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# Functional estimation of endothelin-1 receptor antagonism by bosentan, macitentan and ambrisentan in human pulmonary and radial arteries *in vitro*

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ARTICLE INFO

*Chemical compounds studied in this article:*
Ambrisentan (PubChem CID: 6918493)
bosentan sodium (PubChem CID: 44387533)
endothelin-1 (PubChem CID: 16132423)
and macitentan (PubChem CID: 16004692)

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Endothelin-1
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ABSTRACT

*Background:* Endothelin receptor antagonists are approved for pulmonary arterial hypertension. Development of selective $ET_A$-receptor antagonists over mixed or dual receptor antagonists has depended on a range of receptor binding assays, second messenger assays and functional blood vessel assays. This study compared the 3 clinically-approved endothelin receptor antagonists in assays of human isolated pulmonary and radial arteries *in vitro*.

*Methods:* Human isolated pulmonary (i.d. 5.5 mm) and human radial (i.d. 3.23 mm) artery ring segments were mounted in organ baths for isometric force measurement. Single concentration-contraction curves to endothelin-1 were constructed in the absence or presence of bosentan (1–10 μM), macitentan (0.03–0.3 μM) or ambrisentan (0.1–1 μM).

*Results:* All 3 endothelin antagonists caused competitive rightward shifts in the endothelin-1 concentration-response curves in both arteries. The Clark plot and analysis gave the following $pK_B$ values: bosentan, pulmonary artery 6.28 ± 0.13 and radial artery 6.04 ± 0.10; macitentan, pulmonary artery 8.02 ± 0.13 and radial artery 7.49 ± 0.08; and ambrisentan, pulmonary artery 7.38 ± 0.13 and radial artery 6.96 ± 0.10.

*Conclusions:* Noting the maximum plasma levels attained from recommended oral doses of each antagonist in volunteers, the $pK_B$ findings here show that there would be significant antagonism of endothelin-1 contraction in the pulmonary and radial arteries at therapeutic plasma levels. This functional assay confirms in human tissue that much higher plasma concentrations of endothelin-1 receptor antagonists are required to be effective than those predicted from binding or other biochemical assays.

## 1. Introduction

There is growing interest in the question of whether selectivity of newer endothelin-1 receptor antagonists for the $ET_A$ receptor based on binding studies bears any clinical importance compared with “dual” or “mixed” non-selective $ET_A$ and $ET_B$ receptor antagonists. Indeed, secondary properties such as limiting side effects, longer half-times for once a day dosing and fewer serious drug-drug interactions may prevail as being more important characteristics than receptor selectivity based on binding (Opitz et al., 2008). Historically, much of the quantitative analysis of a vast range of endothelin-1 antagonists from four chemical classes has relied on the determination of $K_i$ values, the dissociation constant of unlabelled ligands in competition binding experiments in human cloned $ET_A$ and $ET_B$ endothelin receptor assays. In “clinically relevant” human cardiovascular tissues such as samples from left ventricle, coronary artery and homogenates of saphenous vein, the binding assay readout for $K_i$ values of a range of endothelin-1 antagonists were compared to the “functional” $K_B$ values, i.e. the antagonist concentration that shifted the agonist endothelin-1 $EC_{50}$ two-fold to the right (Maguire et al., 2012). These authors reported a significant discrepancy between the $K_i$ and $K_B$ values that was not correlated with the structural class of the endothelin antagonists.

In this work we have determined the functional $K_B$ values of bosentan, macitentan and ambrisentan in isolated ring segments of human pulmonary and radial arteries (discarded from surgical procedures) in organ baths and compared these $K_B$ values to the literature-reported plasma levels of these drugs used in the treatment of pulmonary hypertension. One advantage of these two human large conduit vessels is that the $ET_A$ receptor appears to be the primary functional endothelin-1 receptor subclass that mediates the contraction

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(Conant et al., 2002; Hay et al., 1993; Liu et al., 1996; Maguire and Davenport, 1995).

## 2. Materials and methods

The Royal Melbourne Hospital (Melbourne Health) Human Research Ethics Committee approved the use of human discarded tissues for these experiments (HREC ethics number 2011.11). Patients gave informed written consent prior to their cardiothoracic surgery.

### 2.1. Tissue collection

Excess lengths of radial artery (internal diameter, i.d., ≈3.5 mm) or pulmonary artery (i.d. ≈5.0 mm) were obtained in theatre from patients undergoing coronary artery bypass graft surgery or lung resection for cancer, respectively, at the Royal Melbourne Hospital, Melbourne, Australia. These tissue segments were placed in cold physiological salt solution (PSS) with the following composition (mmol/l): NaCI 119; KCl 4.69; $MgSO_4{\cdot}7H_2O$ 1.17; $KH_2PO_4$ 1.18; glucose 5.5; $NaHCO_3$ 25; $CaCl_2{\cdot}6H_2O$ 2.5; EDTA 0.026 and saturated with carbogen ($O_2$ 95%; $CO_2$ 5%) at pH 7.4.

Artery segments were cut into 3 mm long ring segments, trimmed of fat and connective tissue and mounted between 2 L-shaped stainless steel wires (550 μm diameter) on a Perspex® leg driven by a micrometer (Mitutoyo, Kawasaki, Japan) that allowed precision adjustment of the lower wire to stretch the diameter of the artery ring. The upper wire was fixed to a force transducer (FT03C, Grass Instruments, Quincy, MA, USA) connected to a bridge amplifier and data acquisition system (Powerlab, ADInstruments, Sydney, Australia). Organ baths were filled with PSS saturated with carbogen at 37.0 ± 0.1 °C. Tissues were allowed to stabilise for 30 min.

All arteries were subjected to a normalisation procedure where passive stretch of the artery ring was set at the equivalent of 100 mmHg transmural pressure for radial arteries and 20 mmHg for pulmonary arteries and then relaxed to 90% of that internal diameter (see Angus and Wright, 2000). This normalisation procedure allowed each artery to be set at an equivalent passive stretch relative to its internal diameter. Artery rings were allowed to rest at their normalised passive tension before exposure to a maximum depolarising solution of potassium (KPSS; wherein PSS the $K^+$ substitutes for $Na^+$, i.e. 124 mM) which maximally contracts the artery ring. Tissues were then washed with PSS to restore the baseline passive force. Each artery ring was contracted with noradrenaline 1 μM that gave approximately 80% of the contraction previously observed with KPSS. When the contraction was steady, acetylcholine 1 μM was added to test the integrity of the endothelium to release nitric oxide. Normally within < 30 s the artery ring would completely relax the active tension back to the baseline passive tension.

A single concentration-response curve to endothelin-1 (0.1 nM to 1 μM, depending on treatment) was constructed in the absence or presence of a concentration of bosentan (0, 1, 3 or 10 μM), ambrisentan (0, 0.1, 0.3 or 1 μM) or macitentan (0, 0.01, 0.03, 0.1 or 0.3 μM). The endothelin antagonists were added to the organ bath 30 min before commencing the cumulative concentration-response curve to endothelin-1. Endothelin-1 was added in half-$\log_{10}$ M increments allowing time for the contraction to reach a plateau before raising the endothelin-1 concentration.

### 2.2. Drugs

Drugs used were: acetylcholine bromide (Sigma, St Louis, MO, USA); ambrisentan (a gift from GSK, Stevenage, UK); bosentan sodium salt (a gift from Actelion Pharmaceutical Ltd., Allschwil, Switzerland); endothelin-1 (Genscript, Piscataway, NJ, USA); macitentan (a gift from Actelion); and (−)-noradrenaline bitartrate (Sigma). All drugs were dissolved in MilliQ water except for endothelin-1 which was dissolved in 10% dimethylformamide to $10^{-4}$ M, then diluted in MilliQ water, and macitentan which was dissolved in DMSO to $10^{-3}$ M, then diluted in MilliQ water.

**Table 1**
Characteristics of human radial and pulmonary arteries in the control group.

| | Radial artery $n$=9 | Pulmonary artery $n$=8 |
|---|---|---|
| Diameter (mm) | $D_{100}$ 3.20 ± 0.18 | $D_{20}$ 5.71 ± 0.55 |
| KPSS contraction (mN) | 134 ± 18 | 22 ± 3 |

Data are mean ± S.E.M. $D_{20}$, internal diameter at an equivalent transmural pressure of 20 mmHg. $D_{100}$, internal diameter at an equivalent transmural pressure of 100 mmHg. KPSS, maximum contraction to isotonic potassium physiological salt solution ($K^+$124 mM). $n$, number of arteries from separate patients.

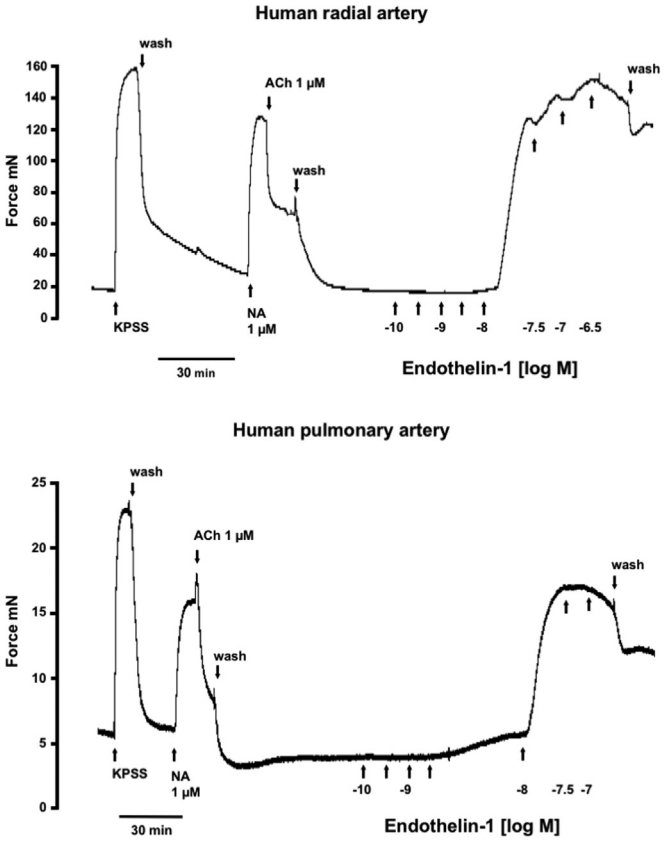


**Fig. 1.** Chart recordings of traces captured of the contraction to KPSS followed by washout and subsequent contraction to noradrenaline (NA 1 μM) before rapid relaxation by the nitric oxide-releasing agent acetylcholine (ACh 1 μM). After washout and stabilisation, a cumulative concentration-contraction curve to endothelin-1 was constructed. Top trace: human radial artery ring segment. Lower trace: human pulmonary artery ring segment.

### 2.3. Statistics and analyses

All data are expressed as mean ± S.E.M. from $n$ experiments. All contraction responses to endothelin-1 were measured as a % of the Emax to KPSS within each artery. Each individual sigmoidal concentration-contraction curve to endothelin-1 in the absence or presence of the endothelin antagonist was fitted using Prism 6 (GraphPad Software, La Jolla, CA, USA). The $pEC_{50}$ ± S.E.M. values (-$\log_{10}$ M $EC_{50}$) were determined for each treatment group.

#### 2.3.1. Clark plot and analyses

To determine the antagonist dissociation constant ($K_B$) for each endothelin antagonist we applied the global regression method (Lew and Angus, 1995) that was simplified from that developed originally by