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A NOVEL CLASS OF NON-PEPTIDIC ENDOTHELIN ANTAGONISTS ISOLATED FROM THE MEDICINAL HERB *PHYLLANTHUS NIRURI*

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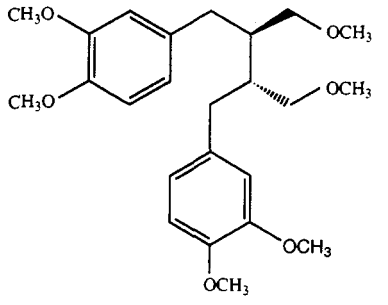
ABSTRACT.—Fractionation of an extract of *Phyllanthus niruri*, based on its ability to inhibit [^{125}I]-ET-1 binding to A10 cells (rat thoracic aortic smooth muscle cells), led to the isolation of three non-peptidic endothelin-1 (ET-1) antagonists, which have been identified as the lignans phyllanthin [**1**], hypophyllanthin [**2**], and nirtetralin [**3**]. These isolates were also found to inhibit [^{125}I]-ET-1 binding to the recombinant human ET_A receptor expressed in Chinese hamster ovary cells (CHO-ET_A), but were inactive against the recombinant ET_B receptor. The most potent compound was **2** with an IC_{50} value of 40 μM. By means of a microphysiometer, **2** was found to attenuate ET-1-induced acceleration in the rate of acid extrusion from CHO-ET_A consistent with ET-1 antagonistic activity. Screening of synthetic 1-phenyl-tetrahydronaphthalene-derived analogues revealed that 5,8-dimethoxy-6-methyl-4-phenyl-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid (BL-4170, **4**) also inhibited [^{125}I]-ET-1 binding.

In 1988, Yanagisawa *et al.* described a potent vasoconstrictor peptide isolated from the supernatant of cultured bovine aortic endothelial cells (1). This peptide was named endothelin (ET, later ET-1) and was subsequently shown to be one of a family of related peptides, ET-1, ET-2, ET-3, and the sarafotoxins (2,3). ET-1 is notable both for its high potency as a vasoconstrictor and for its long duration of action (4). Chronic intravenous infusion of ET-1 in rats can produce sustained vasoconstriction without tachyphylaxis (5). These characteristics of ET-1-induced vasoconstriction suggest that it may be involved in the production of hypertension in man (1). After the discovery of ET-1, it became apparent that there were at least two receptors for the peptide (6). Two of these, termed ET_A and ET_B, have now been characterized pharmacologically and cloned (7–9). The potency of ET analogues in producing vasoconstriction (ET-1≥ET-2>>ET-3) agrees most closely with their binding affinities at the ET_A receptor (10–13). In addition, the ET_A receptor is the more prevalent receptor in arterial tissue (3).

During the course of a high-throughput screening program for potential ET_A inhibitors, an extract of the herb *Phyllanthus niruri* L. (Euphorbiaceae) was found to possess a significant capacity to inhibit [^{125}I]-ET-1 binding. When subjected to activity-guided fractionation, we obtained a group of active compounds of the lignan class [**1**–**3**]. This report describes the isolation of these compounds as well as their biological properties.

RESULTS AND DISCUSSION

Binding of [^{125}I]-ET-1 to A10 cells was inhibited by ET-1 (IC_{50} value of 0.2 nM±0.04, n=8), and by ET-3 (IC_{50}=120 nM±0.2, n=8). These data are consistent with [^{125}I]-ET-1 binding to the ET_A subtype, as has been previously suggested (14). Binding was not inhibited by a number of unrelated peptides such as angiotensin II, bradykinin, and gp 120 (data not shown).



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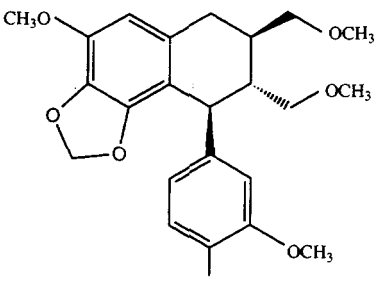

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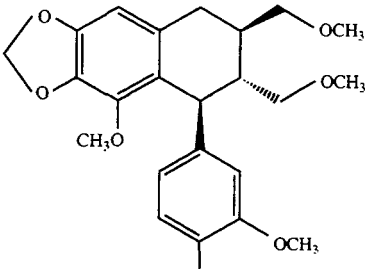

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During the screening of a large number of synthetic compounds and natural products, an extract prepared from the herb *P. niruri* was found to inhibit [^{125}I]-ET-1 binding to A10 cells. The activity was purified by subjecting the crude extract to a bioassay-monitored fractionation that involved solvent-solvent partition and cc. As a result, three active lignans were isolated and identified as phyllanthin [**1**], hypophyllanthin [**2**], and nirtetralin [**3**]. Their identification was achieved by means of spectral determination and by comparison of their physical and spectral data with those published previously (15–17). The apparent IC_{50} values for **2** and **3** were 580 μM and 400 μM, respectively, while **1** was considered inactive because its IC_{50} value was >1 mM. Their actual affinities for the receptor are probably higher than observed, for none of them could be completely solubilized in the assay buffer.

The interactions of compounds **1–3** with the human ET_A receptor were also explored. Similar to our findings with the rat ET_A receptor, compound **1** was inactive at human ET_A (IC_{50} value >1 mM). However, [^{125}I]-ET-1 binding to CHO-ET_A cells was inhibited by **2** and **3** with IC_{50} values of 40 μM±3 and 75 μM±9, respectively (n=2; Figure 1). In contrast, these compounds did not inhibit [^{125}I]-ET-1 binding to the human ET_B receptor expressed in CHO-ET_B cells (IC_{50} value of >>500 μM). These findings suggest that these lignans have a higher affinity to ET_A subtype than to the ET_B subtype.

The structural lead provided by the identification of compounds **2** and **3** as inhibitors of ET-1 was followed by examination of our chemical archives. Approximately 100 synthetic compounds containing the 1-phenyl-tetrahydronaphthalene moiety were selected and tested for their [^{125}I]-ET-1 binding inhibition. They included different analogues of tetrahydronaphthalene, tetrahydronaphthol, naphthylamine, tetrahydronaphthoic acid, and others. Among them, only 5,8-dimethoxy-6-methyl-4-phenyl-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid (BL-4170) [**4**] was active (CHO-ET_A: IC_{50} value of 160 μM±45, n=2).

In order to ascertain if compound **2** functioned as an ET_A receptor agonist or

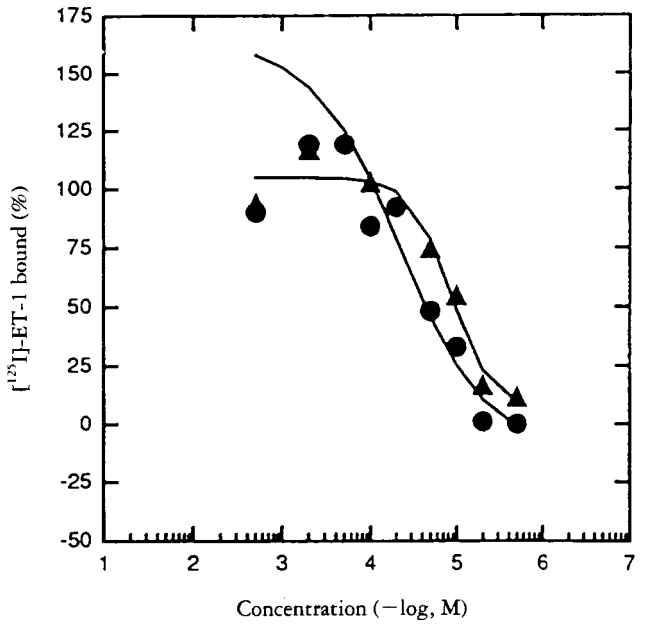
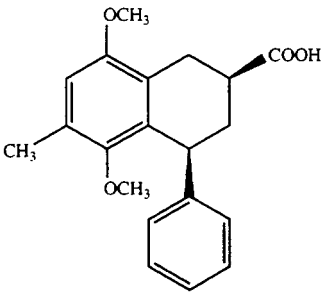


FIGURE 1. Inhibition of [^{125}I]-ET-1 (50 pM) binding to CHO-ET_A cells by hypophyllanthin (**2**, ●) and nirtetralin (**3**, ▲). The figure is a representative of similar experiments, with each point run in duplicate.



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antagonist, we evaluated its effect on extracellular acidification by microphysiometry. ET-1 (1 nM) produced increases in the peak rate of acidification of the medium of CHO-ET_A and CHO-ET_B cells of 79±11% (n=3) and 89±13% (n=4), respectively (Figure 2). Elevated rates of acidification of the medium as a result of receptor stimulation have been demonstrated in a number of systems, and can be attributed to enhanced cellular metabolism and the consequent production and excretion of metabolic acids (18,19). Compound **2**, the most potent in binding assays, was tested at concentrations of 20 μM and 100 μM. It inhibited the elevation in the rate of acidification produced by 1 nM ET-1 by 28±7% (n=3) and 39% (n=1, Figure 2a), respectively. In the absence of ET-1, this compound had no effect on the acidification rate. Compound **2** did not inhibit the effect of ET-1 on CHO-ET_B cells (Figure 2b). These data are consistent with the notion that this compound may function as an ET_A selective binding antagonist.

Historically, the herb *P. niruri* and another closely related species, *Phyllanthus amarus*, have been utilized to cure a wide variety of human ailments, including allergies

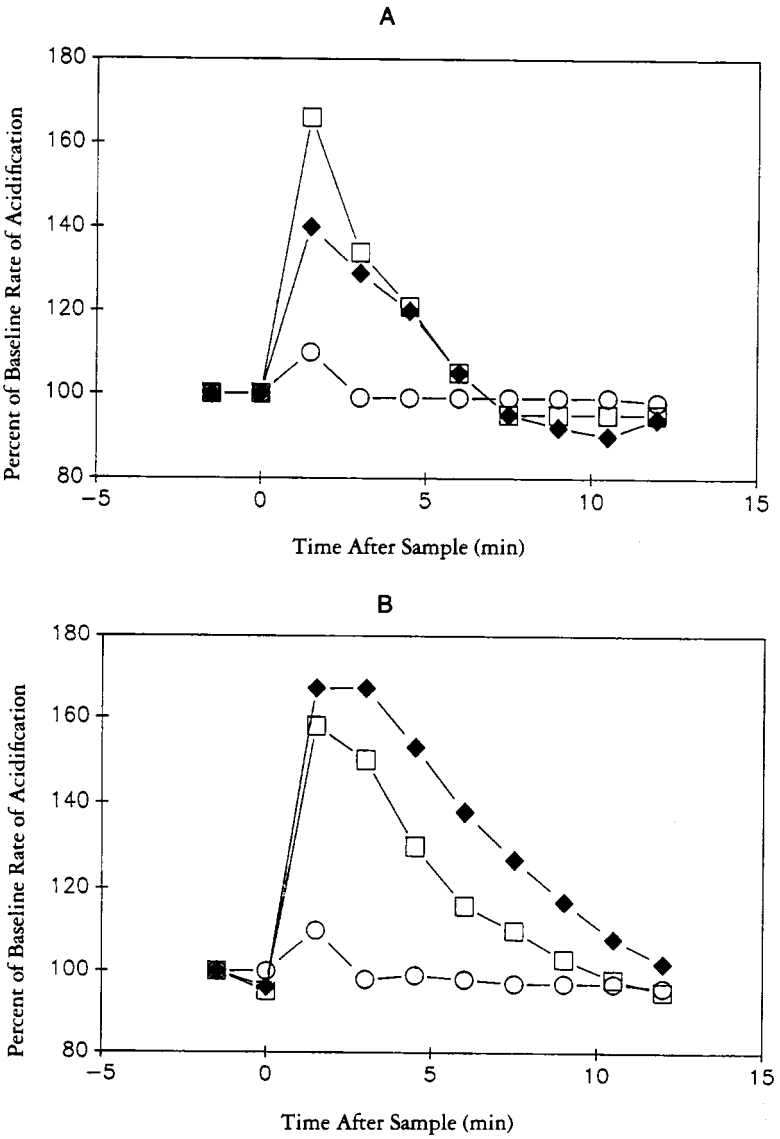

FIGURE 2. Percentage enhancement of the rate of extracellular acidification in CHO-ET_A (panel a) and CHO-ET_B (panel b) cells by ET-1, and reversal of this effect by hypophyllanthin [**2**]. Measurements were made using a microphysiometer, as described in the Experimental. The baseline metabolic rate, taken at −3 min, was −96±8 μV/sec. Cells were treated with 1 nM ET-1 (□), 1 nM ET-1+100 μM **2** (◆), or 100 μM **2** (○) at time 0 for 30 sec. The compound **2** curve did not differ from that seen with the vehicle (data not shown). The figure shows representative results from one of four experiments, with each point run in duplicate, all of which gave similar results.

and hepatitis, a practice that continues to this day (16,20). The discovery of *Phyllanthus* lignans as a new class of ET-1 antagonists supports the importance of traditional medicinal plants as a source of new drug prototypes. Interestingly, other lignans isolated from a Chinese herbal medicine, *Arctium lapa* (Compositae), have been found to exhibit a Ca^{2+}-antagonistic activity; one of these was reported to exert strong and long-lasting

antihypertensive effects on spontaneously hypertensive rats (21). In a similar fashion to a procedure published previously (22), compound **2** demonstrated significant activity at and above 23 μM in the Ca^{++}-stimulated guinea pig ileum contraction assay (Panlabs, Taipei, Taiwan; unpublished results).

During the time that we have been involved in screening for ET_A selective ET-1 inhibitors, other groups also have reported success in obtaining this activity from natural products (23–25). The ET-1 antagonists described in the present study are, however, structurally different from any of those previously reported. As representatives of a new chemotype, they may aid in elucidating the structural requirements of ET_A inhibitors. In addition, other ET-1 receptors besides the ET_A and ET_B subtypes may exist and some of the important cardiovascular effects of ET-1 may be mediated by these novel receptor subtypes (11,12). As more ET-1 binding inhibitor chemotypes become available as pharmacological tools, it should be possible to dissect out any additional ET-1 receptor subtypes.

EXPERIMENTAL

PLANT MATERIAL.—Quantities of *Phyllanthus niruri* were collected by one of us (P.F.) in June 1991, near Hyderabad, India. A voucher specimen is on deposit in our plant depository.

EXTRACTION AND ISOLATION.—Fresh aerial parts were shredded and extracted into MeOH. Thereafter, all solvents were evaporated to dryness *in vacuo*, yielding a dark green residue. The crude extract was subjected to a partition between H_2O and $CHCl_3$, the ET-1 inhibitory activity being extracted into the organic phase. The activity was then purified by chromatography on three successive Si gel columns and eluted by gradient mixtures of toluene/Me_2CO, hexane/Me_2CO, and petroleum ether/EtOAc. Crystallization was affected in hexane and Me_2CO mixtures [**1–2**]. Physical and spectral characteristics (mp, $[\alpha]D$, ir, uv, ms, ^1H- and ^{13}C-nmr) are in full agreement with data published previously (15–17).

BIOLOGICAL TESTING.—*Rat ET_A*: A10 cells (rat thoracic aortic smooth muscle cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% bovine calf serum. On the assay day, confluent cells were removed from the roller bottle by scraping, collected by centrifugation, then resuspended in PBG (PBS, 0.1% glucose, 0.1% BSA). A suspension of 10^5 cells in 80 μl was placed into each well of a microtiter plate and 10 μl of [^{125}I]-ET-1 (2200Ci/mM, NEN) at 0.4 nM was added. In addition, each well contained 10 μl of vehicle [EEB: EtOH-emulphor-25 mM tris buffer (1:1:1), diluted to 0.6% with tris buffer] or vehicle containing ET-1 or compounds **1–4**. The cells were incubated for 3 h at 4°, then were washed with ice-cold PBG, and collected onto a glass fiber filtermat using a cell harvester (Tomtec™). The unbound ligand was removed by washing with cold PBS containing 0.1% BSA, and the bound radioactivity was quantified by scintillation counting (Betaplate™). Non-specific binding, taken as that occurring in the presence of 1 μM of unlabeled ET-1, was subtracted from the total binding to yield specific binding.

Cloned human ET_A and ET_B.—The human ET_A and ET_B receptors were cloned and expressed in Chinese hamster ovary cells (CHO) (9,26). The binding assays were conducted as reported previously and adapted for use with intact cells (27). CHO cells transfected with either the ET_A (CHO-ET_A) or ET_B (CHO-ET_B) human receptors were plated in growth medium in 24-well plates and grown to approximately 50% confluence. The cells were incubated with 50 pM [^{125}I]-ET-1 (220Ci/mM) and either 100 nM ET-1, test compound, or vehicle in a final volume of 0.2 ml assay buffer (50 mM Tris-HCl pH 7.4, 0.1% BSA, 2 μM phosphoramidon). Test compounds were prepared as 5 mM solutions in DMSO, and serially diluted in assay buffer. The mixture was incubated at 4° for 4 h, and the assay was terminated by aspiration and washing with cold PBS. For quantification, 0.2 ml of 0.5 N NaOH was added to each well, the well contents were transferred to Sarstedt tubes, and the radioactivity was measured using a COBRA Auto-gamma counter (Packard™). Data were analyzed by iterative curve fitting, and IC_{50} values calculated and expressed as mean SEM or SD (n).

MICROPHYSIOMETRY.—A10 cells were seeded onto 12-mm polycarbonate capsule cups at 7.5×10^5/well in DMEM supplemented with 10% fetal bovine serum. Cultures were incubated at 37° and 5% CO_2 for 3 h to allow the cells to attach to the membrane. The medium was replaced with serum-free DMEM, and the plates were re-incubated for 16–18 h. The capsule cups were loaded into a microphysiometer (Cytosensor™) and perfused with a modified medium (DMEM without bicarbonate and with 1 mg/ml BSA). In some cases, the modified medium contained either ET-1 or **2** alone, ET-1 with **2**, or the vehicle (DMSO).

The rate of acidification in the extracellular microenvironment was measured as an indicator of cell metabolic activity. Multiple rate determinations were performed for each experiment. The peak rate of acidification following agonist treatment of the cells was used in the quantification of the results.

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