Endothelin Receptor Antagonists: Effect of Serum Albumin on Potency and Comparison of Pharmacological Characteristics

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ABSTRACT
Endothelins (ETs) are 21-amino acid peptides that bind to membrane receptors to initiate pathophysiologic effects. Two types of ET receptors, ETA and ETB, have been identified. Various ET receptor antagonists are being developed as therapeutic agents. This report examines the effects of bovine serum albumin (BSA) on the potency of ET receptor antagonists and compares five ET receptor antagonists. Competition studies show that in the absence of BSA, A-127722 and L-749329 inhibited ET-1 binding to ETα receptor with the same IC50 value of 0.09 nM. Addition of increasing concentrations of BSA incrementally decreased the potency of the antagonists: in the presence of 5% BSA, the IC50 values increased to 4.3 and 820 nM, respectively. Similarly, addition of BSA decreased the potency of antagonists in inhibiting ET-1-stimulated phosphatidylinositol hydrolysis. These results suggest that serum albumin has profound effects on the potencies of ET receptor antagonists. FR139317, PD-156707, L-749329, Ro-47-0203 and A-127722 were then selected for direct comparison under identical experimental conditions with 0.2% BSA. The potency of antagonists was assessed by binding studies for the determination of IC50 and Ki values and by ET-1-stimulated phosphatidylinositol hydrolysis and arachidonic acid release for the determination of IC50 and pA2 values. All five antagonists inhibited ET binding and the biological effects exerted by ET in a competitive mode. The Ki values for A-127722, PD-156707, FR139317, Ro-47-0203 and L-749329 for the ETα receptor were 0.07, 0.38, 0.80, 3.67 and 33.6 nM, respectively. A similar hierarchy was revealed by the functional assays. Our results suggest that the rank order of potency of the antagonists is A-127722 > PD-156707 > FR139317 > Ro-47-0203 > L-749329.

ET, originally isolated from cultured porcine aortic endothelial cells, is a highly potent vasoconstricting peptide with 21-amino acid residues (Yanagisawa et al., 1988). Three distinct members of the ET family, namely ET-1, ET-2 and ET-3, have been identified in humans through cloning (Inoue et al., 1989). The effects of ETs on mammalian organs and cells are initiated by their binding to high-affinity G-protein-linked receptors. ET receptors are found in various tissues and cells, such as brain, lung and mesangial cells (Sokolovsky, 1992). Two types of ET receptors, ETA and ETB, have been characterized, isolated (Kozuka et al., 1991; Wada et al., 1990) and their cDNA cloned (Arai et al., 1992; Sakurai et al., 1993). ETA receptors are selective for ET-1 and ET-2, whereas ETB receptors bind to ET-1, ET-2 and ET-3 with equal affinity. Several antagonists and agonists for ET receptors have been developed (Opgenorth, 1995). Among them, FR139317 (Sogabe et al., 1993), PD-156707 (Reynolds et al., 1995), L-749329 (Walsh, 1995), Ro-47-0203 (Clozel et al., 1994) and A-127722 (Opgenorth et al., 1996) are potent ET receptor antagonists.

Since endothelins were discovered in 1988 (Yanagisawa et al., 1988), research in this field has become one of the most rapidly developing areas in the biological sciences. Since the development of potent antagonists for ET receptors, there has been keen interest in developing these ET receptor antagonists for clinical utilization. Information on the interaction between ET receptor antagonists and plasma proteins is rather limited, despite the potential impact of protein binding on the in vivo efficacy. Previously we have shown that ET-1, ET-3 and ET receptor antagonists (PD-156707, L-749329, Ro-47-0203 and A-127722) exhibit high degrees of binding to human plasma proteins, especially serum albumin (Wu-Wong et al., 1996). Addition of human serum albumin (HSA, 5%) decreases the potency of ET receptor antagonists in inhibiting ET binding to the receptors (Wu-Wong et al., 1996). Because BSA is frequently included in in vitro assays for determination of the potency and efficacy of ET receptor antagonists, it is important to know whether BSA will have effects similar to those previously shown for HSA. In addition, because investigators do not always use the same concentrations of BSA in their studies, it may be important to know whether different amounts of BSA affect the potency to different extents.

The purposes of this report are 1) to examine whether the

ABBREVIATIONS: ET, endothelin; PI, phosphatidylinositol; AA, arachidonic acid; BSA, bovine serum albumin.
presence of BSA affects the potency of ET receptor antagonists and 2) to compare ET receptor antagonists in various assays using a fixed concentration of BSA. We first show that increasing concentrations of BSA in the assay buffer incrementally decrease the potencies of test antagonists in inhibiting ET binding to the receptors. Furthermore, antagonists of distinct structures are affected by BSA to different degrees. In functional assays, BSA also decreases the potency of antagonists in inhibiting ET-1-stimulated PI hydrolysis. Five structurally distinct ET receptor antagonists (FR139317, PD-156707, L-749329, Ro-47-0203 and A-127722) were then compared in binding and functional assays under identical conditions with 0.2% BSA.

Materials and Methods

Materials. [125I]ET-1 (2200 Ci/mmol) and [125I]ET-3 (2200 Ci/mmol) were obtained from Du Pont, NEN (Boston, MA). ET-1 and ET-3 were purchased from American Peptide Company (Sunnyvale, CA). FR139317 (cC6-N-Leu-D-Trp-(Me)-D-2Pya-OH), A-127722 (trans-trans-2-(4-methoxyphenyl)-4-(1,3-benzodioxo-5-yl)-1-(N,N-dibutylamino)carbonylmethyl)pyrrolidine-3-carboxylic acid), PD-156707 (9-bi-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2-dimethyldioxy-1-(2-propyl-4-carboxyphenoxy)-N-dibutylamino)carbonylmethyl)pyrrolidine-3-carboxylic acid), L-749329 ([3',4'-methylenedioxy-1-(2-propyl-4-carboxyphenoxy)-N-(4-isopropyl-phenylsulfonyl)-benzene acetamide), Ro-47-0203 ([4-tet-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bi-pyrimidin-4-yl]-benzenesulfonamide]) and PD-156707 (sodium 2-benzo[1,3]dioxol-5-yl-4-((4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxy-benzyl)-but-2-enoate)) were synthesized in house. Other reagents were of analytical grade. Figure 1 shows the chemical structures of the five ET receptor antagonists evaluated.

Cell culture. MMQ cells were licensed from University of Virginia and were cultured as described previously (Judd et al., 1988). Smooth muscle cells prepared from human pericardium were cultured as described previously (Judd et al., 1988). Chinese hamster ovary (CHO) cells permanently expressing human ET<sub>A</sub> or ET<sub>B</sub> receptor (Magnuson et al., 1994) were cultured in F-12 medium containing 10% fetal bovine serum (FBS) and 500 μg/ml G418 (geneticin). Cell viability was examined by the trypan blue exclusion method.

Preparation of membranes. Membranes were prepared from porcine cerebellum, rat pituitary MMQ cells or CHO cells as previously described (Wu-Wong et al., 1995; 1996). Briefly, cerebella or cells were homogenized in 25 volumes (w/v) of 10 mM HEPES (pH 7.4) containing 0.25 M sucrose and protease inhibitors (3 mM EDTA, 0.1 mM phenylmethyl sulfon fluoride and 5 μg/ml Pepstatin A) by 3 to 10 s polystron at 13,500 rpm with 10-s intervals (for cerebella) or by a micro ultrasonic cell disruptor (Kontes, Vineland, NJ) (for cells). The mixture was centrifuged at 1000 × g for 10 min. The supernatant was collected and centrifuged at 30,000 × g for 30 min (for cerebella) or at 60,000 × g for 60 min (for cells). The precipitate was resuspended in Buffer B-1 (20 mM Tris, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, pH 7.4) containing the aforementioned protease inhibitors and then centrifuged again. The final pellet was resuspended in Buffer B-1 containing protease inhibitors and stored at −80°C until used. Protein content was determined by the Bio-Rad dye-binding protein assay.

Radioligand binding to membranes. Binding assays were performed in 96-well microtiter plates precoated with 0.1% BSA unless otherwise indicated. Membranes were diluted in Buffer B (Buffer B-1 with the aforementioned protease inhibitors plus 0.025% bacitracin and 0.2% BSA) to a final concentration of 0.05 mg/ml of protein. In some experiments, the concentrations of BSA in Buffer B were different as indicated. In competition studies, membranes were incubated with 0.1 nM of [125I]ET in Buffer B (final volume: 0.2 ml) in the presence of increasing concentrations of unlabeled test ligands for an indicated period of time at 25°C. In saturation studies, membranes were incubated with increasing concentrations of [125I]ET in Buffer B (final volume: 0.2 ml) in the presence or absence of unlabeled test ligands for 4 h at 25°C. After incubation, unbound ligands were separated from bound ligands by vacuum filtration using glass-fiber filter strips in PHD cell harvesters (Cambridge Technology, Inc., Watertown, MA), followed by washing of the filter strips with saline (1 ml) three times. Nonspecific binding was determined in the presence of 1 μM ET-1 or ET-3.

For Ki calculation, K<sub>i</sub> values for ET-1 binding in the presence of an antagonist at different concentrations were determined by Scatchard analysis and were designated as K<sub>d</sub>. K<sub>i</sub> was plotted against the concentrations of antagonists (I) for determining the slopes. K<sub>d</sub> = K<sub>i</sub> when [I] = 0. Because K<sub>i</sub> = 1 + [I]/K<sub>i</sub>/K<sub>d</sub>, K<sub>i</sub> = K<sub>i</sub>/slope.

Measurement of PI hydrolysis. The procedure has been reported previously (Wu-Wong et al., 1995, 1996). Briefly, MMQ cells (0.4 × 10<sup>6</sup> cells/ml) prelabeled with 1 μCi/well of [3H]myo-inositol for 16 to 24 h were washed with PBS and then incubated with Buffer A (Earle’s solution: 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub> and 0.8 mM MgSO<sub>4</sub>, 5 mM glucose, buffered with 25 mM HEPES, pH 7.4, with or without 0.2% BSA) containing protease inhibitors (3 mM EDTA, 0.1 mM PMSF, and 5 μg/ml Pepstatin A) and 10 mM LiCl for 60 min before being challenged with ET-1 for an additional 45 min. ET challenge was terminated by the addition of 50 μl of 1 N NaOH, and the mixture was immediately neutralized by adding 50 μl of 1 N HCl. The samples were treated with 1.5 ml of chloroform/methanol (1:2, v/v). Total inositol phosphates were extracted after adding chloroform and water to give final proportions of chloroform/methanol/water of 1:1:0.9 (v/v/v) as described by Berridge et al. (1982). The upper aqueous phase (1 ml) was retained, and a small portion (100 μl) was counted. The rest of the aqueous sample was analyzed by batch chromatography using the anion-exchange resin AG1-X8 (Bio-Rad, Hercules, CA). Total water-soluble inositol phosphates were eluted from the resin by 6 ml of 1 M ammonium formate with 0.1 N formic acid after the resin was washed with 6 ml of 60 mM sodium formate with 5 mM sodium tetraborate.
Measurement of AA release. Human pericardial smooth muscle cells (HPSCM) in 48-well culture plates at 80% to 100% confluency were labeled with 0.4 μCi/well of [3H]AA in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS for 16 to 24 h. To assay AA release, cells were incubated with DMEM plus 0.2% BSA (0.5 ml/well) for 30 min. After the incubation, the medium was removed, and 0.3 ml DMEM with 0.2% BSA was added to each well. Various test agents at different concentrations were added, and finally ET-1 ranging from $10^{-11}$ to $10^{-6}$ M was added. Cell were incubated at 37°C for another 30 min, and the incubation medium was collected for determination of radioactivity.

For pA2 calculation, ET-1-induced AA release from samples treated with or without antagonists was normalized against the basal AA release from cells not treated with ET or antagonists, and the effective concentration of ET-1 that caused 50% maximum response (EC$_{50}$) was determined. Schild analysis of the antagonist-induced EC$_{50}$ shifts yielded a pA2 value as the comparative index of antagonism (Arunlakshana and Schild, 1956).

$$pA_2 = \log(\text{concentration ratio} - 1) - \log(\text{antagonist})$$

where the concentration ratio (CR) is the ratio of EC$_{50}$ values with and without antagonists (I).

Results

Competition binding studies were conducted in the presence of different amounts of BSA for A-127722 and L-749329, two antagonists that have similar potencies for ETA receptor but are different in potencies for ETB receptor as reported in the literature (Walsh, 1995; Opgenorth et al., 1996). Figure 2 shows that the IC$_{50}$ value for A-127722, when assayed using membranes prepared from MMQ cells with predominantly ETA receptor, shifted from 0.09 nM in the absence of BSA to 0.44, 1.26 and 4.30 nM in the presence of 0.2, 1 and 5% BSA, respectively. A similar shift was observed when the binding assay was done using membranes prepared from porcine cerebella with predominantly ETB receptor; the IC$_{50}$ value for A-127722 changed from 0.10 μM in the absence of BSA to...

Fig. 2. The effect of BSA on the potency of A-127722 (panels A and B) and L-749329 (panels C and D) in inhibiting [125I]ET-1 binding to human ETA (panels A and C) and ETB (panels B and D) receptors. Receptor binding was performed as described except that the assay plates were not BSA-coated. Nonspecific binding (ranging from 3.3 to 8.6 fmol/mg), determined in the presence of 1 μM ET-1, was subtracted from total binding to give specific binding. The results are expressed as percent of control, with binding in the absence of the antagonist as 100%. In the absence of antagonists, specific binding for ETA receptor was 50.1, 45.5, 47.3 and 38.1 fmol/mg at 0, 0.2%, 1% and 5% of BSA, respectively. Specific binding for ETB receptor was 434, 392, 390 and 363 fmol/mg at 0, 0.2%, 1% and 5% of BSA, respectively. Each value represents the mean of two (panels A and B) and three (panels C and D) determinations. Results shown are representative of two different experiments. The active enantiomer of A-127722 was used in this study.
0.34, 1.21 and 2.96 μM in the presence of 0.2, 1 and 5% BSA, respectively. Interestingly, when similar studies were conducted for L-749329 using membranes prepared from MMQ cells, the addition of BSA had a much greater impact on the potency of the antagonist. The IC_{50} value for L-749329 shifted from 0.09 nM in the absence of BSA to 45, 181 and 820 nM in the presence of 0.2, 1 and 5% BSA, respectively. When the binding assay was done using membranes prepared from porcine cerebella, the IC_{50} value for L-749329 shifted from 0.026 μM in the absence of BSA to 12, 33 and >100 μM in the presence of 0.2, 1 and 5% BSA, respectively. The above observation suggests that the potency of an ET receptor antagonist is critically dependent on the concentration of BSA used in the assay. This led us to compare directly a number of ET receptor antagonists in various assay systems at a fixed BSA concentration. We selected five ET receptor antagonists for comparison: A-127722, L-749329, FR139317, Ro-47-0203 and PD-156707. Figure 3 shows the results from competition binding studies using cloned human ET\textsubscript{A} and ET\textsubscript{B} receptors permanently expressed in CHO cells. In this binding assay system, 0.2% BSA was used in a buffer containing 20 mM Tris (pH 7.4), 100 mM NaCl, 10 mM MgCl\textsubscript{2}, and protease inhibitors 0.1 mM PMSF, 5 μg/ml Pepstatin A, 0.025% bacitracin and 3 mM EDTA. The IC_{50} values are summarized in Table 2. The results suggest that the potencies of the five antagonists are in the order of A-127722 > PD-156707 > FR139317 > Ro-47-0203 > L-749329 for ET\textsubscript{A} receptor, and A-127722 > Ro-47-0203 > L-749329 > PD-156707 > FR139317 for ET\textsubscript{B} receptor. All the antagonists tested exhibit a preference for ET\textsubscript{A} receptor, although the selectivity for ET\textsubscript{A} vs. ET\textsubscript{B} ranges from 42-fold for L-749329 to 10,686-fold for PD-156707.

To examine further the nature of the interaction between the antagonists and ET receptor, we performed [\textsuperscript{125}I]ET-1 saturation binding studies using membranes prepared from CHO cells stably expressing human ET\textsubscript{A} and ET\textsubscript{B} receptors. As shown in figure 4, A and B, increasing concentrations of L-749329 affected [\textsuperscript{125}I]ET-1 binding by causing successive increases in the apparent K\textsubscript{d} values without having a significant effect on the B\textsubscript{max} values. This result suggests that L-749329 is a competitive inhibitor for ET-1 binding to the ET\textsubscript{A} receptor. The K\textsubscript{i} value of L-749329 was determined to be 33.6 nM from figure 4C. Similar experiments were performed for the other four antagonists, and the results are compared in figure 4C. All five antagonists exhibited competitive behavior for inhibiting ET-1 binding. K\textsubscript{i} values for A-127722 and Ro-47-0203 against human ET\textsubscript{B} receptor were also determined using [\textsuperscript{125}I]ET-3 binding to the cloned human ET\textsubscript{B} receptor, and again, competitive inhibition was observed. Because of the weak potencies of PD-156707, FR139317 and L-749329 for the ET\textsubscript{B} receptor, K\textsubscript{i} values for these three compounds against human ET\textsubscript{B} receptor were not determined. The K\textsubscript{i} values are summarized in table 3. Again, the rank order of potency of the five antagonists is A-127722 > PD-156707 > FR139317 > Ro-47-0203 > L-749329 for ET\textsubscript{A} receptor.

The potency of an antagonist in inhibiting binding is generally expected to translate into comparable potency for antagonizing a functional response. To confirm this notion, we compared these ET receptor antagonists in functional assays. First, the effect of BSA on the potency of receptor antagonists was tested in ET-1-stimulated PI hydrolysis in MMQ cells. We have previously shown that ET-1-stimulated PI hydrolysis in MMQ cells is mediated through the ET\textsubscript{A} receptor (Wu-
Wong et al., 1993). Figure 5 shows that all the test antagonists inhibited ET-1-evoked PI hydrolysis in a dose-dependent manner in the presence or absence of BSA (0.2%). None of the compounds alone showed any effect, which suggests that none has agonist activity. The IC$_{50}$ values were significantly affected by the addition of BSA (table 4); structurally distinct antagonists were affected differently. In the absence of BSA, the potency is in the order of L-749329 > A-127722 > PD-156707 > FR139317 > Ro-47-0203; in the presence of BSA, the rank order of potency is A-127722 > PD-156707 > FR139317 > L-749329 > Ro-47-0203.

ET-1 has also been shown to stimulate AA release in human pericardial smooth muscle cells, an effect mediated through the ETA receptor (Wu-Wong et al., 1994a). The antagonists were compared in the AA release assay with 0.2% BSA included in the buffer. Figure 6A shows that all the test antagonists inhibited ET-1-evoked AA release in a dose-dependent manner. Figure 6B shows that ET-1 stimulated AA release in a dose-dependent manner with an EC$_{50}$ value of 0.6 nM. Increasing concentrations of A-127722 shifted the concentration-dependent curves of ET-1 to the right in a parallel manner without a significant effect on the maximal response, which suggests that A-127722 is a competitive inhibitor of ET-1-induced AA release. The pA$_2$ value for A-127722 was determined to be 10.5 ± 0.3 (n = 3) by Schild analysis (fig. 6C). Similar experiments were performed for the other four antagonists, and the results are compared in figure 6C and table 5. All five antagonists exhibited competitive antagonism of the ET-1-stimulated AA release. The rank order of potency is PD-156707 > FR139317 > A-127722 > Ro-47-0203.

To evaluate further the data from binding studies and functional assays for the five antagonists, we plotted the IC$_{50}$ values from $[^{125}\text{I}]$ET-1 binding to human ET$_A$ receptors and ET$_B$ receptors (table 2) against the IC$_{50}$ values from ET-1-stimulated PI hydrolysis and AA release (tables 4 and 5) as shown in figure 7.

**Discussion**

The studies presented here clearly demonstrate that BSA affects the potency of ET receptor antagonists. BSA affects not only the potency of antagonists in inhibiting ET binding to receptors but also the potency of antagonists in inhibiting ET-1-stimulated PI hydrolysis. Interestingly, the effect of BSA is remarkably different for different antagonists. As shown in figure 2 and table 1, L-749329 and A-127722 exhibit identical potency for inhibition of ET-1 binding to ET$_A$ receptor when no BSA is added in the assay buffer. However, when 5% BSA is added, an approximately 9000-fold drop in potency

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**Table 2**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>IC$_{50}$ (nM)</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET$_A$</td>
<td>ET$_B$</td>
</tr>
<tr>
<td>A-127722</td>
<td>0.09 ± 0.03</td>
<td>8</td>
</tr>
<tr>
<td>PD-156707</td>
<td>0.23 ± 0.07</td>
<td>4</td>
</tr>
<tr>
<td>FR139317</td>
<td>0.99 ± 0.18</td>
<td>11</td>
</tr>
<tr>
<td>Ro-47-0203</td>
<td>7.13 ± 1.67</td>
<td>4</td>
</tr>
<tr>
<td>L-749329</td>
<td>44.59 ± 18.07</td>
<td>4</td>
</tr>
</tbody>
</table>

*Mean ± S.E.*

*Binding studies using cloned human ET$_A$ and ET$_B$ receptors with 0.2% BSA.*
TABLE 3
The $K_i$ values of ET receptor antagonists in inhibiting $^{[125]}$IET-1 binding to human ET$_A$ and ET$_B$ receptors.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>$K_i$ (nM)</th>
<th>$n$</th>
<th>Inhibition Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-127722</td>
<td>$0.07 \pm 0.01$</td>
<td>3</td>
<td>ET$_A$</td>
</tr>
<tr>
<td>PD-156707</td>
<td>$0.38 \pm 0.08$</td>
<td>4</td>
<td>ND$^c$</td>
</tr>
<tr>
<td>FR139317</td>
<td>$0.80 \pm 0.01$</td>
<td>3</td>
<td>ND$^c$</td>
</tr>
<tr>
<td>L-749329</td>
<td>$33.60 \pm 7.85$</td>
<td>4</td>
<td>ND$^c$</td>
</tr>
</tbody>
</table>

$^a$ Mean $\pm$ S.E.
$^b$ Binding studies using cloned human ET$_A$ and ET$_B$ receptors with 0.2% BSA.
$^c$ ND: Not determined.

TABLE 4
The IC$_{50}$ values of ET receptor antagonists in inhibiting ET-1-stimulated PI hydrolysis with or without BSA.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>IC$_{50}$ (nM)</th>
<th>$n$</th>
<th>With BSA$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-127722</td>
<td>$0.10 \pm 0.02$</td>
<td>6</td>
<td>$0.38 \pm 0.08$</td>
</tr>
<tr>
<td>PD-156707</td>
<td>$0.52 \pm 0.10$</td>
<td>3</td>
<td>$0.58 \pm 0.17$</td>
</tr>
<tr>
<td>FR139317</td>
<td>$1.59 \pm 0.68$</td>
<td>6</td>
<td>$21.79 \pm 5.21$</td>
</tr>
<tr>
<td>L-749329</td>
<td>$0.01 \pm 0.004$</td>
<td>3</td>
<td>$44.28 \pm 21.54$</td>
</tr>
<tr>
<td>Ro-47-0203</td>
<td>$38.29 \pm 22.88$</td>
<td>3</td>
<td>$82.54 \pm 54.17$</td>
</tr>
</tbody>
</table>

$^a$ Mean $\pm$ S.E.

Fig. 5. PI hydrolysis with or without BSA. Rat pituitary MMQ cells were prelabeled with $[3H]$myo-inositol (1 $\mu$Ci/well) for 16 h. Cells were incubated with or without 1 nM ET-1 $\pm$ antagonists for 45 min at 37°C. Control: no addition of ET-1 or test agents. A) Without BSA. B) With 0.2% BSA in the buffer. Each value represents the mean $\pm$ S.E. of $n$ determinations as indicated in Table 4.

for ET$_A$ receptor is observed for L-749329, whereas only a 47-fold decrease in potency for ET$_A$ receptor is observed for A-127722. A similar observation was made in functional assays. When BSA was not added, the IC$_{50}$ value for L-749329 was 0.01 nM in the PI hydrolysis assay (fig. 5 and table 4). However, in the presence of 0.2% BSA, the IC$_{50}$ value for L-749329 was more than 4000-fold greater (fig. 5 and table 4). These results are consistent with our previous observation with human serum albumin (Wu-Wong et al., 1996). The reason why the activity of L-749329 is more affected by BSA than that of A-127722 is not immediately apparent.

Why does BSA have such an impact on the potency of ET receptor antagonists? Our previous studies show that ET receptor antagonists exhibit strong binding to plasma proteins, especially serum albumin (Wu-Wong et al., 1996). The finding is not surprising, because albumin readily binds to lipophilic acids, a common characteristic of the ET antagonists tested in this report. Thus it is likely that BSA acts as a “pseudo-receptor” to bind ET receptor antagonists and that antagonists that bind to BSA are no longer free to bind to ET receptors. As a result, a decrease in the potency is observed. The hypothesis that plasma proteins, such as serum albumin, act as “pseudo-receptors” for ET receptor antagonists may also explain why a disparity between the in vitro and in vivo potencies is often observed for ET antagonists (Sogabe et al., 1993; Clozel et al., 1994; Reynolds et al., 1995; Opgenorth et al., 1996). The following example is an exercise to demonstrate this point. From in vitro binding assays, the IC$_{50}$ value at 0% BSA for A-127722 is $\sim$0.1 nM. A concentration of 100 nM is required to inhibit ET-1 binding completely. On the basis of the plasma elimination half-life of 3.5 h and the peak plasma concentration of 1.1 $\mu$g/ml at 5 mg/kg in the rat (Opgenorth et al., 1996), we can estimate that the dose of A-127722 required to reach a plasma concentration of 100 nM is $\sim$0.25 mg/kg. At this dosage, A-127722 should completely inhibit the increase in arterial blood pressure induced by ET-1. However, we have shown that approximately 10 mg/kg of A-127722 is required to completely inhibit an ET-1-induced increase in arterial blood pressure (Opgenorth et al., 1996). The potency difference (40-fold) between in vitro and in vivo is similar to the IC$_{50}$ difference observed in binding assays at 0% and 5% BSA. It is worth mentioning that the protein concentration in human blood is 7% and that 55% to 60% of that is serum albumin. Similar calculations can be done for other antagonists. Clearly, serum albumin, and perhaps other plasma proteins, greatly impacts the potency of ET receptor antagonists.

Both this report and our previous studies (Wu-Wong et al., 1996) show that the effect of serum albumin on antagonist pharmacology is different for different antagonists. Why do the antagonists behave differently in their sensitivity to serum albumin? It is unlikely that these differences can be explained by different inhibiting modes (e.g., competitive vs.
non-competitive), because the $K_i$ and $pA_2$ studies in this report suggest that all these compounds are fully competitive inhibitors. A second possibility is that each antagonist interacts with different regions of the receptor. Indeed, it has been shown that binding sites for Ro-47-0203 are different from those for BQ-123 (Breu et al., 1995). The advent of radiolabeled antagonists may make it possible to investigate the particular interacting sites on the receptor for each antagonist. A third explanation is that the more reversible the binding of an antagonist to ET receptors is, the greater the impact BSA has on its potency. We have previously shown that ET receptor agonists and antagonists, once bound to the receptor, are difficult to wash away and that the binding of some antagonists is more reversible than that of others (Wu-Wong et al., 1994b, c). The difference in the “stickiness” of binding may shed some light on why different ET receptor antagonists respond to BSA differently. In fact, our preliminary results show that the binding of L-749329 to ET receptors is more reversible than that of A-127722, an outcome coincidental with results in the current study that the effect of BSA on L-749329 is more profound than its effect on A-127722. Finally, because these antagonists have distinct structures, it is possible that the “stickiness” of binding is linked to the chemical features of each compound. So far, development of small-molecule ET receptor antagonists has concentrated on improving potency and pharmacokinetic profiles, and there has been no reported attempt to evaluate the structure-activity relationship of protein binding.

Reports in the literature on ET receptor antagonists utilize various serum albumin concentrations in the assays. For example, Williams et al. (1995) and Sogabe et al. (1993) used

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**Table 5**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>$IC_{50}$ (nM)</th>
<th>n</th>
<th>$pA_2$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-156707</td>
<td>0.17 ± 0.03</td>
<td>3</td>
<td>10.9 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>FR139317</td>
<td>0.41 ± 0.16</td>
<td>3</td>
<td>10.4 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>A-127722</td>
<td>0.91 ± 0.22</td>
<td>4</td>
<td>10.2 ± 0.1</td>
<td>4</td>
</tr>
<tr>
<td>Ro-47-0203</td>
<td>5.53 ± 0.99</td>
<td>3</td>
<td>9.1 ± 0.5</td>
<td>3</td>
</tr>
<tr>
<td>L-749329</td>
<td>25.87 ± 3.35</td>
<td>3</td>
<td>8.7 ± 0.6</td>
<td>3</td>
</tr>
</tbody>
</table>

*a Mean ± S.E.*
of 0.82. If BSA at different concentrations indeed affects the potency of antagonists to different degrees, and if the effect of BSA is different for different antagonists, it is of significant importance to compare ET receptor antagonists directly in assays using a fixed concentration of BSA. Because of our previous studies on ET receptor antagonists (Opgenorth et al., 1996), we chose to continue the practice of using 0.2% BSA in the assays for the comparison study. It would be ideal to compare all known ET receptor antagonists directly, but because of practical considerations, five antagonists were chosen on the basis of the following information: FR139317 is one of the earliest ET receptor antagonists and has been tested in various animal disease models. Ro-47-0203 (Bosentan) is furthest along in clinical development and therefore is of particular interest to researchers in the ET field. And A-127722, PD-156707 and L-749329 are among the most potent antagonists reported in the literature and appear to have clinical utility.

In summary, we show that BSA affects the potency of ET receptor antagonists in binding and functional assays and that the effect of BSA is remarkably different for different antagonists. The comparison study shows that although there is some variation among assays, in general, the rank order of potency of the five antagonists tested in various assays is A-127722 ≥ PD-156707 > FR139317 > Ro-47-0203 > L-749329 when 0.2% BSA is included in the assay buffer. The correlation between binding studies and functional assays is reasonably good with a correlation coefficient of 0.82.

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References


