

the concentration-response curve for endothelin-1 were examined. Compounds were added 15 min prior to the addition of endothelin-1.

4. Assay for identifying compounds that have antagonistic activity against ET<sub>B</sub> receptors

Since endothelin-1 stimulates the release of prostacyclin from cultured bovine aortic endothelial cells, the cyclic peptides are screened for their ability to inhibit endothelin-1 induced prostacyclin release from such endothelial cells by measuring 6-keto PGF<sub>1α</sub> substantially as described by Filep et al. (1991) *Biochem. Biophys. Res. Commun.* 177 171-176. Bovine aortic cells are obtained from collagenase-treated bovine aorta, seeded into culture plates, grown in Medium 199 supplemented with heat inactivated 15% fetal calf serum, and L-glutamine (2 mM), penicillin, streptomycin and fungizone, and subcultured at least four times. The cells are then seeded in six-well plates in the same medium. Eight hours before the assay, after the cells reach confluence, the medium is replaced. The cells are then incubated with a) medium alone, b) medium containing endothelin-1 (10 nM), c) cyclic peptide alone, and d) cyclic peptide+endothelin-1 (10 nM).

After a 15 min incubation, the medium is removed from each well and the concentrations of 6-keto PGF<sub>1α</sub> are measured by a direct immunoassay. Prostacyclin production is calculated as the difference between the amount of 6-keto PGF<sub>1α</sub> released by the cells challenged with the endothelin-1 minus the amount released by identically treated unchallenged cells. Cyclic peptides which stimulate 6-keto PGF<sub>1α</sub> release possess agonist activity and those which inhibit endothelinol 1 6-keto PGF<sub>1α</sub> release possess antagonist activity.

#### B. Assay Results

1. The test results from binding assay #1 are set forth in Table 1:

TABLE 1

TEST COMPOUND	IC <sub>50</sub> (μM)
(6) cyclo(D-Asp—Ala-D-Ala—Ala-D-Trp)	7.3
(7) cyclo(D-Tyr—Ala-D-Ala—Ala-D-Trp)	52
(2) cyclo(D-Tyr—Phe-D-His-β-Ala-D-Trp)	100
(9) cyclo(D-Asp—Ac <sub>3</sub> c-D-Val—Leu-D-Trp)	12

Membrane was prepared as described above for the ET<sub>A</sub> receptors. Using the membrane preparation diluted with binding buffer to a concentration of 1 μg/100 μl, the binding assay was performed as described above for the ET<sub>A</sub> receptors.

TABLE 2

CYCLIC PEPTIDE	% Inhib 100 μM	
	ET <sub>A</sub>	ET <sub>B</sub>
D-Leu-L-Val-D-Pro-L-Asp-L-Trp	96	ND
D-Leu-L-Val-D-Pro-L-Tyr-L-Trp	18	ND
D-Leu-L-Val-D-Pro-L-Ser-L-Trp	49.5	ND
D-Leu-L-Val-D-Pro-L-Glu-L-Trp	25	0
D-Leu-L-Val-D-Ala-L-Asp-L-Trp	26	15
D-Leu-L-Val-L-Gly-L-Asp-L-Trp	14	17
D-Leu—Aib-L-Pro-L-Asp-L-Trp	9.5	11
D-Leu-L-Gly-L-Pro-L-Asp-L-Trp	7.1	12
D-Leu-D-Val-L-Pro-D-Asp-L-Trp	80	2.2
D-Leu-D-Val-L-Pro-D-Glu-L-Trp	23	0

Peptides with the preferred backbone structure exhibit the best activity.

2. The test results from binding assay #2 are set forth in Table 3:

TABLE 3

COMPOUND	IC <sub>50</sub> (μM)
(2) cyclo(D-Tyr—Phe-D-His-β-Ala-D-Trp)	90
(6) cyclo(D-Asp—Ala-D-Ala—Ala-D-Trp)	>100
(7) cyclo(D-Tyr—Ala-D-Ala—Ala-D-Trp)	>100
(9) cyclo(D-Asp—Ac <sub>3</sub> c-D-Val—Leu-D-Trp)	45

10 Cyclic peptide (9), cyclo(D-Asp-Ac<sub>3</sub>c-D-Val-Leu-D-Trp), is, thus, an ET<sub>B</sub> antagonist.

3. Inhibition of Endothelin-1 induced contraction

Compounds (6) cyclo(D-Asp-Ala-D-Ala-Ala-D-Trp) and (7) cyclo(D-Tyr-Ala-D-Ala-Ala-D-Trp) showed no agonistic activity in the contraction assay. Compound (6) cyclo(D-Asp-Ala-D-Ala-Ala-D-Trp) at a concentration of 10 μM caused 75% inhibition of the contraction induced by 100 nM endothelin-1. Compound (7) cyclo(D-Tyr-Ala-D-Ala-Ala-D-Trp) at a concentration of 10 μM caused a 75% inhibition of the contraction induced by 100 nM endothelin-1.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

We claim:

25 1. A substantially pure peptide of formula (III):



or a pharmaceutically acceptable salt thereof, wherein:

30 X<sup>1</sup> is D-Leu, D-Val, D-Ile, D-Ala, Gly, Aib, D-Nva, D-Nle or D-Alle; X<sup>2</sup> is L-Val, L-Ile, L-Leu, L-Ala, L-Gln, L-Gly, Aib, L-Nva, L-Nle or L-Alle; X<sup>3</sup> is D-Pro, D-Hyp, D-Ala, D-Val, D-Ile, Gly, Aib, D-Nva, D-Nle or D-Alle; and X<sup>4</sup> is L-Asp, L-Glu, L-Tyr, L-Ser, L-Thr, L-Cys(O<sub>3</sub><sup>H</sup>) or L-Pen(O<sub>3</sub>H).

35 2. A peptide of claim 1, wherein

X<sup>1</sup> is D-Leu, D-Val, D-Ile or D-Ala;

X<sup>2</sup> is L-Val, L-Ile, L-Leu or L-Ala;

X<sup>3</sup> is D-Pro, D-Ala, D-Val or D-Ile; and

40 X<sup>4</sup> is L-Asp, L-Glu, L-Tyr or L-Ser.

3. The peptides of claim 2, wherein:

X<sup>1</sup> is D-Leu;

X<sup>2</sup> is L-Val, L-Ile, L-Leu or L-Ala;

45 X<sup>3</sup> is D-Pro, D-Ala, D-Val or D-Ile; and

X<sup>4</sup> is L-Asp, L-Glu, L-Tyr or L-Ser.

4. The peptides of claim 3, wherein:

X<sup>1</sup> is D-Leu;

50 X<sup>2</sup> is L-Val;

X<sup>3</sup> is D-Pro, D-Ala, D-Val or D-Ile; and

X<sup>4</sup> is L-Asp, L-Glu, L-Tyr or L-Ser.

5. A peptide of claim 4 selected from the group consisting of cyclo(D-Leu-L-Val-D-Pro-L-Tyr-L-Trp), cyclo(D-Leu-L-Val-D-Pro-L-Ser-L-Trp), cyclo(D-Leu-L-Val-D-Pro-L-Asp-L-Trp), cyclo(D-Leu-L-Val-D-Pro-L-Glu-L-Trp), cyclo(D-Leu-L-Val-D-Ala-L-Asp-L-Trp) and cyclo(D-Leu-L-Val-Gly-L-Asp-L-Trp).

60 6. A peptide of claim 5 that is D-Leu-L-Val-D-Pro-L-Asp-L-Trp or D-Leu-L-Val-D-Pro-L-Ser-L-Trp.

7. A pharmaceutical composition, comprising a peptide of claim 1, in a pharmaceutically acceptable vehicle.

8. The composition of claim 7, that is formulated for 65 single dosage administration and that contains an amount of the peptide that is effective for ameliorating the symptoms of hypertension, cardiovascular disease, asthma, ophthalmo-