[¹²⁵I]-PD151242: a selective radioligand for human ET_A receptors

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Our aim was to synthesize a new endothelin ET_A selective radioligand, [¹²⁵I]-PD151242 and characterize the compound in human vascular tissue. Binding of [¹²⁵I]-PD151242 to sections of human aorta was time-dependent and reached equilibrium after 120 min at 23°C with an association rate constant of $1.26 \pm 0.17 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ ($n = 3$ individuals $\pm$ s.e.mean). The binding was reversible at 23°C with an observed dissociation rate constant of $0.0025 \pm 0.0006 \text{ min}^{-1}$ ($n = 3$). Saturation binding assays using [¹²⁵I]-PD151242 revealed a single population of high affinity ET receptors ($n = 3$) in aorta ($K_D = 0.76 \pm 0.17 \text{ nM}$; $B_{\text{max}} = 5.98 \pm 1.56 \text{ fmol mg}^{-1}$ protein), pulmonary ($K_D = 1.75 \pm 0.20 \text{ nM}$; $B_{\text{max}} = 12.78 \pm 1.39 \text{ fmol mg}^{-1}$ protein) and coronary arteries ($K_D = 0.51 \pm 0.07 \text{ nM}$; $B_{\text{max}} = 44.9 \pm 1.67 \text{ fmol mg}^{-1}$ protein). ET_A selective ligands competed for [¹²⁵I]-PD151242 binding in aorta with nanomolar affinity (BQ123, $K_D = 0.41 \pm 0.26 \text{ nM}$; FR139317, $K_D = 0.55 \pm 0.11 \text{ nM}$) whereas the ET_B selective compound, BQ3020, competed with micromolar affinity ($K_D = 1.36 \pm 0.25 \mu\text{M}$). In isolated coronary arteries, PD151242 was a functional antagonist and caused a significant, parallel rightward shift of the ET-1 dose-response curve with a pA_2 value of 5.92 ($n = 5$) and a slope of unity. The high affinity and selectivity of [¹²⁵I]-PD151242 for ET_A receptors will facilitate the characterization of this sub-type in human tissues.

Keywords: PD151242; endothelin; FR139317; BQ123; BQ3020; ET_A receptor; human aorta; pulmonary artery; coronary artery

Introduction Two endothelin (ET) receptor sub-types, ET_A and ET_B have been isolated and cloned from human tissue. They are classified at present by the relative potency of the three ET isoforms. ET-1 and ET-2 have similar affinities for the ET_A receptor whereas that of ET-3 is much lower. All three isoforms are thought to be equipotent for the ET_B sub-type (see Miller *et al.*, 1993). Classification of receptors has been advanced by the development in animals of sub-type selective agonists for the ET_B receptor (Doherty, 1992). Radiolabelled versions of these compounds, [¹²⁵I]-[Ala^{1,3,11,15}]ET-1 and [¹²⁵I]-BQ3020, are selective for human ET_B receptors (Molenaar *et al.*, 1992) and the peptides have been used to determine the distribution of this sub-type in human tissues (Molenaar *et al.*, 1993; Davenport *et al.*, 1993; Karet *et al.*, 1993).

ET_A-selective antagonists have also been developed in animal models including BQ123 (Ihara *et al.*, 1992) and FR139317 (Nirei *et al.*, 1993) but these are unsuitable for direct iodination. We have therefore synthesized PD151242, which contains a tyrosine residue and has structural similarity to FR139317, and tested the ability of the iodinated compound to bind to ET receptors in human blood vessels. *In vitro* pharmacological assays were used to test for functional antagonism in human isolated blood vessels.

Methods Cardiovascular tissue was obtained from recipient patients (age range 43–58 years) undergoing heart transplants for ischaemic heart disease. Immediately on removal from the patient, the endothelial layer and intima were removed from the vessels (aorta, pulmonary and epicardial coronary arteries) and the media snap frozen in liquid nitrogen and stored at -70°C until use. Sections (10 μm thick) were cut on a cryostat microtome and mounted onto gelatin coated microscope slides.

For saturation experiments, tissue sections were pre-

incubated for 15 min in HEPES buffer as previously described (Davenport *et al.*, 1989). Sections were then incubated with increasing concentrations (8 pM–8 nM) of [¹²⁵I]-PD151242 in incubation buffer for 2 h at 23°C. Non-specific binding was defined by use of 1 μM unlabelled PD151242. Sections were rinsed in Tris-HCl buffer (0.05 M, pH 7.4) at 4°C (3 $\times$ 5 min) and the amount of radioactivity measured in a gamma counter. Under these washing conditions (4°C), used to separate tissue bound [¹²⁵I]-PD151242 from free, less than 7% of the label had dissociated after 15 min. Association experiments were carried out using aorta as described above, except that sections were incubated for increasing time periods (0–240 min) with [¹²⁵I]-PD151242. For dissociation experiments, sections were incubated with [¹²⁵I]-PD151242 for 2 h at 23°C before incubating sections in an excess of buffer at 23°C for increasing time periods (0–240 min). In competition assays, sections were incubated with 100 pM [¹²⁵I]-PD151242 or [¹²⁵I]-ET-1 and increasing concentrations of unlabelled peptides (20 pM–100 μM). Non-specific binding was defined by use of 1 μM unlabelled PD151242 or ET-1 respectively.

The results of binding experiments were analysed using EBDA and LIGAND programmes as previously described (Molenaar *et al.*, 1992; 1993; Davenport *et al.*, 1993). The presence of 1 or 2 sites was tested by the *F*-ratio test in LIGAND. The model adopted was that which provided the best fit ($P < 0.05$).

To test for functional antagonism of PD151242, endothelium-denuded rings (2 mm) of epicardial coronary arteries were mounted in 25 ml organ baths in continuously oxygenated Krebs solution at 37°C as previously described (Davenport *et al.*, 1993). Dose-response curves were determined for ET-1 (100 pM–1 μM) in the absence (control) and presence of PD151242 (1–30 μM) added to the medium 30 min prior to addition of ET-1. The responses to ET-1 were expressed as a percentage of the maximum contraction to 50 mM KCl used to terminate the experiment and pA_2 values calculated by Schild analysis.

PD151242, (N-[(hexahydro-1-azepinyl)carbonyl])L-Leu(1-Me) D-Trp-D-Tyr; FR139317 (N-[(hexahydro-1-azepinyl)

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carbonyl)-L-Leu(1-Me)-D-Trp-3(2-pyridyl)-D-Ala; BQ123, cyclo [D-Asp-L-Pro-D-Val-L-Leu-D-Trp] and BQ3020, [Ala^{11,15}]Ac-ET-1(6-21), were synthesized by solid phase t-Boc chemistry. Peptide concentration was determined by u.v. spectrophotometry. [¹²⁵I]-PD151242 (2000 Ci mmol⁻¹) was synthesized (Amersham International plc, Amersham, Bucks) from the unlabelled material by mild oxidation using an enzymatic method and purified to be carrier-free. [¹²⁵I]-ET-1 (2000 Ci mmol⁻¹) was obtained from Amersham and unlabelled ET-1 from Novabiochem, Nottingham.

Results Binding of [¹²⁵I]-PD151242 to sections of human aorta was time-dependent and reached equilibrium after 120 min at 23°C with an association rate constant of $1.26 \pm 0.17 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ ($n = 3 \pm \text{s.e.mean}$). The binding was reversible at 23°C with an observed dissociation rate constant of $0.0025 \pm 0.0006 \text{ min}^{-1}$ ($n = 3$). [¹²⁵I]-PD151242 binding to sections of aorta, pulmonary and coronary arteries was concentration-dependent and saturable (Table 1) with affinities ranging from 0.5–1.8 nM. The Hill coefficients were close to unity, and in each case a one site was preferred to a two site model.

Unlabelled PD151242 competed for the binding of 0.1 nM [¹²⁵I]-ET-1 to aorta in a biphasic manner: $K_D \text{ ET}_A = 3.27 \pm 0.93 \text{ nM}$ and $K_D \text{ ET}_B = 4.85 \pm 1.65 \mu\text{M}$ with a ratio of $\text{ET}_A:\text{ET}_B$ of 84:16%. ET_A and ET_B -selective compounds were tested further in human aorta for their ability to compete for the binding of [¹²⁵I]-PD151242. The ET_A -selective ligands, FR139317 and BQ123, competed with [¹²⁵I]-PD151242 binding to aorta with high affinity in the sub-nanomolar range (Table 1). The ET_B -selective ligand, BQ3020 competed only at high concentrations. In all cases, a one site fit was preferred to a two site model. Vasoactive non-endothelin peptides (including calcitonin gene-related peptide, angiotensin II, bradykinin and atrial natriuretic peptide) did not compete for binding of [¹²⁵I]-PD151242 when tested at a concentration of 1 μM (data not shown). Binding of [¹²⁵I]-PD151242 (up to 0.1 nM) could not be detected to cultures of human umbilical vein endothelial cells that have previously been shown by reverse-transcriptase polymerase chain reaction assays to express only ET_B mRNA (Molenaar *et al.*, 1993).

In human isolated epicardial coronary arteries, PD151242 (1–30 μM) produced significant, parallel rightward shifts of the ET-1 dose-response curve. A pA_2 value of 5.92 ($n = 5$ individuals) was derived by Schild analysis and the slope of the regression was not significantly different ($P > 0.05$) from unity.

Discussion [¹²⁵I]-PD151242 has a high affinity and selectivity for human ET_A receptors in aorta, pulmonary and coronary arteries. Hill slopes were close to unity suggesting that the ligand binds to a single population of receptors or to multiple binding sites with similar affinities. In each vessel, affinities were comparable to those previously obtained for [¹²⁵I]-ET-1 in saturation binding assays: aorta ($K_D = 0.51 \text{ nM}$), pulmonary ($K_D = 0.85 \text{ nM}$) and coronary arteries ($K_D = 0.14 \text{ nM}$) (Davenport *et al.*, 1993). We have previously shown that the media of all three vessels express mRNA encoding both ET_A and ET_B sub-types by reverse-transcriptase polymerase chain reaction and *in situ* hybridization assays. Competition binding assays have confirmed that human vascular tissue contains predominantly the ET_A sub-type with a smaller population of ET_B receptors. For example, BQ123 competes with [¹²⁵I]-ET-1 binding to human coronary artery in a biphasic manner with $K_D \text{ ET}_A = 0.85 \text{ nM}$, $K_D \text{ ET}_B = 7.6 \mu\text{M}$, ratio 87%:13% (Davenport *et al.*, 1993). In

Table 1 A Saturation binding experiments: dissociation constants (K_D), maximal density of receptors (B_{max}) and Hill coefficients (nH) for [¹²⁵I]-PD151242 binding to the media from human arteries

	n	K_D (nM)	B_{max} (fmol mg ⁻¹ protein)	nH
Aorta	3	0.76 ± 0.17	5.98 ± 1.56	1.00 ± 0.01
Pulmonary	3	1.75 ± 0.20	12.78 ± 1.39	1.00 ± 0.03
Coronary	3	0.51 ± 0.07	44.9 ± 1.67	0.84 ± 0.09

B Competition binding experiments: comparison of ET_A and ET_B ligands competing for the binding of [¹²⁵I]-PD151242 to the media of human aorta

	n	K_D
ET_A		
PD151242	3	$1.20 \pm 0.88 \text{ nM}$
BQ123	3	$0.41 \pm 0.26 \text{ nM}$
FR139317	3	$0.55 \pm 0.11 \text{ nM}$
ET_B		
BQ3020	3	$1.36 \pm 0.25 \mu\text{M}$

Values are the mean $\pm$ s.e.mean of three individuals in both A and B.

support of these results, the density of ET_A receptors estimated by [¹²⁵I]-PD151242 (Table 1) was lower than those obtained with the non-selective ligand [¹²⁵I]-ET-1 where B_{max} values of 9, 15 and 71 fmol mg⁻¹ protein were found in aorta, pulmonary and coronary arteries respectively (Davenport *et al.*, 1993). Bax *et al.* (1993) have observed that in *post-mortem* coronary artery, both BQ123 and [Ala^{1,3,11,15}]ET-1 compete monophasically for [¹²⁵I]-ET-1 binding suggesting an atypical ET receptor but our results thus far are consistent with the presence of the two known receptor sub-types.

PD151242 antagonized ET-1 constrictor responses in human isolated coronary arteries and the regression slope of unity indicated the compound acts in a competitive manner. These data support results obtained with other ET_A antagonists, suggesting that vasoconstriction in the human vasculature is predominantly mediated via the ET_A sub-type (Maguire & Davenport, 1993). The difference between the affinity of [¹²⁵I]-PD151242 measured by ligand binding and the potency of the unlabelled compound observed *in vitro* is intriguing but consistent with results from other studies using established ET_A -selective antagonists. For example, the K_D for BQ123 competing for [¹²⁵I]-ET-1 binding in the media of human coronary artery was 0.85 nM (Davenport *et al.*, 1993) but has a pA_2 value of 7.0 in the same tissue *in vitro* (Maguire & Davenport, 1993). Similar pA_2 values of BQ123 have been reported for example, in pig coronary artery (Ihara *et al.*, 1992) and rat thoracic artery (Summer *et al.*, 1992).

The results show the new iodinated ligand, PD151242, has high affinity and selectivity for ET_A receptors and the availability of this radioligand will facilitate the further characterization of this sub-type in human tissues.

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