Greater Functional ET<sub>B</sub> Receptor Antagonism With Bosentan Than Sitaxsentan in Healthy Men

Iain M. MacIntyre, Neeraj Dhaun, Pajaree Lilikarntakul, Vanessa Melville, Jane Goddard, David J. Webb

Abstract—Endothelin (ET)-1 is implicated in the development of hypertension and a role for endothelin receptor antagonists (ETRAs) in the management of hypertension is emerging. ETRAs are classified as selective or mixed depending on their degree of ETA:ETB receptor blockade. As yet, there are no comparative studies in humans that measure biochemical and functional ET<sub>B</sub> blockade achieved by currently licensed ETRAs. We therefore investigated the effects of bosentan, a mixed ETRA, and sitaxsentan, an ETA selective ETRA, on plasma ET-1 concentrations and ET<sub>B</sub>-mediated vasodilatation to ET-3. In a randomized, double-blind, 3-way crossover study, 10 healthy subjects received 7 days of placebo, bosentan 250 mg, and sitaxsentan 100 mg daily. Plasma ET-1 concentrations were measured at baseline and 3 hours on day 1 and predose on day 7. Subjects also underwent forearm blood flow measurements on day 7 of each period with brachial artery infusion of ET-3 (60 pmol/min for 5 minutes). Bosentan, but not placebo or sitaxsentan, significantly increased plasma ET-1 concentrations at day 7 (+0.70±0.20 pg/mL; P<0.005). Maximal ET-3–mediated vasodilatation was seen at 2 minutes following placebo (30±6%) and sitaxsentan (21±11%); however, this was abolished by bosentan, with a reduction in forearm blood flow of 8±3% (P<0.01 versus placebo and sitaxsentan). Bosentan but not sitaxsentan increases circulating plasma ET-1 levels and abolishes acute ET-3–mediated vasodilatation, confirming that the mixed ETA<sub>B</sub> antagonist bosentan, but not the selective ETA<sub>A</sub> antagonist sitaxsentan, causes functional ET<sub>B</sub> blockade at clinically relevant doses in healthy human subjects. (Hypertension. 2010;55:1406-1411.)

Key Words: endothelin ■ endothelin receptors ■ selectivity ■ bosentan ■ sitaxsentan

The endothelin (ET) system comprises a family of 21-aa peptides with powerful vasoconstrictor and pressor properties. ET-1, the predominant isopeptide, is generated by the vascular endothelium and plays an important role in the maintenance of vascular tone and blood pressure (BP). ET-1 acts via 2 specific receptors, the ETA and the ETB receptors. Within the vasculature, ETA receptors are expressed predominantly on vascular smooth muscle cells (VSMCs), cardiomyocytes, and fibroblasts. Their activation results in sustained vasoconstriction, cell proliferation, and fibroblast activation. In contrast, ETB receptors are predominantly expressed on endothelial cells and mediate vasodilatation primarily through nitric oxide (NO). The role of the ETB receptor, however, is more complex, because they are also present on VSMCs and fibroblasts where they too contribute to vasoconstriction, proliferation, and fibrosis. In addition, ETB receptors, particularly in the pulmonary circulation, act as the primary clearance mechanism for circulating ET-1. Furthermore, out with vasculature ET-1 appears to play an important role in sodium handling. In vitro and in vivo data suggest that, within the kidney, ET-1, acting through ETB receptors, plays an important role in mediating natriuresis and diuresis, opposing salt-induced increases in BP. Conversely ET-1 also appears to be a potent stimulator of aldosterone. In the rat, this effect occurs solely via the ETB receptor, although both receptors have been implicated in humans.

The potent pressor and mitogenic properties of ET-1 support the hypothesis that the ET system plays an important role in the pathophysiology of essential hypertension. In many experimental models of hypertension (particularly salt-dependent types), production of vascular ET-1 is increased and associated with vascular remodeling. Furthermore, selective ETA<sub>A</sub> or mixed ETA<sub>A/B</sub> antagonism in these animals causes reductions in BP and regression of vascular growth. In keeping with animal models, vascular ET-1 is also increased in the small resistance vessels of patients with severe hypertension and chronic dosing with both selective ETA<sub>A</sub> or mixed ETA<sub>A/B</sub> antagonists have been shown to significantly lower BP. Despite the hypothesized benefits of an unblocked ETB receptor in hypertension, which include preserved endothelial-dependent NO-mediated vasodilatation,
ET-1 clearance, natriuresis and diuresis, to date no differences have been demonstrated in vivo between selective ET_A and mixed ET_A/B endothelin receptor antagonists (ETRAs).

We therefore examined biochemical and functional markers of ET_B receptor antagonism in vivo in healthy subjects following 7-day oral dosing with either the selective ET_A antagonist sitaxsentan or the mixed ET_A/B antagonist bosentan. We hypothesized that bosentan, but not sitaxsentan, would affect biomarkers for ET_B receptor function, specifically to increase plasma ET-1 and reduce ET-3–mediated vasodilatation through functional ET_B receptor blockade.

Methods

Subjects

The study was undertaken in 10 healthy male volunteers recruited from the local community and had the approval of the local research ethics committee. Written informed consent was obtained from each subject before entry into the study. All subjects were allocated to a randomized treatment sequence of placebo, sitaxsentan and bosentan.

Drugs

Bosentan and sitaxsentan were chosen because they have the lowest (20:1) and highest (6500:1) ETA receptor affinities, respectively, of the currently licensed ETRAs. Bosentan (Actelion Pharmaceuticals Ltd, Basel, Switzerland) was given orally at a dose of 125 mg twice daily, the current recommended maintenance dose in pulmonary arterial hypertension. Sitaxsentan (Pfizer Ltd, New York) was given orally at the standard dose of 100 mg once daily. When taking sitaxsentan, subjects were also given placebo tablets at night. In the placebo group placebo tablets were given orally twice daily. To ensure blinding, all tablets were double encapsulated in gelatin and compliance was assessed by tablet count at the end of each period.

ET-3 has a greater affinity for the ET_B than the ET_A receptor (Ki 0.06 versus 140 nmol/L) compared to ET-1, which has equal affinity for the ET_A and ET_B receptor (Ki 0.6 and 0.12 nmol/L, respectively). Hence, ET-3 can be used as a relatively selective ET_B agonist. ET-3 (Merck Chemicals Ltd), dissolved in physiological saline, was administered intraarterially at a dose of 60 pmol/min for 5 minutes, based on previous work showing, in vivo, that this dose causes significant early forearm vasodilatation, in keeping with functional stimulation of the endothelial ET_B receptor.

Plasma ET-1 Assessment

Venous blood (10 mL) was collected into an EDTA tube and centrifuged immediately at 2500g for 20 minutes at 4°C. Samples were stored at −80°C until analysis. After extraction,21 ET-1 was determined by radioimmunoassay using rabbit antihuman endothelin-1 (Bachem UK Ltd, St Helens, UK).22 The mean recovery of ET-1 from extraction to assay was >90%. The intra- and interassay variations were 6.3% and 7.2%, respectively. The cross-reactivity of the antibody was 100% with ET-1, 7% for both ET-2 and ET-3, and 10% with big ET-1.

Forearm Blood Flow Measurement

Forearm blood flow (FBF) studies were performed with subjects resting supine in a quiet, temperature-controlled room (23°C to 25°C). Subjects were asked to abstain from caffeine-containing drinks, alcohol, and cigarette smoking on the day of the study and to fast from the midnight before the study. On study days, the brachial artery of the nondominant arm of each subject was cannulated with a 27-SWG needle, under local anesthesia (1% lidocaine; Hameln Pharmaceutical Ltd, Gloucester, UK) and connected to a constant-rate infusion pump (IVAC P7000; ALARIS Medical Systems Inc) via a 16-gauge epidural catheter (Portex Inc).

The rate of intraarterial infusion was maintained constantly throughout all intraarterial studies at 1 mL/min.

FBF was measured by venous occlusion plethysmography with mercury-in-silastic strain gauges applied to the widest aspect of each forearm.21,24 The hand was excluded during periods of blood flow study by inflation of wrist cuffs to 220 mm Hg. An upper arm cuff was intermittently inflated to 40 mm Hg for 10 seconds every 15 seconds to temporarily prevent forearm venous outflow and obtain plethysmographic recordings.

Blood Pressure

BP was measured with an appropriate sized cuff, by use of a validated oscillometric sphygmomanometer (Dinamap Compact TS; Critikon Ltd, Newport, UK).25

Study Protocol

This was a 3-way, randomized, double-blind placebo-controlled trial. The study consisted of three 7-day treatment periods with placebo, sitaxsentan, and bosentan. There was a minimum 14-day washout between periods. On day 1 of each study period, subjects were required to attend the research center fasted at 9:00 AM. Following baseline blood sampling, the study drug was administered. A further blood sample was then taken at 3 hours after dosing. Subjects were then allowed to return home and continued to receive the study drug for 7 days (Figure 1A).

On day 7, patients attended the research center at 9:00 AM. Following baseline BP and plasma ET-1 sampling, the last dose of study drug was taken. Patients then rested for 3 hours before FBF measurements at peak plasma concentrations of the study drugs.21,26 Following left brachial arterial cannulation saline was infused for 30 minutes during which 2 measurements of FBF were made (at −20 and −10 minutes). ET-3 was then infused via the brachial artery at 60 pmol/min for 5 minutes, followed by physiological saline for 60 minutes. FBF was recorded from 3 minutes before to 10 minutes after the ET-3 infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes (Figure 1B).

Data Analysis

Plethysmographic data were extracted from Chart (version 5.0.2 PowerLab 2003) data files, and FBFs were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 2004 for Macintosh; Microsoft Corp). The last 5 measurements from each 3-minute recording period were averaged for the infused and noninfused arm. However, to detect early, transient changes in blood flow, every recording during the 13 minutes of continuous FBF measurement was analyzed. To reduce variability of
blood flow data, the ratio of flows in the 2 arms was calculated for each time point. FBF results were calculated as the ratio of flow between the infused and noninfused arms and shown as percentage change from baseline.\(^2\) The percentage change in FBF following drug administration was calculated as follows:

\[
100\% \times \frac{F(i)_t / F(n)_t - F(i)_b / F(n)_b}{F(i)_b / F(n)_b}
\]

where \(F(i)\) and \(F(n)\) represent measured blood flows in the infused and noninfused arms respectively during periods of drug \((d)\) and vehicle \((v)\) administration.\(^2\)

**Statistical Analysis**

Based on previous data,\(^2\) the probability is 80% that the study will detect a treatment difference at a 2-sided 5% significance level, if the true difference in the ratio of flow between the infused and noninfused arms between the treatments is 14%. This is based on the assumption that the SD of the difference in the response variables is 10%. We expect to see a difference between the bosentan arm versus placebo and sitaxsentan arms of approximately 20%.\(^2\)

The coprimary end points were change from baseline of plasma ET-1 and maximal forearm vasodilatation to ET-3. Three comparisons of interest were preidentified: placebo versus sitaxsentan, placebo versus bosentan, and sitaxsentan versus bosentan. The absolute change from baseline plasma ET-1 concentrations for each group were analyzed using Student’s \(t\) test. FBF results were analyzed by repeated-measures ANOVA with Bonferroni correction. These statistical analyses were performed with Excel (Excel 2004 for Macintosh). Statistical significance was taken at the 5% level. All results are presented as means±SEM.

**Results**

Ten healthy men with a mean age of 36±16 years (range, 20 to 66 years) were recruited and completed all 3 phases of the study. Three of the 10 subjects were cigarette smokers. Overall, subjects had a mean systolic BP of 121±11 and diastolic BP of 68±7 mm Hg. Mean body mass index was 23±2 kg/m\(^2\). No adverse effects of treatment were reported.

**Plasma ET-1**

Baseline plasma ET-1 concentrations were not significantly different between study periods (placebo: 3.44±0.27 pg/mL; sitaxsentan: 3.42±0.21 pg/mL; bosentan: 2.92±0.22 pg/mL). Following pretreatment with either placebo or sitaxsentan, there was no significant change in plasma ET-1 concentrations from baseline to 3 hours or on day 7. Following pretreatment with bosentan, however, there was a trend to a rise in plasma ET-1 concentrations at 3 hours (+0.51±0.26 pg/mL; \(P=0.07\)) and a significant increase at day 7 (+0.70±0.20 pg/mL; \(P<0.005\)) (Figure 2).

**FBF Study**

Baseline BP, heart rate, and FBF were similar during the study days, and there was no significant difference in basal FBF between infused and noninfused arms (Table). BP, heart rate, and FBF in the noninfused arm did not significantly change after infusion of ET-3, confirming that the effects of ET-3 were confined to the infused arm (Table).

Following pretreatment with placebo, intraarterial infusion of ET-3 caused significant local vasodilatation with a maximal increase in FBF of 30±6% at 2 minutes (\(P<0.01\) versus baseline). This dilatation persisted for 5 minutes. Similar results were seen following pretreatment with sitaxsentan, with ET-3 causing a peak increase in FBF of 21±11% at 2 minutes (\(P=0.44\) versus placebo). Pretreatment with bosentan, however, abolished ET-3–induced vasodilatation and was associated with a reduction of FBF at 2 minutes of 8±3% (\(P<0.01\) versus placebo and sitaxsentan) (Figure 3A and 3B).

**Discussion**

We have shown that 7 days of treatment with bosentan, a mixed ET\(_A/B\) receptor antagonist, increases plasma ET-1 concentrations and abolishes acute ET-3–mediated vasodilatation. Neither of these effects was seen with the selective ET\(_A\) receptor antagonist sitaxsentan, confirming that bosentan, but not sitaxsentan, causes functional ET\(_B\) blockade at the standard clinically licensed doses in healthy humans. This is the first study to show that selective and mixed antagonists differ in their biochemical and functional effects in humans.

**Table. Hemodynamic Data**

<table>
<thead>
<tr>
<th>Hemodynamic Data</th>
<th>Placebo (n=10)</th>
<th>Sitaxsentan (n=10)</th>
<th>Bosentan (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>83±2</td>
<td>83±3</td>
<td>81±2</td>
</tr>
<tr>
<td>3 minutes ET-3</td>
<td>88±2</td>
<td>85±3</td>
<td>83±2</td>
</tr>
<tr>
<td>60 minutes post ET-3</td>
<td>60±3</td>
<td>65±3</td>
<td>62±4</td>
</tr>
<tr>
<td>HR, bpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>60±3</td>
<td>59±3</td>
<td>61±3</td>
</tr>
<tr>
<td>3 minutes ET-3</td>
<td>60±3</td>
<td>65±3</td>
<td>62±4</td>
</tr>
<tr>
<td>Infused FBF, mL/100 mL·min(^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>2.3±0.3</td>
<td>2.7±0.4</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>3 minutes ET-3</td>
<td>3.2±0.4</td>
<td>3.3±0.5</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td>60 minutes post ET-3</td>
<td>2.9±0.4</td>
<td>3.1±0.5</td>
<td>2.6±0.4</td>
</tr>
<tr>
<td>Control FBF, mL/100 mL·min(^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>2.0±0.2</td>
<td>2.4±0.4</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>3 minutes ET-3</td>
<td>2.2±0.2</td>
<td>2.7±0.5</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>60 minutes post ET-3</td>
<td>2.6±0.4</td>
<td>2.7±0.3</td>
<td>2.4±0.3</td>
</tr>
</tbody>
</table>

Values are means±SEM.
which may be relevant to the benefits and harms of these drugs.

The ETB receptor has an important role in clearing circulating ET-1. Thus, blockade of this receptor would be expected to cause a rise in plasma ET-1.9 Indeed, ET-1 concentrations are elevated in animal ETB receptor knockout models.29,30 Furthermore, studies with both selective ETB receptors and mixed ETA/B receptor antagonists23,31 have shown that plasma ET-1 concentrations rise following their administration. In contrast, the highly selective ETB receptor antagonists BQ-123, sitaxsentan, and ZD4054 (ZD4054 has no measurable affinity for the ETB receptor) do not increase ET-1 concentrations.23,33–35 In the present study, we have shown that plasma ET-1 concentrations increase significantly following bosentan, but not sitaxsentan, therapy, in keeping with previous acute studies that have shown that concomitant ETB blockade impairs natriuresis and renal blood flow.23,41 Conversely, mixed ETA/B receptor antagonists may be required to block the secretagogue effect of ET-1 on aldosterone within the zona glomerulosa.42 It is, therefore, important to be able to clearly classify ETRAs as selective or mixed based on in vivo rather than in vitro models before study.

### Study Limitations

As a limitation, the present studies were performed in healthy volunteers. Therefore, the full clinical relevance of our findings in various pathophysiological conditions cannot be determined because endothelin receptors may be modified under these circumstances. However, the principle that blocking the ETB receptor with a mixed ETA/B antagonist has effects different from an ETA selective approach is clearly shown. Also if mixed antagonists inhibit 2 ETB-mediated effects of ET-1, it is likely that they may affect others. This may be therapeutically useful in target tissues, such as SMCs and adrenal cortex, or deleterious if inhibiting natriuresis through the ETB receptor in the kidney. Effects on
natriuresis, in particular, should be the subject of further investigation, given the propensity for ETRAs to cause fluid retention. It must also be remembered that, to date, no study has shown any clinical advantage of using selective over mixed ETRA antagonists and that any presumed benefits of selective ET<sub>A</sub> receptor antagonism may not be borne out in clinical practice.

In summary, we have described a novel method of assessing in vivo ETRA selectivity using both plasma ET-1 concentrations and ET-3 as selective ET<sub>B</sub> receptor agonist. This method provides additional important clinical information above that of in vitro receptor assays and could be used to examine the selectivity of other ETRAs at clinically relevant doses in humans. This would be particularly important for those ETRAs that have previously been shown to increase plasma ET-1, where the doses used may not be ET<sub>A</sub>-selective. Our data confirm, for the first time, that selective and mixed ETRAs differ functionally in healthy subjects, a finding that may be relevant to clinical outcomes, affecting both benefits and harms.

**Perspectives**

There is a theoretical benefit in selective ET<sub>A</sub> antagonism in a number of conditions, such as hypertension, by preserving beneficial actions of the ET<sub>B</sub> receptor including vasodilatation, nitric oxide production and natriuresis. However, to date, no clinical differences have been demonstrated between selective and mixed ETRAs. This has led some to question whether current ‘selective’ ET<sub>A</sub> receptor antagonists are indeed truly selective in vivo.

We examined functional and biochemical markers of ET<sub>B</sub> receptor activation in healthy volunteers following 7-day treatment with oral selective and mixed ETRAs. Our findings confirm for the first time, in vivo, that the ET<sub>B</sub> receptor remains functionally active following selective ET<sub>A</sub> receptor, but not mixed ET<sub>AB</sub> receptor, antagonism. This has a number of scientific and clinical implications. Firstly, other oral ETRAs can now be tested using this technique, giving investigators and clinicians a clearer understanding of the true functional actions, this study supports a call for larger head-to-head trials of selective and mixed antagonist to be performed in important therapeutic indications, including hypertension.

**Acknowledgments**

We thank Drs Neil Davie and Simon Teal for their support throughout the study, particularly during the amalgamation of Encysive Pharmaceuticals into Pfizer. We also acknowledge the support provided by the British Heart Foundation CoRE at the University of Edinburgh.

**Sources of Funding**

This study was funded by a research grant from Encysive Pharmaceuticals/Pfizer.

**Disclosures**

I.M.M., N.D., J.G., and D.J.W. have all received research grants from Pfizer. N.D. and J.G. have held academic research fellowships funded by educational grants from Pfizer. J.G. has acted as a consultant to Encysive, Pfizer, and Speedel. D.J.W. has acted as a consultant to Abbott, Encysive, GlaxoSmithKline, Pfizer, and Speedel.

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*Hypertension.* 2010;55:1406-1411; originally published online April 19, 2010;
doi: 10.1161/HYPERTENSIONAHA.109.148569
*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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