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RESEARCH PAPER

Selective endothelin A receptor antagonism with sitaxentan reduces neointimal lesion size in a mouse model of intraluminal injury

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BACKGROUND AND PURPOSE

Endothelin (ET) receptor antagonism reduces neointimal lesion formation in animal models. This investigation addressed the hypothesis that the selective ET_A receptor antagonist sitaxentan would be more effective than mixed $ET_{A/B}$ receptor antagonism at inhibiting neointimal proliferation in a mouse model of intraluminal injury.

EXPERIMENTAL APPROACH

Antagonism of ET_A receptors by sitaxentan (1–100 nM) was assessed in femoral arteries isolated from adult, male C57Bl6 mice using isometric wire myography. Neointimal lesion development was induced by intraluminal injury in mice receiving sitaxentan (ET_A antagonist; 15 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), A192621 ($ET_B$ antagonist; 30 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), the combination of both antagonists or vehicle. Treatment began 1 week before, and continued for 28 days after, surgery. Femoral arteries were then harvested for analysis of lesion size and composition.

KEY RESULTS

Sitaxentan produced a selective, concentration-dependent parallel rightward shift of ET-1-mediated contraction in isolated femoral arteries. Sitaxentan reduced neointimal lesion size, whereas ET_B and combined $ET_{A/B}$ receptor antagonism did not. Macrophage and α-smooth muscle actin content were unaltered by ET receptor antagonism but sitaxentan reduced the amount of collagen in lesions.

CONCLUSIONS AND IMPLICATIONS

These results suggest that ET_A receptor antagonism would be more effective than combined ET_A/ET_B receptor antagonism at reducing neointimal lesion formation.

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Abbreviations

ET, endothelin; ET_A, endothelin A receptor; ET_B, endothelin B receptor

Tables of Links

TARGETS
ET_A receptor
ET_B receptor

LIGANDS		
A192621	Bosentan	Phenylephrine (PE)
ACh	Darusentan	Sitaxentan
Ambrisentan		

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Percutaneous revascularization is well established as a treatment for flow-limiting arterial narrowing, with over 80 000 percutaneous coronary interventions performed in the UK in 2009 (Ludman *et al.*, 2011). The evolution of this technique, including the introduction of drug-eluting stents, has reduced the rates of restenosis but re-narrowing remains a significant clinical concern (Dangas *et al.*, 2010). Activation of the endothelin (ET) system, which is implicated in a number of cardiovascular diseases, has a recognized role in the development of restenosis (reviewed in Kirkby *et al.*, 2008; Kitada *et al.*, 2012). Consequently, pharmacological targeting of ET-1 and its receptors has therapeutic potential in the prevention of the neointimal remodelling that contributes to restenosis.

ET-1 is a potent vasoconstrictor and mitogen that acts by stimulation of endothelin A (ET_A) and endothelin B (ET_B) receptor subtypes (reviewed in Kirkby *et al.*, 2008). In the arterial wall, ET_A receptors are present on smooth muscle cells (Arai *et al.*, 1990), whereas ET_B receptors are found both on endothelial and smooth muscle cells (Molenaar *et al.*, 1993). The therapeutic potential of targeting ET receptors has been supported by the demonstration that non-selective ET_A/ET_B receptor antagonism reduces lesion formation in a variety of models (Douglas *et al.*, 1994; Reel *et al.*, 2005). However, mixed ET receptor antagonism does not always inhibit lesion formation (Kirkby *et al.*, 2012), and selective antagonism of the ET_A receptor subtype has been suggested as a preferable therapeutic option (Murakoshi *et al.*, 2002; Kitada *et al.*, 2009; Kirkby *et al.*, 2012), although some results are not consistent with this proposal (Douglas *et al.*, 1995).

The case for selective ET_A receptor antagonism seems logical in light of the mechanisms regulated by the ET receptor subtypes (Kirkby *et al.*, 2008). Activation of ET_A receptors on smooth muscle cells causes responses (vasoconstriction (Yanagisawa *et al.*, 1988), cell proliferation (Komuro *et al.*, 1988), fibroblast activation, reactive oxygen species generation, adhesion molecule production (Li *et al.*, 2003; Amiri *et al.*, 2004) that are likely to exacerbate arterial narrowing. In contrast, activation of ET_B receptors on endothelial cells mediates responses [vasodilatation through the NO pathway (de Nucci *et al.*, 1988; Hirata *et al.*, 1993); clearance of ET-1 from the plasma (Fukuroda *et al.*, 1994; Kelland *et al.*, 2010)] that would be predicted to inhibit arterial remodelling. Consistent with these roles, selective antagonism (Murakoshi *et al.*, 2002; Kitada *et al.*, 2009; Kirkby *et al.*, 2012) or global genetic deletion (Murakoshi *et al.*, 2002; Kitada *et al.*, 2009) of ET_B receptors increases neointimal lesion size in mice and rats.

The ability of ET_A 'receptor-selective' ET antagonists to inhibit neointimal lesion formation may depend on their degree of selectivity. Indeed, reported receptor selectivity is clearly assay- dependent, with evidence that ET_A receptor antagonists with <100-fold selectivity in cloned receptors lose their selectivity in human ventricle (which expresses both ET_A and ET_B receptors; Maguire *et al.*, 2012b). This suggests that ET receptor antagonists may need at least 1000× ET_A : ET_B selectivity in *in vitro* assays to retain selectivity *in vivo* (Maguire *et al.*, 2012b). Sitaxentan has far greater reported selectivity (7000-fold; Wu *et al.*, 1997; ~200 000 fold; Maguire *et al.*, 2012a) than many other antagonists (e.g. atrasentan, 2000-fold; Opgenorth *et al.*, 1996; darusentan, 170-fold; Riechers *et al.*, 1996; ambrisentan, 893-fold; BQ123, 33288-fold Maguire *et al.*, 2012a) for the ET_A receptor. This investigation used a murine model of neointimal proliferation to address the hypothesis that sitaxentan would be more effective than combined ET_A/ET_B receptor antagonism in inhibiting lesion formation.

Methods

Animals

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Experiments were performed using male, C57Bl/6J mice (22–30 g), purchased from Charles River Laboratories (Tranent, UK), under the auspices of UK Home Office Project and Personal Licences and in accordance with the Animals (Scientific Procedures) Act (UK), 1986. Experiments were approved by the local Ethical Review Committee. Every effort was made to limit animal suffering and reduce the number of animals used. Mice were maintained at 21–22°C and 50% humidity with a 12 h diurnal light/dark cycle and free access to food and water. Mice were killed by asphyxiation in CO_2 or by perfusion fixation, as appropriate. The total number of mice used in the experiments was 54.

Ex vivo *analysis of ET-1-mediated arterial contraction*

Functional analyses were performed using mouse isolated femoral arteries, as described previously (Opgenorth *et al.*, 1996). Briefly, rings (~2 mm in length) of femoral artery were suspended on two intraluminal 40 μm tungsten wires in a myograph (model 610M Multimyograph; JP Trading, Aarhus, Denmark) chamber. Arteries were equilibrated at their optimum resting force (8 mN) in physiological salt solution (PSS; 119 mM NaCl, 14.9 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 1.6 mM $CaCl_2$, 0.026 mM EDTA, 5.5 mM glucose), aerated (95% O_2, 5% CO_2) and maintained at 37°C. Each vessel was then exposed to a high potassium PSS (KPSS; 123.7 mM KCl, 14.9 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 1.6 mM $CaCl_2$, 0.026 mM EDTA, 5.5 mM glucose) three times. The vessels were then incubated either with sitaxentan (1 nM, 10 nM and 100 nM) or vehicle (distilled water) for 15 min before cumulative concentration-response curves to phenylephrine (PE, 1×10^{-9}–3×10^{-5}M), ACh (1×10^{-9}–3×10^{-5}M) and ET-1 (1×10^{-11}–3×10^{-7}M) were acquired. Concentration-responses to ACh were performed after pre-contraction with a submaximal concentration of PE (3×10^{-6}M) to achieve ~50% E_{max}. All responses were measured and recorded with Powerlab software (AD Instruments, Oxford, UK).

In vivo *ET receptor antagonism*

The orally active selective ET_A receptor antagonist sitaxentan (15 mg·kg^{-1}·day^{-1}), the selective ET_B receptor antagonist A192621 (30 mg·kg^{-1}·day^{-1}), or the combination of both, were administered by admixture with chow. Each compound was prepared by dispersion in 0.2% methylcellulose and bound with RM1 mouse chow using beef gelatine (Supercook, UK) and fresh diet was provided daily. Drug administration was started 1 week before surgery for wire injury and continued for a further 28 days. Body weights were recorded throughout the study to ensure adequate food intake.

Measurement of BP

BP was measured using tail cuff plethysmography. Animals were familiarized with the technique for two weeks before drug administration and BP was measured twice a week before the surgery. Following surgery, the mice were allowed to recover for one week after which BP was measured weekly, until the animals were killed.

Femoral artery injury

An intraluminal injury was performed as described previously (Kirkby *et al.*, 2012). Briefly, general anaesthesia was induced by inhalation of isoflurane (5% in medical oxygen) and maintenance was achieved using 2–3% isoflurane in medical oxygen, as required. Depth of anaesthesia was confirmed by loss of the pedal withdrawal response. A 0.014′ diameter straight sprung angioplasty guide wire was advanced ~1.5 cm proximally into the isolated femoral artery through an arteriotomy in the popliteal branch. After withdrawal, the popliteal branch was ligated to allow re-perfusion of the injured femoral artery. Mice were then allowed to recover (28 days) to allow lesion development.

Perfusion fixation

After the recovery period, mice were killed by perfusion fixation. Under terminal anaesthesia (sodium pentobarbital, Ceva Animal Health, Amersham, UK, 60 mg·kg^{-1}; i.p.), thoracotomy and transverse sternotomy were performed to allow introduction of a 23-gauge needle into the left ventricle. An incision was made in the right ventricle and PBS containing heparin (Leo Laboratories, Hurley, UK; 10 U·mL^{-1}) was administered (6 mL·min^{-1}) until blood was washed out. This was followed by perfusion with 10% neutral buffered formalin (Sigma, Poole, UK) until adequate fixation occurred (indicated by the development of rigidity of the body). Following perfusion fixation, femoral arteries, liver, heart and kidneys were removed. Organs were weighed and all tissues were left in formalin for a further 48 h before processing to paraffin for histological assessment.

Histological assessment of neointimal lesions

Sections (4 μm) were cut from paraffin-embedded femoral arteries at 80 μm intervals with a Leitz 1512 microtome (Leica Microsystems, Milton Keynes, UK), and mounted onto Superfrost glass slides. Every 10th slide was selected for staining (Shandon Varistain Gemini automated slide stainer) with US Trichrome, as described previously (Hadoke *et al.*, 1995). Images were taken using an Axioskop KS300 stage microscope (Carl Zeiss Inc., Cambridge, UK) and a CCD camera (Photometrics, Tucson, AZ, USA) with a liquid crystal filter (Mirco-Color, CRI Inc, Woburn, MA, USA). Image analysis was performed using MCID basic 7.0 software (Imaging Research, St. Catharine's, Ontario, Canada). The location of the maximal lesion was determined and serial sections were used for compositional analysis, including picro-sirius staining for the quantification of collagen content.

Immunohistochemistry

De-waxed and re-hydrated sections were blocked with goat serum before incubation with primary antibodies to α-smooth muscle actin (1:400; 30 min; Sigma) or Mac2 (1:6000; overnight; Cedarlane, Burlington, NC, USA). Sections were then washed and incubated with a secondary antibody (goat anti-mouse or goat anti-rat, respectively; 1:400, 30 min; Vector Labs, Peterborough, UK). This was followed by incubation with streptavidin-conjugated HRP (Extravidin; 30 min; Sigma). Slides were developed by addition of 3,3-diamidobenzidine (DAB peroxidase staining kit, Vector Lab) for 1 min. Images were taken as before and analysed with ImageJ software (ImageJ, NIMH, Bethesda, MD, USA).

Statistics

All results are mean ± SEM, where n indicates the number of mice used. Analyses were performed using one-way ANOVA with Dunnett's *post hoc* test. Significance was assumed when $P < 0.05$. Schild analysis (Arunlakshana and Schild, 1959) was performed using GraphPad Prism software (GraphPad, SanDiego, CA, USA). Measurements and analyses were performed by operators blinded to treatment. αSMA, Mac-2 and collagen content of lesions are expressed as percentage of neointimal area. n = 6 for myography and n = 6–16 for femoral artery injury.

Results

Sitaxentan is a competitive antagonist of ET-1 receptor-mediated contraction in murine femoral arteries

Mouse isolated femoral arteries used for functional investigation relaxed in response to ACh, indicating the presence of a functional endothelium (Figure 1A; Table 1). ET-1 (Figure 1B) or PE (Figure 1C) caused concentration-dependent contractions of isolated mouse femoral arteries. Incubation with sitaxentan (1–100 nM) caused a concentration-dependent rightward shift of the contractile response to ET-1 without affecting maximal force generation, yielding a pA_2 of 8.0 and a slope of 1.1 (Figure 1B; Table 1). In contrast, exposure to sitaxentan did not alter PE-mediated contraction (Figure 1C; Table 1).

Physical impact of ET receptor antagonism in vivo

Tail cuff plethysmography did not detect any changes in BP in response to any of the ET receptor antagonist treatment regimens (Figure 2). Similarly, body weight and the weights of heart and kidneys were unaffected by treatment (Table 2). Administration of the ET_B receptor-selective antagonist A192621 was associated with a reduction ($P < 0.01$) in liver weight (1.02 ± 0.04 g) compared with vehicle-treated controls (1.34 ± 0.07 g), which was not seen with selective ET_A or dual ET_A/ET_B receptor antagonism (Table 2).

ET_A receptor antagonism reduced lesion size

Wire injury caused the development of concentric neointimal lesions in all groups (Figure 3A). Administration of the ET_A receptor-selective antagonist sitaxentan reduced lesion size (Figure 3B; 23 ± 4% vs. 51 ± 4%; $P < 0.05$). An apparent increase in lesion size following ET_B receptor-selective antagonism did not achieve significance (61 ± 7%; $P > 0.05$) and dual antagonism did not change lesion size (51 ± 7%; $P > 0.05$). These results were mirrored by changes in the arterial lumen (Figure 3C), which showed that ET_A receptor-selective antagonism increased lumen size (66 ± 9% vs. 34 ± 5%; $P < 0.01$), while ET_B receptor-selective antagonism caused an apparent decrease in lumen size that did not achieve significance (21 ± 5%; $P > 0.05$). Dual antagonism did not change lumen size (33 ± 6%; $P > 0.05$).

Effect of ET receptor antagonism on lesion composition

Collagen content in lesions (Figure 4A) was lower in animals treated with sitaxentan (14 ± 2% vs. 44 ± 6% vehicle; $P < 0.01$). Dual antagonism also lowered collagen content (17 ± 3%; $P < 0.05$), but ET_B receptor antagonism (A192621) had no effect (30 ± 6%; $P > 0.05$). Immunohistochemistry indicated that lesions in all groups stained strongly for αSMA (Figure 4B), with a much lower percentage of the lesion immunoreactive for the macrophage marker, Mac-2 (Figure 4C). None of the treatment regimens altered the αSMA content (43 ± 7% for vehicle, 36 ± 6% for sitaxentan, 35 ± 5% for A192621 and 30 ± 2% for dual antagonism) of the lesions. There was a trend towards decreased macrophage

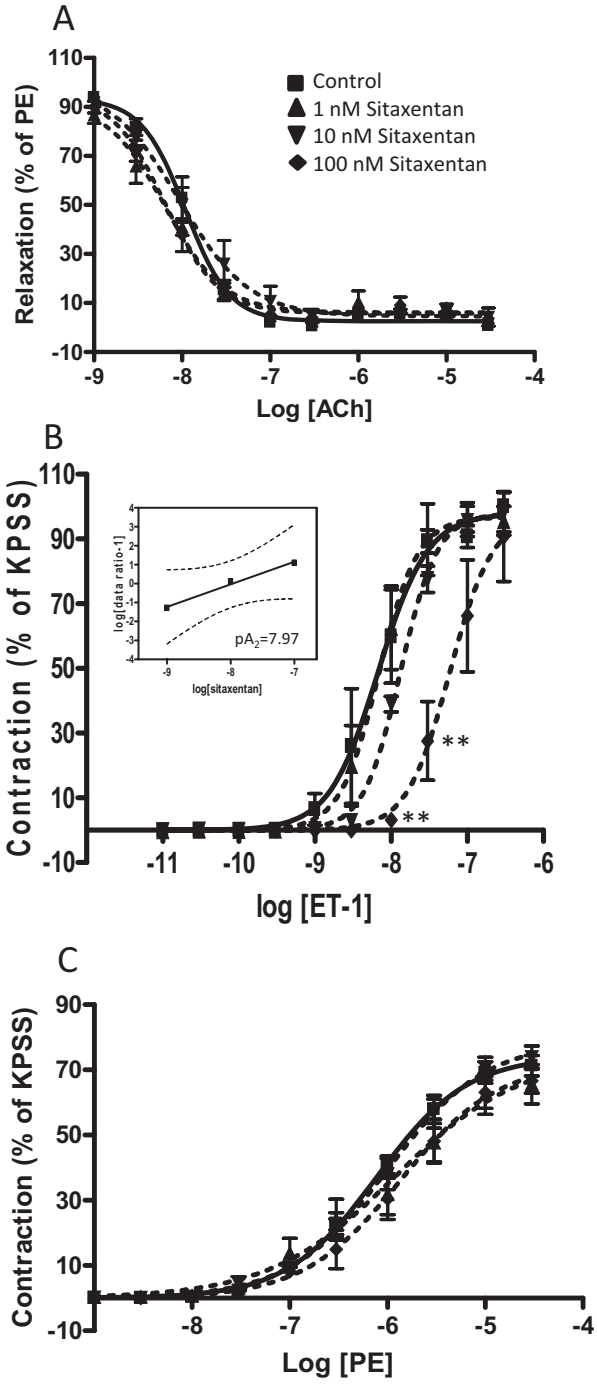

Figure 1

ET_A receptor antagonism with sitaxentan. In murine isolated femoral arteries, (A) ACh produced a concentration-dependent relaxation, indicating the presence of an intact endothelium in control arteries and in those exposed to 1 nM, 10 nM or 100 nM sitaxentan. (B) ET-1 produced a concentration-dependent contraction. Incubation with 1 nM, 10 nM or 100 nM sitaxentan caused a parallel rightward shift (without reducing maximal force generation), compared with vehicle. Schild analysis (inset) yielded a pA_2 of 7.97 with a slope of 1.1. (C) PE-induced contraction was unaffected by incubation with 1 nM, 10 nM or 100 nM sitaxentan. Each point represents mean ± SEM, $n = 6$. $^{**}P < 0.01$.

Table 1

Sitaxentan selectively blocks ET-1-mediated contraction of mouse femoral arteries

			Sitaxentan		
		Control	1 nM	10 nM	100 nM
PE	E_{max}	74.3 ± 2.6	74.2 ± 8.8	78.7 ± 3.8	72.4 ± 6.0
	pD_2	6.1 ± 0.1	5.9 ± 0.2	6.0 ± 0.1	5.9 ± 0.1
ACh	E_{max}	97.4 ± 1.5	93.9 ± 1.5	95.4 ± 2.2	94.2 ± 1.3
	pD2	8.00 ± 0.1	8.2 ± 0.1	8.0 ± 0.1	8.2 ± 0.1
ET-1	E_{max}	99.9 ± 5.4	96.9 ± 1.7	97.9 ± 1.8	97.9 ± 1.5
	pD_2	8.2 ± 0.1	8.2 ± 0.1	7.9 ± 0.0	7.2 ± 0.1**

E_{max} data are given as percentage of the contraction induced by KPSS (PE, ET-1) or percentage relaxation (ACh). Values are mean ± SEM (n = 6). **P < 0.01 compared with control. One-way ANOVA with Dunnett's *post hoc* test.
ET-1, endothelin-1; E_{max}, maximum response; KPSS, high (125 mM) potassium PSS; PE, phenylephrine.

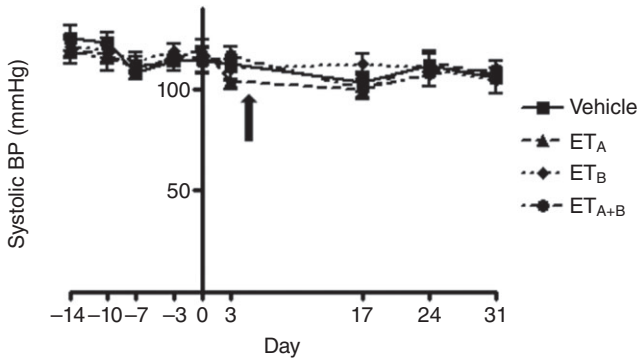

Figure 2

Endothelin receptor antagonism did not alter systolic BP. BP was measured in conscious, restrained mice using tail cuff plethysmography. Administration of sitaxentan (ET_A receptor antagonist; 15 $mg \cdot kg^{-1} \cdot day^{-1}$), A192621 ($ET_B$ receptor antagonist; 30 $mg \cdot kg^{-1} \cdot day^{-1}$) or a combination of both antagonists, had no effect on systolic BP. Each point represents mean ± SEM, n = 5–8.

content in mice exposed to sitaxentan (0.8 ± 0.4% sitaxentan vs. 1.8 ± 0.7% vehicle) that did not achieve significance.

Discussion

This investigation addressed the hypothesis that the selective ET_A receptor antagonist sitaxentan would be more effective than mixed receptor antagonism at inhibiting neointimal lesion formation in mouse femoral arteries. Sitaxentan was shown to antagonize ET-1 receptor-mediated arterial contraction *ex vivo*. A recent investigation showed that sitaxentan is a competitive antagonist of ET-1 receptor-mediated contraction in human saphenous vein (Maguire *et al.*, 2012a), but the authors are unaware of any previous use of sitaxentan in isolated arteries. Administration of sitaxentan reduced neointimal lesion formation following intraluminal injury to the mouse femoral artery, whereas ET_B receptor-selective or combined antagonism did not.

ET-1-induced contraction is mediated by the ET_A receptor in the smooth muscle cells of many different blood vessels [human coronary artery, internal mammary artery, saphenous vein (Maguire and Davenport, 1995), rat mesenteric artery (Matsumoto *et al.*, 2009)]. There is also evidence that ET_B receptors contribute to ET-1-mediated contraction of veins and some arteries (McCulloch *et al.*, 1996; Mickley *et al.*, 1997) but the physiological significance of this pathway is unclear. We have shown previously that contractions in response to ET-1 in the mouse femoral artery are blocked by ET_A, but not by ET_B receptor, antagonism (Kirkby *et al.*, 2012). Thus, the rightward shift in ET-1-induced contraction (without a reduction in maximum contraction) caused by sitaxentan is consistent with competitive antagonism at the ET_A receptor. This is supported by the demonstration that the effect was selective for ET-1 (with no alteration in PE-mediated contraction). Furthermore, the effects of sitaxentan could not be attributed to impaired endothelial cell function because ACh-mediated relaxation was unaltered and there was no evidence of the increased maximum contraction to ET-1 reported following removal of the vascular endothelium or ET_B receptor antagonism (Kirkby *et al.*, 2012).

The effect of sitaxentan on arterial contraction does not appear to have been reported before. Previous evidence that sitaxentan is a selective antagonist of ET_A receptors was obtained from ligand binding in membrane preparations from a human rhabdomyosarcoma (TE 671) cell line (Wu *et al.*, 1997), as well as from competition binding assays in human left ventricle and a vasoconstriction functional assay in human saphenous vein (Maguire *et al.*, 2012a,b). The pA_2 reported in TE 671 cells is consistent with the value determined in isolated femoral arteries (both pA_2 = 8.0), which is slightly larger than that reported for human saphenous vein (K_B 64.6 nM equivalent to pA_2 7.2; Maguire *et al.*, 2012a,b). This value is consistent with a number of other ET_A receptor antagonists (which have pA_2 values in the range 7.2–9.2; Sogabe *et al.*, 1993; Webb *et al.*, 1995; Reynolds *et al.*, 1995) but suggests a lower affinity for the ET_A receptor than atrasentan (pA_2 9.2 in isolated rat aorta; Opgenorth *et al.*, 1996). Therefore, these results suggest that sitaxentan, while more selective than atrasentan for ET_A receptors, is less potent. It is,

Table 2

Effect of ET receptor antagonism on body and organ weights

	± Treatment groups			
	Control	Sitaxentan	A192621	Sitaxentan + A192621
Body	28.1 ± 0.4 g	28.9 ± 0.91 g	29.1 ± 0.7 g	28.9 ± 0.6 g
Liver	1.3 ± 0.1 g	1.5 ± 0.1 g	1.0 ± 0.0 g**	1.2 ± 0.0 g
	(4.7 ± 0.2%)	(5.2 ± 0.3%)	(3.5 ± 0.2%)**	(4.4 ± 0.1%)
Heart	0.18 ± 0.01 g	0.19 ± 0.02 g	0.20 ± 0.02 g	0.19 ± 0.01 g
	(0.63 ± 0.04%)	(0.67 ± 0.03%)	(0.67 ± 0.06%)	(0.66 ± 0.04%)
Left kidney	0.21 ± 0.01 g	0.22 ± 0.01 g	0.23 ± 0.02 g	0.21 ± 0.01 g
	(0.75 ± 0.03%)	(0.76 ± 0.04%)	(0.78 ± 0.04%)	(0.73 ± 0.02%)
Right kidney	0.22 ± 0.01 g	0.23 ± 0.01 g	0.22 ± 0.01 g	0.22 ± 0.02 g
	(0.78 ± 0.02%)	(0.79 ± 0.05%)	(0.67 ± 0.01%)	(0.76 ± 0.58%)

Weights are expressed as mean ± SEM. Organ weights are expressed in g and as a percentage of body weight (parentheses). n = 7–8; **P < 0.01.

however, important to note the influence of assay systems on the results produced by these antagonists; in competition binding assays using human left ventricle, sitaxentan generated a higher affinity (K_D, 1.65 ± 0.8 nM) than seen in isolated veins and a higher ET_A receptor selectivity (~200 000-fold; Maguire *et al.*, 2012a,b) than reported in TE 671 cells (7000-fold; Wu *et al.*, 1997).

Consistent with several previous studies using selective ET_A receptor antagonists (Ferrer *et al.*, 1995; Burke *et al.*, 1997; Kirkby *et al.*, 2012), sitaxentan reduced neointimal lesion formation. The magnitude of the reduction was comparable with that reported previously with atrasentan (10 mg·kg^{-1}; Kirkby *et al.*, 2012). The mechanism for this reduction has not been established but ET_A receptor antagonism may inhibit mitogenesis of neointimal smooth muscle cells (Burke *et al.*, 1997; Azuma *et al.*, 1999). Alternatively, reduced lesion formation may be secondary to the improved endothelial cell function that has been reported with the use of ET_A receptor-selective antagonists (atrasentan; Reriani *et al.*, 2010). Notably, as in our previous investigation (Kirkby *et al.*, 2012), sitaxentan did not reduce the proportion of the lesion consisting of smooth muscle cells. The trend towards reduced macrophage content in lesions from sitaxentan-treated mice may suggest a reduced inflammatory response to injury. To determine whether this was the case, it would be necessary to directly assess the effect of sitaxentan on the acute (1–7 days) inflammatory response induced by wire insertion. The reduction in the amount of collagen in lesions from animals treated with sitaxentan (either alone or in combination with an ET_B receptor antagonist) is consistent with our previous observations with atrasentan (Kirkby *et al.*, 2012) and with the demonstration that ET_A receptor antagonism reduced collagen deposition in a rat model of myocardial infarction (Fraccarollo *et al.*, 2002). While reduced collagen content has been associated with increased lesion vulnerability in a model of tandem arterial injury in atherosclerosis-prone ($ApoE^{-/-}$) mice (Chen *et al.*, 2013), and in the setting of plaque development, more collagen clearly indicates a more stable lesion; there was no evidence that reduced collagen content increased lesion vulnerability to rupture in the current investigation.

Previous studies reported that mixed ET receptor antagonism reduces neointimal lesion formation (Douglas *et al.*, 1994; Reel *et al.*, 2005; Kitada *et al.*, 2009). However, the failure of mixed antagonism to reduce lesion size in the current investigation is consistent with our previous experience in this model (Kirkby *et al.*, 2012). The explanation for this lack of effect was suggested by increased lesion formation in the presence of ET_B receptor antagonism (Kirkby *et al.*, 2012). A similar (but non-significant) trend towards increased lesion size following administration of A192621 was observed in the current study. Indeed, pharmacological antagonism (Murakoshi *et al.*, 2002; Kitada *et al.*, 2009) and deletion (Murakoshi *et al.*, 2002; Kitada *et al.*, 2009) of ET_B receptors have both been shown to increase lesion formation, consistent with the proposal that ET_B receptor antagonism should be avoided in models of neointimal lesion formation. It is unclear why the effect of ET_B antagonism did not achieve significance in the current investigation; this situation was not altered by increasing group sizes to exclude the possibility of type II errors. It seems likely that ET_B receptor antagonism produces a small increase in lesion size but this is difficult to detect given the inherent variability in lesion size following wire injury. A note of caution should be included when extrapolating these data to patients, as the model used in the present study is of neointimal proliferation, not restenosis. In humans, restenosis following percutaneous revascularization is associated with up-regulation of ET_A and ET_B receptors in vascular smooth muscle. This may indicate a role for ET_B receptor up-regulation in the remodelling process (Wackenfors *et al.*, 2004; Shirai *et al.*, 2006). Future investigations need to apply intraluminal injury to atherosclerosis-prone mice to provide a setting closer to that in patients. Furthermore, appropriate techniques should be used (e.g. laser capture microdissection) to determine whether there is any ET receptor up-regulation in this model.

Reduced lesion size cannot be attributed to reduced arterial BP as there was no evidence that ET receptor antagonism

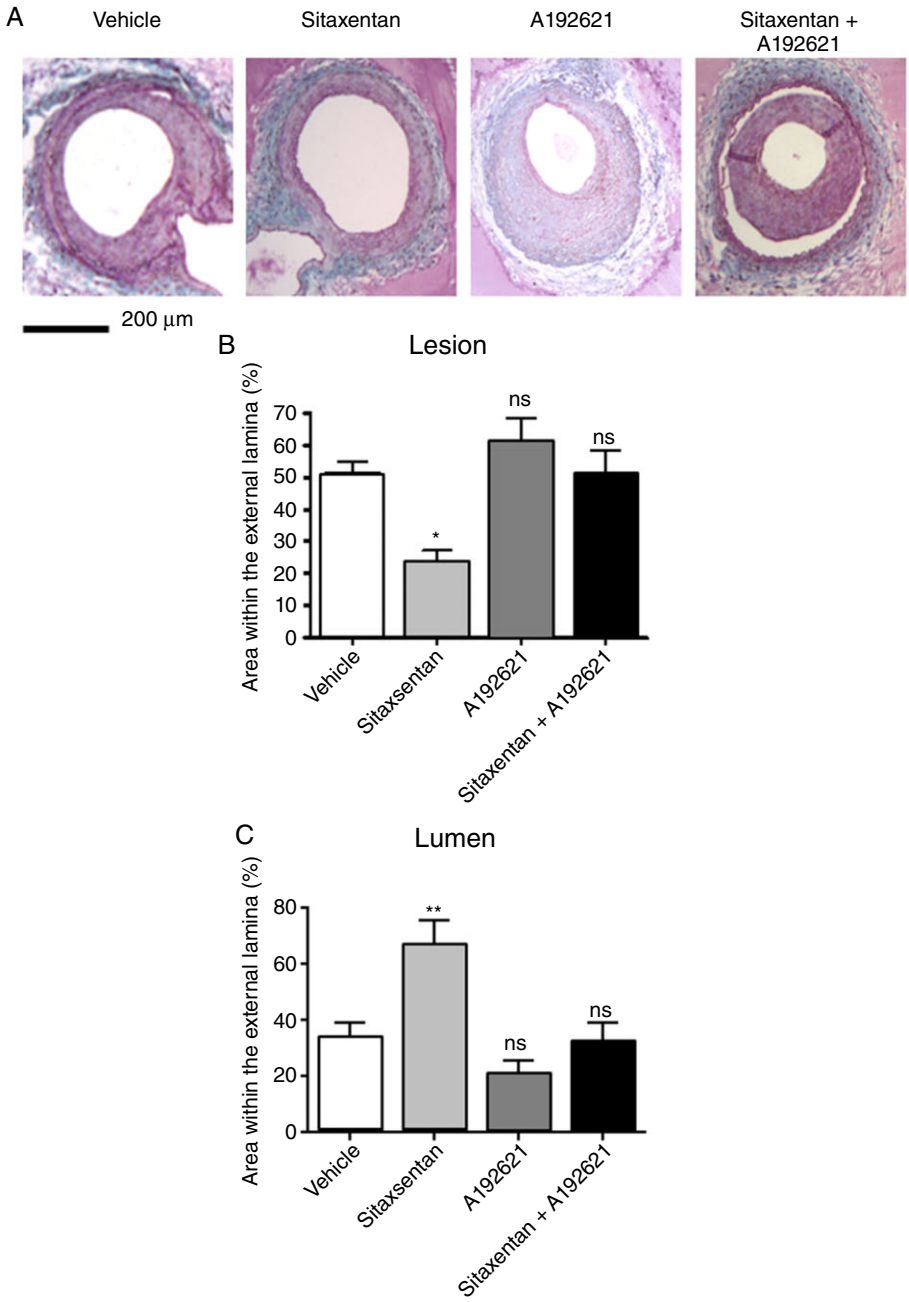

Figure 3

Selective ET_A receptor antagonism reduces neointimal lesion size in mouse femoral artery. Femoral arteries were isolated 28 days after the mice had been subjected to intraluminal-wire injury. US trichrome staining (A) indicated the presence of extensive, concentric neointimal lesions. Lesion (B) and lumen (C) size were measured in the section from each artery with greatest cross-sectional narrowing. There was a decrease in lesion size after treatment with sitaxentan and an apparent increase in lesion size that did not achieve significance ($P > 0.05$) with A192621. The combination of both drugs had no effect on lesion size. The differences in lesion size were mirrored in the lumen size, with sitaxentan administration resulting in increased luminal area (**$P < 0.01$), whereas the combination of both antagonists had no effect and an apparent decrease in luminal area with A192621 did not achieve significance ($P > 0.05$). Columns represent mean ± SEM, where $n = 6–16$. *$P < 0.05$, **$P < 0.01$.

altered BP in this investigation. This in itself was surprising as we previously demonstrated that atrasentan reduced, and A192621 increased, BP in this model (Kirkby *et al.*, 2012). The lack of effect on BP in the current investigation may reflect a lack of sensitivity in the technique (tail cuff plethysmography), which produces a stressed BP recording. It is worth noting, however, that while sitaxentan decreased systolic BP in spontaneously hypertensive hamsters (Rubinstein, 2006), it had no effect in rats (Tilton *et al.*, 2000).

Despite its promise as a treatment for pulmonary arterial hypertension (Barst *et al.*, 2002), sitaxentan (Thelin) was withdrawn from clinical practice because of serious and

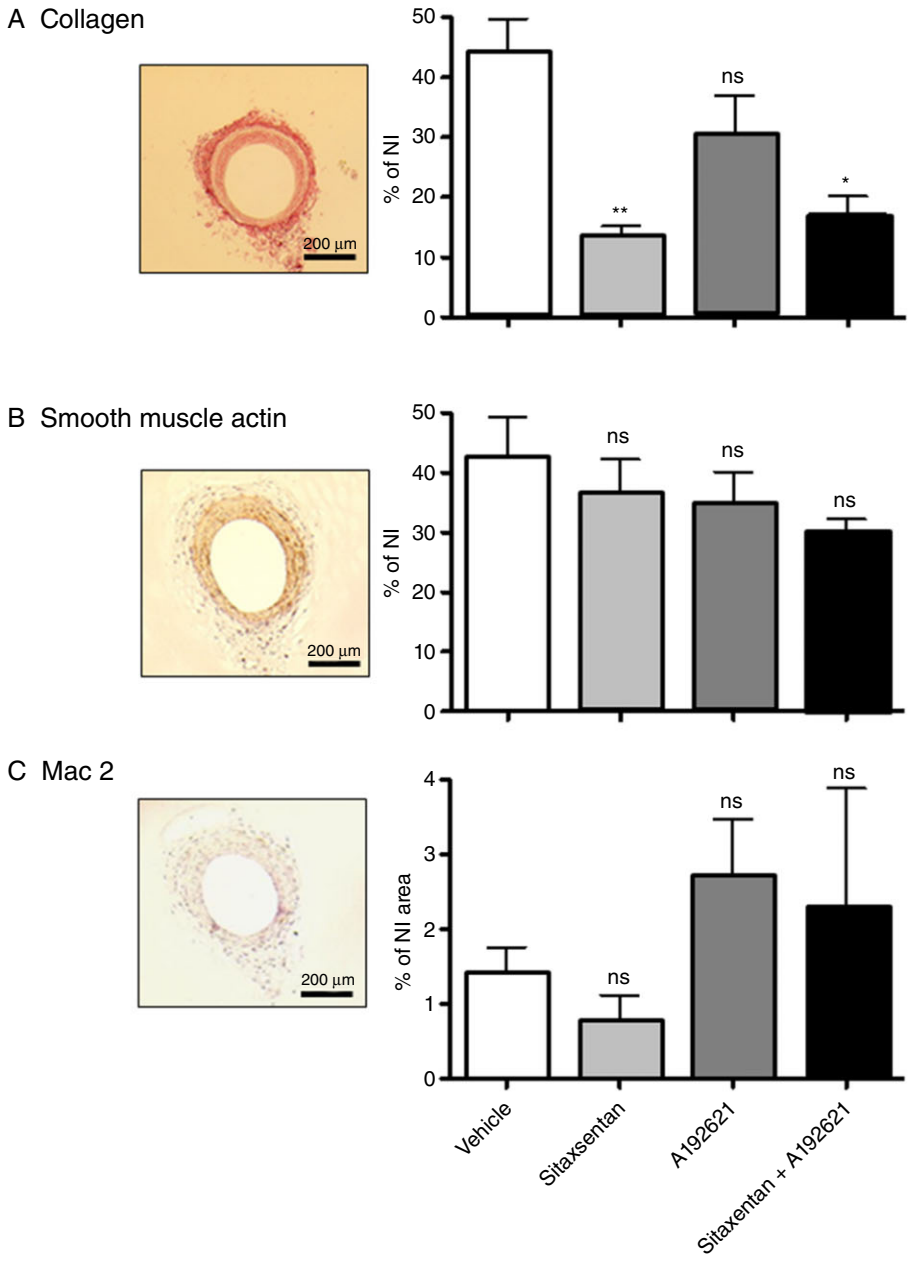

Figure 4

ET receptor antagonism alters neointimal lesion composition. Serial sections from arteries with maximal cross-sectional narrowing were stained for collagen using picro-sirius red (A), for smooth muscle using α-actin (B) and for macrophages using an antibody to Mac-2 (C). Collagen content was reduced by administration of sitaxentan and by the combination of sitaxentan and A192621, but not by A192621 alone. The proportion of the lesion staining positive for smooth muscle α-actin was not altered by any of the treatment regimens. There was an apparent decrease in macrophage content after treatment with sitaxentan but this failed to reach statistical significance ($P > 0.05$). Columns represent mean ± SEM, where $n = 6–16$. *$P < 0.05$, **$P < 0.01$.

fatal hepatotoxicity. The exact cause of this liver toxicity has not been established, but it seems to be related to its sulphonamide structure and does not occur with some other ET-1 receptor antagonists in clinical use (MacIntyre *et al.*, 2008; Kenna *et al.*, 2015). Indeed, a recent *in vitro* investigation suggested that a number of different mechanisms contribute to the rare, severe liver injury associated with sitaxentan (Kenna *et al.*, 2015). The effect of bosentan on these mechanisms was much less dramatic and ambrisentan showed little evidence of toxicity *in vitro*. In the current investigation, administration of sitaxentan had no apparent detrimental effects. The mice continued to thrive and showed a similar increase in body weight to control mice. Furthermore, major organs were normal on gross inspection (heart, kidney) and were comparable weight to controls. No symptoms of liver disease were

observed and treatment did not alter liver weight. The absence of any evidence of side effects with this treatment in mice suggests that further studies may be warranted in other animal models to give a clearer understanding of the mechanisms underlying the toxic effects of ET_A receptor antagonists.

An unexpected observation was that ET_B receptor antagonism with A192621 decreased liver weight. This contrasts with a previous demonstration that A192621 did not alter liver weight in mice with polycystic kidney disease (Chang *et al.*, 2007). However, an effect of ET_B receptors on the liver has been described, with ET_B receptor knockout in mice leading to altered liver morphology (reduced number and diameter of sinusoids) and red blood cell congestion in large intrahepatic veins; Ling *et al.*, 2012). This, combined with altered ET_B receptor expression in patients, suggested a role for the ET system in hepatic cirrhosis, and indicated that sinusoidal constriction could contribute to hepatotoxicity associated with some ET antagonists.

In conclusion, this investigation supports the hypothesis that selective ET_A receptor antagonism is more effective than mixed or ET_B receptor-selective antagonists in preventing neointimal lesion formation. This is consistent with the suggestion that ET_A receptor stimulation contributes to lesion formation, whereas ET_B receptors prevent lesion development. With the development of newer more selective ET_A receptor antagonists without liver toxicity, this approach has potential clinical benefit for the prevention of neointimal lesion formation (Yoon *et al.*, 2013). Further investigations are warranted to identify the optimum regimen (dose, timing of administration) to reduce lesion formation without producing systemic side effects.

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Author contributions

P. W. F. Hadoke, K. M. Duthie, N. S. Kirkby, E. Miller J. R. Ivy, J. F. McShane, W. G. Lim performed the research. P. W. F. Hadoke, K. M. Duthie and D. J. Webb designed the research study. P. W. F. Hadoke, K. M. Duthie, E. Miller, J. R. Ivy and J. F. McShane analysed the data.

P. W. F. Hadoke, K. M. Duthie and D. J. Webb wrote the paper.

Conflict of interest

This work was partially funded by a grant and provision of Sitaxentan from Pfizer. D. J. W. is a member of an independent data monitoring committee for AbbVie in the endothelin field. None of the authors have any conflicts of interest.

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