock into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (4% glucose, 0.5% yeast extract, 0.0001% $FeSO_4$, 0.05% $MgSO_4$, 0.05% KCl, 0.1% KH_2PO_4 and 0.3% $NaNO_3$). This seed culture was incubated at 28°C on a rotary shaker (250 rpm). After 72 hours, 4-ml aliquots were transferred to 500-ml Erlenmeyer flasks containing 100 ml of production medium (8% potato flakes and 2% glucose). The production cultures were incubated for 6 days at 28°C on a rotary shaker (250 rpm).

Isolation and Characterization of 7-Chloro-1-*O*-methylemodin

The isolation of 7-chloro-1-*O*-methylemodin was monitored by the ET_B receptor binding assay, and the procedure is illustrated in Fig. 1. Briefly, the fermentation broth of *F. aquaeductuum* WC-5228 was extracted with ethyl acetate. The extract was dissolved in aqueous methanol and partitioned against pre-equilibrated hexane, carbon tetrachloride, and chloroform sequentially. The chloroform extract was purified by Sephadex LH-20 column chromatography. The bioactive fractions were collected, concentrated and crystallized in alcohol to give brown needles (5.0 mg). The compound has the following physico-chemical pro-

perties: High resolution MS: Found 318.0295, Calcd. for $C_{16}H_{11}ClO_5$ 318.0303; UV λ_{max} nm (MeOH): 248, 278, 310 (sh), 440; ^1H NMR (DMSO-d_6) δ: 2.49 (C-CH_3), 3.94 (OCH_3), 7.22 (5-H), 7.44 (2-H), 7.59 (4-H), 14.02 (8-OH); ^{13}C NMR (DMSO-d_6) δ: 21.8 (q), 56.5 (q), 106.5 (d), 110.1 (s), 113.1 (s), 117.4 (s), 119.9 (d), 120.2 (d), 131.2 (s), 134.5 (s), 147.5 (s), 159.9 (s), 160.2 (s), 160.8 (s), 182.0 (s), 186.3(s); CI-MS (*m/z*): 319 (MH^+, 100%), 285 (24%). Based on these data the compound was identified as 7-chloro-1-*O*-methylemodin. The localization of 1-*O*-methyl group was further confirmed by ^1H-^1H NOE experiment. A yellow powder (12 mg) was also obtained from the Sephadex LH-20 column chromatography in the fraction prior to those containing 7-chloro-1-*O*-methylemodin. The compound was identified as 1-*O*-methylemodin based on its spectral data.

Preparation of Haloemodin Derivatives

Chlorinated emodins were prepared from emodin (Aldrich). Briefly, emodin dissolved in a mixture of $CHCl_3$-MeOH (10:1) was added to an ice-chilled solution of $CHCl_3$ containing an equal molar amount of chlorine. After stirring 6 hours at room temperature, the reaction mixture was concentrated *in vacuo* to give a red oil. The oil was purified by flash column chromatography of silica gel with a solvent mixture of CH_2Cl_2-MeOH-H_2O (100:2:0.1 to 100:5:0.5). 5-Chloroemodin was first and 7-Chloroemodin then eluted as major products, followed by 5,7-dichloroemodin. The three products were crystallized in alcohol as brown powders. 5-Chloroemodin, ^1H NMR (DMSO-d_6) δ: 2.42 (CH_3), 6.78 (7-H), 7.16 (CH), 7.44 (CH), 11.76 (OH), 12.78 (OH); CI-MS (*m/z*): 307 (41%), 305 (MH^+, 100%), 271 (18%); 7-chloroemodin, ^1H NMR δ: 2.41 (CH_3), 7.20 (CH), 7.32 (CH), 7.50 (CH), 11.84 (OH), 12.88 (OH); CI-MS (*m/z*): 307 (38%), 305 (MH^+, 100%), 271 (10%); 5,7-dichloroemodin, ^1H NMR δ: 2.42 (CH_3), 7.14 (CH), 7.24 (CH), 11.92 (OH), 13.66 (OH); CI-MS (*m/z*): 341 (72%), 339 (MH^+, 100%), 307 (24%), 305 (66%), 271 (27%). 4,5,7-Trichloroemodin was prepared in a similar manner by reacting emodin with an excess amount of chlorine, ^1H NMR δ: 2.46 (CH_3), 7.54 (CH), 11.75 (OH), 12.38 (OH); CI-MS (*m/z*): 375 (100%), 373 (MH^+, 95%). To prepare brominated derivatives, emodin was added to an ice-chilled CH_2Cl_2 solution containing an equal molar amount of bromine. The solution was stirred 3 hours at room temperature and a reddish-yellow precipitate was formed. The precipitate was filtered and washed with MeOH. The product was identified as 5-bromoemodin, ^1H NMR δ: 2.42 (CH_3), 7.18 (CH), 7.29 (CH), 7.49 (CH), 11.82 (OH), 12.89 (OH); CI-MS (*m/z*): 351 (100%), 349 (MH^+, 98%), 271 (32%). 5-Bromoemodin was further reacted in a similar fashion with an equal molar amount of bromine for 3 hours to give a brown precipitate. After filtering and washing with MeOH, the product was characterized as 5,7-dibromoemodin, ^1H NMR δ: 2.42 (CH_3), 7.15 (CH), 7.47 (CH), 11.69 (OH), 13.74 (OH); CI-MS (*m/z*): 451 (55%), 429 (100%), 427 (MH^+, 40%), 351 (12%), 349 (11%), 271 (2%).

Acknowledgments

We thank Drs. K. COLSON, S. HUANG and S. KLOHR for part of the spectral data measurements.

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