Affinity and Selectivity of PD156707, A Novel Nonpeptide Endothelin Antagonist, for Human ET\textsubscript{A} and ET\textsubscript{B} Receptors\textsuperscript{1}

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ABSTRACT

We have determined the affinity and selectivity of a new nonpeptide antagonist PD156707 (sodium 2-benzo(1,3)dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enoate) for human endothelin (ET)\textsubscript{A} and ET\textsubscript{B} receptors. In human coronary artery and saphenous vein the affinity of the ETA receptor for PD156707 was 0.15 ± 0.06 nM and 0.5 ± 0.13 nM, respectively. Competition experiments in human left ventricle and kidney revealed that PD156707 had 1,000- to 15,000-fold selectivity for the ETA receptor over the ET\textsubscript{B} receptor. This selectivity was confirmed autoradiographically. In human coronary artery, mammary artery and saphenous vein PD156707 (3–300 nM) potently antagonized the vasoconstrictor responses to ET-1. The pA\textsubscript{2} values estimated from the Gaddum-Schild equation were 8.07 ± 0.09, 8.45 ± 0.11 and 8.70 ± 0.13, respectively. The concentration-response curves to ET-1 were shifted to the right in parallel fashion, without reduction of the maximum response. However, the regression lines fitted to the resulting Schild data deviated significantly from one. PD156707 appeared to be a more effective antagonist at lower concentrations than at the higher ones. It is possible that PD156707, a sodium salt, was reverting to a less soluble form which results in underestimation of its potency. These data show that PD156707 is a potent and selective antagonist at human ET\textsubscript{A} receptors and will be useful in clarifying the role of the endothelins in human cardiovascular disease.

The endothelins, a family of three peptides designated ET-1, ET-2 and ET-3 (Masaki et al., 1991; Yanagisawa et al., 1988), are the most potent constrictors of the human vasculature yet described (Davenport et al., 1989). Both of the ET receptors, ETA and ETB (Arai et al., 1990; Sakurai et al., 1990), mediate vasoconstriction but the ETA receptor predominates (>80%) in the medial layer of human blood vessels (Davenport et al., 1995). The ETA receptor is therefore responsible for the majority of the profound constrictor response elicited by these peptides both in vitro (Godfraind, 1993; Hay et al., 1993; Maguire et al., 1994; Maguire and Davenport, 1995; Opgaard et al., 1994; Riezebos et al., 1994) and in vivo (Haynes et al., 1995). These data strongly imply a therapeutic role for ETA-selective antagonists in conditions of pathological vasospasm, e.g., subarachnoid hemorrhage (Masaoka et al., 1989), in which plasma levels of ET-1 are raised. Indeed ETA selective antagonists such as BQ123 are effective in limiting tissue damage in some animal models of vasospasm (e.g., Clozel and Watanabe, 1993).

Nonpeptide ET antagonists have been developed recently which exhibit a range of receptor selectivities. We have previously reported that the orally active, nonpeptide, butenolide PD155080 (Doherty et al., 1995), had up to 1,000-fold selectivity for human ET\textsubscript{A} receptors compared with ET\textsubscript{B} receptors (Maguire et al., 1995). With human cloned receptors a second compound in this series, PD156707 (fig. 1) (Doherty et al., 1995), has been shown to possess enhanced selectivity for the ET\textsubscript{A} over the ET\textsubscript{B} receptor and improved affinity for both receptors compared with PD155080. After oral administration, PD156707 blocked the ETA pressor response to infused ET-1 without affecting the ET\textsubscript{B} depressor response, which indicated good bioavailability.

As species differences in the ET receptors have been reported with respect to their pharmacological profiles (Reynolds et al., 1995a; Russell and Davenport, 1996), we have measured the affinity of PD156707 for human ET\textsubscript{A} receptors in preparations of artery and vein. The ETA subtype comprises more than 80% of the total ET receptor population on human vascular smooth muscle cells (Davenport et al., 1995). Competition data from coronary artery and saphenous vein will therefore establish whether or not PD156707 has high affinity for this receptor subtype. Selectivity for one subtype over another is best demonstrated in tissues that express both ETA and ET\textsubscript{B} receptors. We have therefore used sections

ABBREVIATIONS: ET, endothelin; PD156707, sodium 2-benzo(1,3)dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enoate; PD155080, sodium 2-benzo(1,3)dioxol-5-yl-3-benzyl-4-(4-methoxy-phenyl)-4-oxobut-2-enoate; BQ123, cyclo(D-Asp-L-Pro-D-Val-L-Leu-D-Trp); Tris, 2-amino-2-(hydroxymethyl)-1,3-propan diol; HEPES, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid].
of human left ventricular free wall and kidney. The ratio of ET<sub>A</sub>:ET<sub>B</sub> in ventricle is approximately 75%:25% (Molenaar et al., 1992), whereas this is reversed in kidney, with ET<sub>B</sub> receptors predominating (30% ETA:70% ETB) (Karet et al., 1993). The potency of PD156707 as an antagonist of ET-1 constrictor responses was determined in human isolated coronary artery, internal mammary artery and saphenous vein.

**Methods**

**Competition binding experiments.** Human tissues were obtained with local ethical approval. The studies reported in this manuscript were carried out in accordance with the Declaration of Helsinki and/or with Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

For the binding experiments epicardial coronary arteries (n = 3, male, 21–50 years of age) and left ventricular free wall (n = 3, male, 54–58 years of age) were obtained from patients undergoing coronary artery bypass grafts (n = 43, 38 male and 5 female, 42–81 years of age). Sections of nondiseased kidney were obtained from patients (n = 3, male, 32–74 years of age) undergoing nephrectomy for nonobstructing tumors. All tissues were frozen in liquid nitrogen at the time of the operation and stored at −70°C until required.

Sections (10 µm) were cut from the media (smooth muscle layer) of coronary arteries and from blocks of left ventricle and kidney (cortex and medulla). Saphenous veins were dissected free of connective tissue, ground at −70°C with a freezer mill (Glen Creston Ltd, Midddex, U.K.) and homogenized with ice-cold Tris-HCl buffer (50 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, 5 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 100,000 IU/ml aprotinin, pH 7.4). The homogenate was centrifuged (1,000 × g, 1 min, 4°C), the pellet discarded and the supernatant further purified by centrifugation at 40,000 × g for 30 min (4°C). The resulting pellet was resuspended in Tris-HCl buffer and subjected to two additional centrifugation steps (40,000 × g, 30 min, 4°C) with the final pellet resuspended in HEPES buffer (50 mM HEPES, 5 mM MgCl<sub>2</sub>, 0.3% bovine serum albumin, 4°C, pH 7.4). The concentration of protein was determined (Biorad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.), the homogenate diluted to give a final concentration of 6 mg protein/ml and stored at −70°C. When required the saphenous vein homogenate was thawed, centrifuged (20,000 × g, 10 min, 4°C) and resuspended in HEPES buffer (23°C).

For competition binding experiments sections of coronary artery, left ventricle and kidney and aliquots of saphenous vein homogenate (final assay concentration, 2 mg/ml) were incubated for 2 h at 23°C with 0.1 nM [125I]ET-1 (2000 Ci/mmol, Amersham International plc, Buckinghamshire, U.K.) and increasing concentrations (20 pM–100 µM) of PD156707. Nonspecific binding was determined by the inclusion of 1 nM ET-1. Sections were then washed in ice-cold Tris-HCl buffer (3 × 5 min) and the homogenates were centrifuged (20,000 × g, 10 min, 4°C); the resulting pellets were washed and recentrifuged in ice-cold Tris-HCl buffer (20,000 × g, 10 min). All tissues were then counted for [125I] content. The amount of total binding for [125I]ET-1 was typically 10,000 to 30,000 dpm per tissue section or aliquot. In all instances specific binding was 85% to 95% of the total. Data files from separate experiments generated by EBDA (McPherson, 1983) were coanalyzed by the nonlinear iterative curve-fitting programme LIGAND (Munson and Rodbard, 1980) to calculate values of equilibrium dissociation constant (K<sub>D</sub>, expressed as nanomolar) and maximum receptor density (B<sub>max</sub>, expressed as femtomoles bound per milligram of protein). The presence of one, two or three sites was determined by the F<sub>R</sub> ratio test. Selectivity of PD156707 for the ET receptor subtypes was estimated by the comparison of the derived K<sub>D</sub> values.

**Autoradiography.** To visually demonstrate the receptor selectivity of PD156707 for ET<sub>A</sub> receptors additional kidney sections were incubated for 2 h at 23°C, with 0.1 nM [125I]ET-1 to label both ETA and ETB receptors. Adjacent sections were incubated with 0.1 nM [125I]ET-1 to which the selective agonist BQ3020 ([Ala<sup>11,15</sup>]Ac-ET-1 (6–21), 200 nM) was added. This concentration of BQ3020 was calculated from saturation data (Karet et al., 1993) to block more than 99% of ET<sub>B</sub> receptors, but less than 3% of ET<sub>A</sub> receptors, and therefore reveal ETA receptor distribution. Similarly BQ123 was added at 100 nM, calculated to block more than 90% of ET<sub>A</sub> receptors and less than 3% ET<sub>B</sub> receptors, and so identify the latter. Finally, for comparison, the pattern of receptor distribution was determined for [125I]ET-1 in the presence of PD156707 (53 nM). This concentration of PD156707 was calculated from the competition data to block more than 98% of ET<sub>A</sub> receptors but less than 8% of ET<sub>B</sub> receptors. The sections were washed in ice-cold Tris-HCl buffer (3 × 5 min), air-dried and exposed to radiation-sensitive Hyperfilm β-max (Amer sham International plc, Buckinghamshire, U.K.) for 5 days.

**In vitro pharmacological experiments.** For in vitro pharmacological experiments blood vessels were collected in ice-cold Krebs’ solution at the time of the operation and transported back to the laboratory. Coronary arteries were obtained from 12 patients (10 male, 2 female, 42–59 years of age) who were undergoing cardiac transplantation, and internal mammary arteries from 13 patients (11 male, 2 female, 46–71 years of age) and saphenous vein from 13 patients (10 male, 3 female, 47–74 years of age) were from patients receiving coronary artery bypass grafts.

Sections of coronary artery, mammary artery and saphenous vein were cut into 3- to 4-mm lengths, the endothelium was gently rubbed away with a blunt metal seeker (verified histologically), and the rings were suspended between two metal L-shaped hooks (verified histologically), and the rings were suspended between two metal L-shaped hooks in 5-ml tissue baths containing oxygenated Krebs-Henseleit solution maintained at 37°C. Contractile responses were measured isometrically (F30 force transducers, Hugo Sachs Elektronik, March-Hugstetten, Germany) and recorded on a Graphtec chart recorder (Linton Instrumentation, Diss, Norfolk, UK). The vessels were allowed to equilibrate for 1 h, and then responses were elicited to 50 mM KCl at increasing levels of resting tension until no further increase in the magnitude of the KCl response was obtained. The preparations were then allowed to relax to their own resting tension before the effect of PD156707 was determined. PD156707 (3–300 nM) or vehicle (control) was added to the bathing medium for 30 min, then cumulative concentration-response curves were constructed to ET-1 (10<sup>−10</sup>–10<sup>−6</sup> M). When addition of a higher concentration of ET-1 elicited no
further contractile response, 50 mM KCl was added to determine the maximum possible contractile response for each preparation. ET-1 responses were subsequently expressed as a percent of this KCl response. Data for Schild analysis (Arunlakshana and Schild, 1959) were derived from the graphs of ET-1 concentration (log10) plotted against response (percent KCl response) in the absence and presence of increasing concentrations of PD156707. The slope of the resulting Schild regressions, if not significantly different from one, were constrained to one to determine the value of pKB for PD156707 in each of the three blood vessels used.

Additional experiments were designed to show whether or not PD156707 (100 nM) could reverse a constrictor response to 30 nM ET-1. Rings of saphenous vein (n = 3) were contracted with 30 nM ET-1 and the response allowed to develop fully over 30 to 40 min. Once the maximum response was established, PD156707 (100 nM) was added to the bath and the response was recorded. The effectiveness of PD156707 was compared with that of BQ123 (3 μM).

Fig. 2. Competition curves for the inhibition of specific [125I]ET-1 binding by PD156707 (20 pM–100 μM) in (a) 10-μm sections of human coronary artery media and (b) homogenates of human saphenous vein. From analysis of the competition data, a one-site fit was preferred in coronary artery which indicated that only ETα receptors could be detected. In saphenous vein a biphasic competition curve was obtained with PD156707 and a two-site fit was preferred which revealed a majority of ETα receptors and a smaller population of ETβ receptors. Data points are the mean ± S.E.M. from three individuals.

Fig. 3. Competition curves for the inhibition of specific [125I]ET-1 binding by PD156707 (20 pM–100 μM) in 10-μm sections of (a) human left ventricle and (b) human kidney. Biphasic competition curves were obtained for PD156707 in both tissues with high-affinity ETα receptors predominating in the left ventricle and low-affinity ETβ receptors most abundant in the kidney. Data points are the mean ± S.E.M. from three and four individuals, respectively.

| TABLE 1 | Affinity (Kd) and selectivity of PD156707 for human ETα and ETβ receptors |
|---|---|---|---|---|
|  | ETα | ETβ | ETα:ETβ | n |
| Coronary artery | 0.15 ± 0.06 | – | 100.0 | 3 |
| Saphenous vein | 0.50 ± 0.13 | 1.42 ± 0.02 | 83.17 | 2,840 | 3 |
| Left ventricle | 0.92 ± 0.38 | 13.3 ± 2.09 | 75.25 | 14,457 | 3 |
| Kidney | 0.53 ± 0.49 | 0.57 ± 0.04 | 22.78 | 1,076 | 4 |

Equilibrium dissociation constants (Kd) were derived by use of LIGAND from data pooled from the individual experiments. The ratio of ETα to ETβ receptors identified in these tissues by PD156707 was obtained by comparison of the Bmax values. Receptor selectivity for PD156707 was calculated by comparison of the Kd values of ETα and ETβ in each case. n refers to the number of individuals from whom tissue was obtained.
Affinity and selectivity of PD156707 for human ET<sub>A</sub> and ET<sub>B</sub> receptors. PD156707 competed with subnanomolar affinity for the majority of sites labeled by 0.1 nM [125I]ET-1 in coronary artery (fig. 2a) and saphenous vein (fig. 2b). A small low-affinity component was detected only in the saphenous vein. These data suggest that PD156707 has high affinity for human ET<sub>A</sub> receptors (table 1).

This was confirmed by competition experiments in left ventricle and kidney. In ventricle PD156707 competed with subnanomolar affinity for 75% of the binding sites and micromolar affinity for the remaining 25% of sites (fig. 3a). Conversely, in the kidney PD156707 exhibited micromolar affinity for almost 80% of the specific [125I]ET-1 binding and subnanomolar affinity for the remaining 20% (fig. 3b). These data verify that PD156707 has very high affinity for human ET<sub>A</sub> receptors and reveal a 1,000- to 15,000-fold selectivity for this subtype compared with the ET<sub>B</sub> receptor (table 1).
TABLE 2

Antagonism of ET-1-mediated vasoconstriction by PD156707 in human isolated blood vessels

For each concentration of PD156707 data are the pA2 values (mean ± S.E.M., from n individuals) estimated from the Gaddum-Schild equation assuming a slope of 1, where pA2 = log10[concentration ratio - 1]/concentration PD156707 (M).

<table>
<thead>
<tr>
<th>PD156707</th>
<th>Coronary Artery</th>
<th>Mammary Artery</th>
<th>Saphenous Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.23 ± 0.26 (n = 6)</td>
<td>9.17 ± 0.25 (n = 8)</td>
<td>9.23 ± 0.27 (n = 5)</td>
</tr>
<tr>
<td>10</td>
<td>8.42 ± 0.18 (n = 9)</td>
<td>8.24 ± 0.11 (n = 8)</td>
<td>8.62 ± 0.22 (n = 8)</td>
</tr>
<tr>
<td>30</td>
<td>8.30 ± 0.14 (n = 8)</td>
<td>8.05 ± 0.14 (n = 6)</td>
<td>8.64 ± 0.35 (n = 5)</td>
</tr>
<tr>
<td>100</td>
<td>7.94 ± 0.13 (n = 8)</td>
<td>7.91 ± 0.31 (n = 3)</td>
<td>8.11 ± 0.30 (n = 6)</td>
</tr>
<tr>
<td>300</td>
<td>7.62 ± 0.11 (n = 5)</td>
<td>8.05 ± 0.09</td>
<td>8.45 ± 0.11</td>
</tr>
<tr>
<td>Mean</td>
<td>8.07 ± 0.09</td>
<td>8.45 ± 0.11</td>
<td>8.70 ± 0.13</td>
</tr>
</tbody>
</table>

From the autoradiograms it is clear that both receptor subtypes are present throughout the kidney medulla and cortex (fig. 4A). The pattern of ETA (fig. 4B) and ETB (fig. 4C) receptor distribution is consistent with that which we have previously demonstrated with use of specific ETA and ETB radioligands (Davenport et al., 1994) and microautoradiographical techniques (Karet et al., 1993). ETA receptors are localized to the large arcuate arteries and afferent veins at the corticomedullary junction and the resistance arteries present particularly in the cortex (fig. 4B), whereas ETB receptors have a nonvascular distribution (fig. 4C). The results also demonstrate that the pattern of receptor distribution remaining after incubation with PD156707 (fig. 4D) is identical with that achieved with the highly ETA-selective antagonist BQ123 (fig. 4C) and different to that obtained with the ETB agonist BQ3020 (fig. 4B).

**Antagonism of ET-1 constriction in human isolated blood vessels.** ET-1 potently contracted rings of isolated coronary artery (n = 12), mammary artery (n = 13) and saphenous vein (n = 13) with EC50 values of 9.1 nM (5.6–15.0 nM), 4.3 nM (2.8–6.7 nM) and 1.7 nM (0.8–3.7 nM), respectively (geometric mean with 95% confidence intervals). The maximum response to ET-1 in each preparation was compared with that to 50 mM KCl added at the end of the experiment. For coronary artery, mammary artery and saphenous vein the mean KCl responses were 5.07 ± 0.87 g, 4.64 ± 1.10 g and 2.01 ± 0.43 g with maximum responses to ET-1 of 80.91 ± 4.32%, 79.22 ± 3.78% and 92.97 ± 1.91% respectively.

In each of the three preparations the response to ET-1 was antagonized by PD156707 which produced a rightward shift of the ET-1 concentration-response curve without significant diminution of the maximum response. This suggested that PD156707 was acting in a competitive manner; however, it was apparent that some of the lowest concentrations tested were as effective at displacing the control ET-1 concentration-response curve as some of the higher concentrations (fig. 5). From the Schild data it is clear that the resulting regression slopes deviated significantly from one (P < 0.05, Student’s t test) and were 0.53 ± 0.17 in coronary artery, 0.37 ± 0.14 in mammary artery and 0.48 ± 0.17 in saphenous vein (fig. 6).

Since pKB values could not be derived from the Schild regressions, pA2 values were estimated for each concentration of PD156707 from the Gaddum-Schild equation, which assumes a slope of one. In this way the variation in potency of PD156707 as an ET antagonist could be compared for each of the concentrations tested (table 2) and this ranged from 7.62 calculated for 300 nM PD156707 in coronary artery to 9.23 for 3 nM PD156707 in saphenous vein.

Saphenous vein preparations were contracted by 30 nM ET-1, with the response well maintained for more than 80 min (fig. 7a). Some reversal (~50%) of this established ET-1 contraction was achieved with 3 μM BQ123 (fig. 7b), whereas more than 80% reversal was achieved with 100 nM PD156707 (fig. 7c).
Discussion

Among the known vasoactive peptides ET possesses a unique pharmacological profile. Not only is it the most potent vasoconstrictor yet described (Yanagisawa et al., 1988), but it also has an unusually prolonged duration of action (Clarke, et al., 1989; Ide et al., 1989; Weitzberg, et al., 1991). In addition to its direct effects on vascular smooth muscle, subthreshold concentrations of ET will enhance the response to other vasospastic agents such as noradrenaline and 5-hydroxytryptamine (Chester et al., 1992; Yang et al., 1990). Since plasma ET levels are raised in a number of vascular disorders (see Huggins et al., 1993, for review) it is possible that this peptide is responsible for, or exacerbates, the vasospasm which may accompany conditions such as subarachnoid hemorrhage and ischemic heart disease.

The clinical potential of ET antagonists has encouraged the design and synthesis of orally active, nonpeptide antagonists such as the recently described antagonist PD156707 (Doherty et al., 1995). In the present study we have extended this pharmacological characterization by evaluating the affinity and selectivity of PD156707 for native human ET receptors and determining its potency as an antagonist of ET-1-mediated vasoconstriction in human isolated blood vessel preparations.

In all of the human tissues investigated the ET_A receptor exhibited subnanomolar affinity for PD156707 whereas the ET_B receptor had only micromolar affinity. These data confirm the selectivity of PD156707 for human ET_A receptors. We find a 1,000- to 15,000-fold higher affinity for ET_A compared with ET_B receptors, which is comparable to that reported for PD156707 in animal tissue and cloned human receptors (Reynolds et al., 1995b).

In functional experiments PD156707 potently inhibited the contractile response to ET-1 observed in human isolated vascular preparations. The concentration-response curves for ET-1 were shifted to the right in parallel fashion without reduction of the maximum response which indicates competitive antagonism. This concurred with observations by Reynolds and colleagues in the rabbit femoral artery (Reynolds et al., 1995b). However the ability of PD156707 to antagonize ET-1 in these human blood vessel preparations did not appear to be completely concentration-dependent. The Schild regressions deviated significantly from one and it was apparent that the compound was more potent at the lower concentrations tested compared with the higher concentrations (fig. 5b). We do not have an explanation for the reduced potency of PD156707 observed at the higher concentrations tested. One possibility that requires investigation is that PD156707 may be reverting to a less soluble form (Doherty et al., 1995) leading to an underestimation of the antagonist’s potency.

It is noteworthy that the pA_2 values estimated for PD156707 in human tissues at their most conservative (pA_2 of 7.6–8.1 at 300 nM) are better than that reported for the antagonism of ET-1 in rabbit femoral artery (Schild-derived pA_2 = 7.5; Reynolds et al., 1995b). We find that PD156707 is up to 50 times more potent as an antagonist of ET-1 contractions in human vasculature in vitro (pA_2 = 9.2 at 3 nM) than in the rabbit preparation. We are not able to give a reason for this discrepancy as yet, although in binding experiments it appears that members of this series of butenolide analogs have higher affinity for human ET_A receptors than rabbit ET_A receptors, whereas the converse is true for ET_B receptors (Doherty et al., 1995).

PD156707 has been shown to be effective in limiting tissue damage in animal models of ischemia. After intravenous administration, PD156707 (3 μmol/kg bolus + 5 μmol/kg/h infusion) restored cerebral blood flow to normal and reduced by 45% the volume of cerebral ischemic damage in a cat model of focal ischemia (Patel et al., 1996). Crucially we were able to demonstrate the complete reversal of the constrictor response to ET-1 by PD156707 in human saphenous vein in vitro. This suggests that ET_A-selective antagonists such as PD156707 would be effective in conditions of established vasospasm in man.

The high affinity and excellent selectivity of PD156707 demonstrated for human vascular ET_A receptors suggests a potential therapeutic role for this and related compounds in conditions of pathological vasospasm in which the ET system is thought to be important.

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References


Novel Human ETA Receptor Antagonist 1107


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