

REVIEW

New Drugs and Emerging Therapeutic Targets in the Endothelin Signaling Pathway and Prospects for Personalized Precision Medicine

A. P. DAVENPORT¹, R. E. KUC¹, C. SOUTHAN², J. J. MAGUIRE¹

¹Experimental Medicine and Immunotherapeutics, University of Cambridge, Addenbrooke's Hospital, Cambridge, United Kingdom, ²Deanery of Biomedical Sciences, University of Edinburgh, Edinburgh, United Kingdom

Received January 26, 2018

Accepted March 29, 2018

Summary

During the last thirty years since the discovery of endothelin-1, the therapeutic strategy that has evolved in the clinic, mainly in the treatment of pulmonary arterial hypertension, is to block the action of the peptide either at the ET_A subtype or both receptors using orally active small molecule antagonists. Recently, there has been a rapid expansion in research targeting ET receptors using chemical entities other than small molecules, particularly monoclonal antibody antagonists and selective peptide agonists and antagonists. While usually sacrificing oral bio-availability, these compounds have other therapeutic advantages with the potential to considerably expand drug targets in the endothelin pathway and extend treatment to other pathophysiological conditions. Where the small molecule approach has been retained, a novel strategy to combine two vasoconstrictor targets, the angiotensin AT₁ receptor as well as the ET_A receptor in the dual antagonist sparsentan has been developed. A second emerging strategy is to combine drugs that have two different targets, the ET_A antagonist ambrisentan with the phosphodiesterase inhibitor tadalafil, to improve the treatment of pulmonary arterial hypertension. The solving of the crystal structure of the ET_B receptor has the potential to identify allosteric binding sites for novel ligands. A further key advance is the experimental validation of a single nucleotide polymorphism that has genome wide significance in five vascular diseases and that significantly increases the amount of big endothelin-1 precursor in the plasma. This observation provides a rationale for testing this single nucleotide polymorphism to stratify patients for allocation to treatment with endothelin agents and highlights the potential to use personalized precision medicine in the endothelin field.

Key words

Allosteric modulators • Biased signaling • G-protein coupled receptors • Endothelin-1 • Monoclonal antibodies • Pepducins • Single nucleotide polymorphisms

Corresponding author

A. P. Davenport, Experimental Medicine and Immunotherapeutics, University of Cambridge, Addenbrooke's Hospital, Cambridge, CB2 0QQ, United Kingdom. Fax: 01223 762576. E-mail: apd10@medschl.cam.ac.uk

Introduction

During the last thirty years since the discovery of endothelin-1 (ET-1), the therapeutic strategy that has evolved in the clinic, mainly in the treatment of pulmonary arterial hypertension (PAH), is to block the action of the peptide at the ET_A subtype using ambrisentan (Letairis, Volibris, LU208075) or by blocking both receptors using bosentan (Tracleer, Ro47-0203) and macitentan (Opsumit, ACT-064992). All three drugs are orally active small molecule antagonists (Maguire and Davenport 2014). The timelines for key discoveries in the ET signaling pathway (the three ET peptides, two receptors and the endothelin converting enzymes (ECE), ECE-1 and ECE-2 are shown in Figure 1, together with the most widely used selective peptide ligands used to characterize the receptors *in vitro* and *in vivo*.

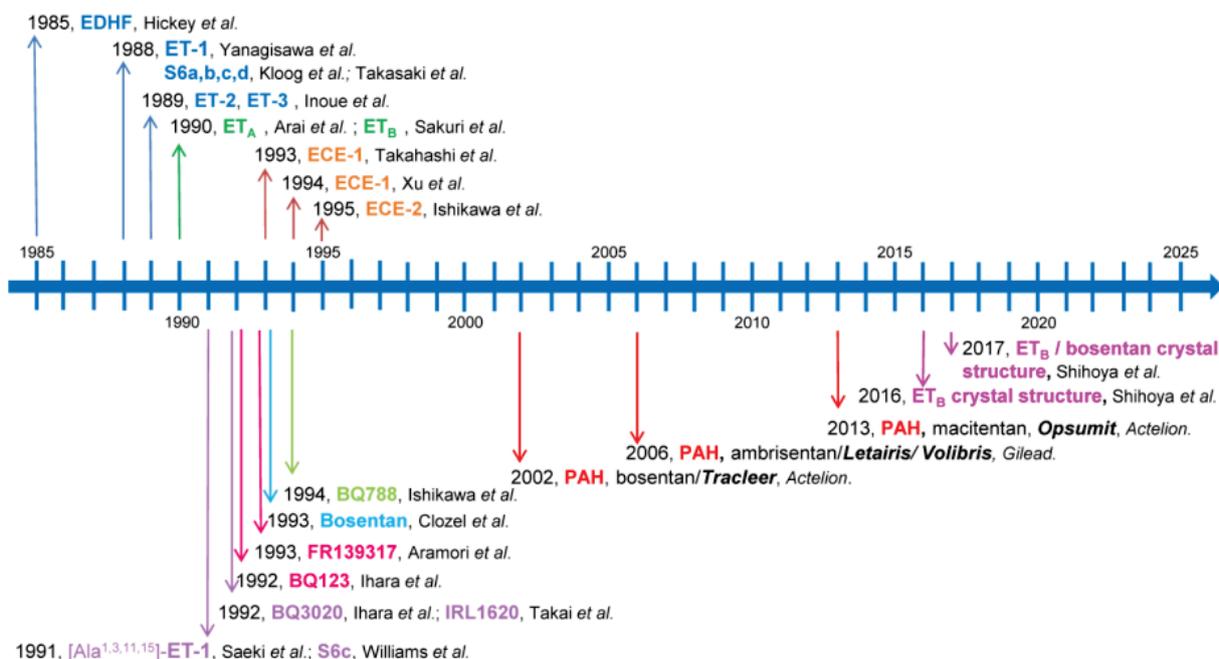


Fig. 1. The timelines for key discoveries in the ET signaling pathway: the three ET peptides, ET_A and ET_B receptors and endothelin converting enzymes, ECE-1 and ECE-2. The twenty-one amino acid sarafotoxins S6a, b, c, d, identified in the venom of the snake *Atractaspis engaddensis* that have a high degree of sequence similarity, are also shown. Timelines for the identification of key pharmaceutical agents are listed: ET_B agonists [Ala^{1,3,11,15}]-ET-1, BQ3020 and sarafotoxin S6c; ET_A antagonists BQ123 and FR139317; bosentan, the first ET_A/ET_B antagonist approved for clinical use; ET_B antagonist BQ788; ET_A antagonist ambrisentan; the next generation of ET_A/ET_B antagonists, macitentan; the crystal structure of the ET_B receptor.

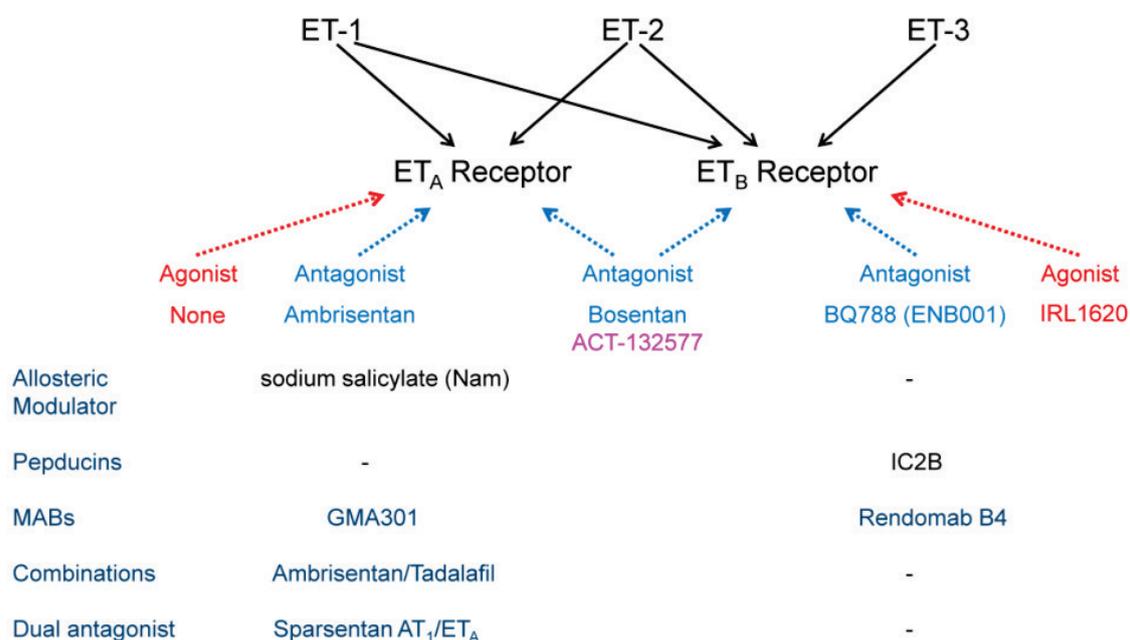


Fig. 2. ET signaling pathway, showing established and emerging pharmacological targets together with corresponding agents.

Recently, there has been a rapid expansion in research targeting ET receptors using chemical entities other than small molecules, namely monoclonal antibody antagonists and selective peptide agonists and antagonists (Fig. 2). While usually sacrificing oral bio-availability,

these compounds have other therapeutic advantages with the potential to considerably expand drug targets in the ET pathway and extend treatment to other pathophysiological conditions. Where the small molecule approach has been retained, the novel strategy is to

combine two vasoconstrictor targets, the angiotensin AT₁ as well as the ET_A receptors in a dual antagonist, sparsentan (Komers *et al.* 2017). A second emerging strategy is to use two separate drugs, the ET_A antagonist ambrisentan with the phosphodiesterase (PDE) 5 inhibitor tadalafil, to improve the treatment of PAH (Galie *et al.* 2015) (Fig. 2). For endothelin research the most recent milestones in scientific progress are shown in Figure 1 culminating in the solving of the crystal structure of the ET_B receptor that has the potential to identify allosteric binding sites for further novel ligands (Shihoya *et al.* 2016, Shihoya *et al.* 2017). Finally, a further key advance is the experimental validation of a single nucleotide polymorphism (SNP) that has genome wide significance in five vascular diseases that significantly increases the amount of big ET precursor in the plasma (Gupta *et al.* 2017). This study provides a rationale for testing for this SNP to stratify patients for allocation to treatment with ET compounds and demonstrates the potential for the use of precision medicine in the ET field. This review focusses on a concise update of drugs in the ET field and other reviews should be consulted for more details on ET signaling pathways (Davenport *et al.* 2016, Houde *et al.* 2016, Czopek *et al.* 2016).

ET_B peptide antagonist BQ788 (ENB001, ENB Therapeutics) in melanoma

BQ-788 has been established as a highly selective peptide ET_B antagonist both *in vitro* and *in vivo* in the clinic and is now being explored in a range of clinical conditions (Davenport *et al.* 2016). The ET signaling pathway has been implicated in a number of cancers. In particular, the ET_B receptor is overexpressed in melanoma (Asundi *et al.* 2011), a cancer that develops from pigment-containing melanocytes where ET has a role in proliferation and melanin synthesis and typically occurs in the skin. It is an aggressive cancer, characterized by its capacity to metastasize, leading to an increase in mortality rates and affects about 340,000 patients in the US and Europe. The transformation of melanocytes to melanoma cells is often associated with an increased capacity to proliferate. It is thought that ET_B receptors are a driver of melanoma progression and marker of aggressive phenotype (Bittner *et al.* 2000). BQ788 inhibits melanoma cell progression (Bagnato *et al.* 2004) and the growth of xenograft human melanoma tumors in mice which is thought to resemble spontaneous human melanoma regression (Lahav 2005). In a small

clinical study, the local action of intra-lesion administration of BQ-788 in five melanoma patients found that excised treated lesions exhibited decreased anti-apoptotic markers and ET_B receptors compared with saline controls. In one patient, treated for longer than one week, lesion growth was inhibited (Wouters *et al.* 2015). In this setting BQ788 was tolerated. These results suggest that ET_B receptors may play a key role in melanoma and represents a new therapeutic target.

The MAP-kinase (MAPK) pathway is deregulated in the majority of malignant melanomas but therapeutic targeting of the primary driver of hyper-active MAPK signaling, BRAF, while initially effective in patients, owing to patient heterogeneity can lead to resistance. ET-1 gene expression was found to be enhanced in tumors of patients on BRAF treatment and this was proposed as a mechanism of resistance. Treatment with bosentan overcomes the ET-1 signaling pathway and prolongs BRAF-inhibitor responses (Smith *et al.* 2017).

Therapeutic monoclonal antibodies directed against the ET_B receptor: Rendomab-B1 and B4

Class A G-protein coupled receptors (GPCRs) that include the ET sub-types are targets of about one third of all current medicines used in the clinic. The development of monoclonal antibodies (MABs) has been a major growth area with over fifty approved for clinical use. In addition to being highly selective for the target protein, MABs can have less off-target actions. They have a very long plasma half-life compared with small molecules, leading to a prolonged time course of action of several months and to improved patient compliance. The majority of MABs to GPCRs tend to bind to extracellular regions and therefore can stabilize different conformational states compared with small molecule ligands that bind to transmembrane domains. MABs may cause an immunogenic response in human patients, reducing their therapeutic efficacy, but this can be mitigated by using human-derived sequences (Hutchings *et al.* 2017). The main disadvantage of MABs is that there can be significantly greater costs of manufacturing large proteins with post-translational modifications compared with small molecules and the need to penetrate tissues, for example, to reach cell surface expressed receptors. MABs potentially offer a wider range of therapeutic strategies compared with small molecules,

particularly when the X-ray structure is known, as is the case of the ET_B receptor, to discover regions distinct from the ligand binding site (allosteric modulators). MABs can

bind and stabilize the inactive state of a receptor blocking any change in the conformational structure needed to activate intracellular transduction pathways.

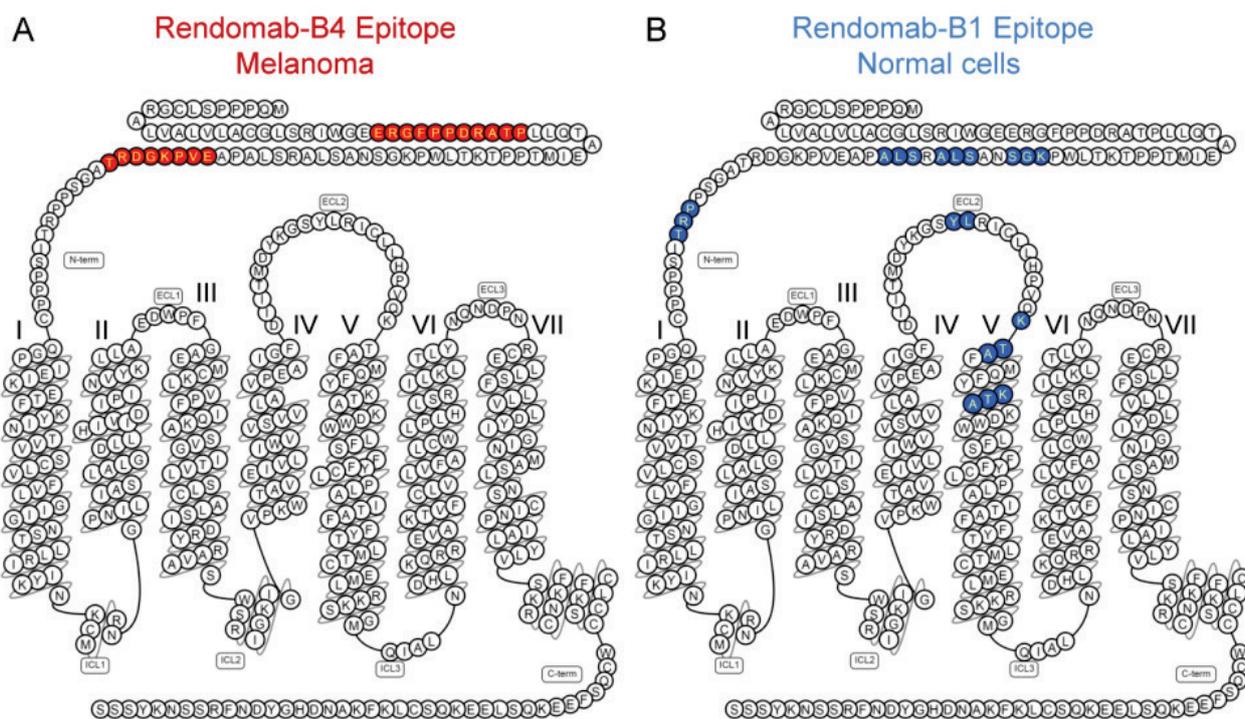


Fig. 3. Structure of the ET_B receptor showing amino acids determined by epitope mapping interacting with the MAB Rendomab-B4 in melanoma (**A**) compared to Rendomab-B1 interacting with different regions with native ET_B receptors (**B**). Roman numerals indicate the transmembrane spanning domains (Pándy-Szekeres *et al.* 2018).

The development of MABs to GPCRs has proved to be challenging. Recently, the first monoclonal antibody to be developed with sub-nanomolar affinity at the ET_B receptor was reported. Rendomab-B1 was found to be more effective than BQ788 in competing for ET-1 at the ET_B receptor and represents a significant advance for the ET field and GPCRs in general (Allard *et al.* 2013). Rendomab-B1 was proposed to function as an antagonist, based on the observed inhibition of ET-1-induced IP₃-calcium signaling. Epitope mapping suggested Rendomab-B1 recognized two discontinuous regions in the N-terminal extracellular domain and one in the extracellular loop E2 (Fig. 3).

Surprisingly when tested in melanoma cells, Rendomab-B1 had only low affinity for cell surface ET_B receptors expressed in this cancer. This finding suggested structural heterogeneity among the ET_B sub-type in tumors. The authors (Borrull *et al.* 2016) went on to discover a new MAB Rendomab-B4 that was able to bind to ET_B receptors in three melanoma cell lines (UACC-257, WM-266-4 and SLM8). In one of these (UACC-257) Rendomab-B4 was internalized and found

to co-localize with the early endosomal protein EEA-1. This is consistent with the known properties of ET_B receptors being sorted from endosomes to lysosomes and degraded, resulting in signal termination. In contrast, ET_A receptors are dephosphorylated in the endosomes and recycled back to the cell surface to signal again. Intriguingly, Rendomab-B4 behaved as a biased allosteric modulator able to inhibit G protein-dependent signaling (ET-mediated phospholipase C pathway) but was less effective at inhibiting the β -arrestin dependent pathway (ERK1/2 phosphorylation induced by ET).

Epitope mapping showed Rendomab-B4 recognizes two non-contiguous sequences in the N-terminal of the receptor. Rendomab-B4 did not bind to ET_B receptors expressed in HEK or native receptors in human umbilical vein endothelial cells. The binding properties were thus opposite to Rendomab-B1. Borrull *et al.* (2016) suggest possible explanations for these differences. The most plausible is that there could be differences in post-translational modifications, where ET_B receptors have a surprising number (Davenport *et al.* 2016). The most likely is a potential glycosylation site at

Asn⁵⁹ (N⁵⁹) in the N-terminus located between the two sequences that are thought to bind the MAB. However, there is no evidence yet that this is important for binding, although in other GPCRs at least one glycosylation site is often critical for receptor expression at the cell membrane and for function. Alternative splice variants have been identified encoding ET_B receptors but to date when artificially expressed, display no change in binding characteristics and their physiological or pathophysiological significance remain unclear (Davenport and Maguire 2006). A detailed experimental study using three antisera site-directed to three different regions of the ET_B receptor to ensure visualization of both wild type and splice variants found no differences in distribution in human kidney compared with ET_B receptors visualized by autoradiography using radiolabeled ET_B ligands (Kuc and Davenport 2004). Splice variants seem an unlikely reason for differences in these MABs. These MABs have not been tested *in vivo* and the pharmacokinetic properties are not yet known. Based on the published pharmacodynamics profile, Rendomab-B1 may mimic the action of the ET_B antagonist BQ788 to block endothelial ET_B receptors, inhibiting autocrine-paracrine mediated release of endothelium derived relaxing factors and therefore cause vasoconstriction. In contrast, if the binding properties of Rendomab-B4 are retained *in vivo*, this MAB would be predicted to bind ET_B receptors present on melanoma intra-cranially xenografted into mice but to have little or no effect on normal cells expressing ET_B receptors and therefore provide an elegant strategy for selectively targeting tumors.

These studies provide proof of principle for the generation of a pharmacologically active MABs against the ET_B receptor. This is important as MABs may be further engineered to extend therapeutic targets, particularly in cancer. For example, MABs can be conjugated to a chemotherapy drug or to a radioactive compound and this toxic payload may be used to selectively treat tumors in which ET_B receptors are over-expressed. Bispecific MABs can also be developed that bind, for example, to ET_B in target tumors and to CD3 expressed on T cells to promote the immune system to attack the cancer cells.

Therapeutic monoclonal antibodies directed against the ET_A receptor

Antibodies have been generated to the ET_A receptor but these have largely been restricted to

immunohistochemical localization of the protein rather than directed to the development of MABs and exploring their pharmacological properties, particularly antagonism of ET-1 responses. Recently, preliminary data have been reported on Rendomab-A63 (Herbet *et al.* 2018), a MAB directed against the human ET_A receptor that binds with sub-nanomolar affinity (K_D of 0.3 nM). A humanized ET_A MAB, GMA301, has been reported in a patent and scheduled to be tested in a clinical trial by Gmaxbiopharm but no further information is available as a publication (<http://www.gmaxbiopharm.com/pipeline3.html>). An ET_A selective small molecule or peptide analogue has not been discovered and an ET_A MAB agonist could be exploited as a tool compound for further exploration of the pharmacology of this sub-type. To date it has been assumed that because of ET_A mediated vasoconstriction, a selective ET_A MAB agonist would not have a therapeutic application. However, as discussed later, Gupta *et al.* (2017) identified vascular diseases such as migraine headache, where elevated ET-1 levels associated with the minor allele G/G may be beneficial in preventing the condition, suggesting a possible but highly speculative application for a selective ET_A MAB agonist in individuals with the A/A allele and therefore lower ET-1 levels in the plasma. Conversely, some insight into the potentially deleterious actions of an ET_A MAB agonist is provided by the identification of autoantibodies to the ET_A receptor. In one study, in most patients with systemic sclerosis, in which augmented ET signaling has been implicated, ET_A receptor autoantibodies were detected and were functionally active, that is acted as agonists. Surprisingly, the author reported autoantibodies specifically bound to endothelial cells although these cells are widely considered to express ET_B but not ET_A (Riemekasten *et al.* 2011).

ET-1 remains one of the most potent vasoconstrictors identified, with a usually long lasting action, so ET_A receptor MABs may be an attractive therapeutic strategy if they display very long half-lives compared with small molecule antagonists. It has been suggested that the long lasting action of ET-1 is the result of irreversible binding to ET_A receptors. However, dissociation of labelled ET-1 from cloned ET_A receptors was found to be slower compared with other vasoactive agents but was not irreversible (Blandin *et al.* 2000) with about a fifth of specific [¹²⁵I]-ET-1 binding dissociated from native ET_A receptors human vessels after 20 min (Maguire *et al.* 1996). However, it is crucial that MABs

are able to reverse established ET-1 constrictor responses in pathophysiological conditions such as cerebral vasospasm. Long lasting ET-1 constrictor responses have been shown to be reversible by small molecule antagonists *in vitro* and *in vivo* (Warner *et al.* 1994, Pierre and Davenport 1999).

ET_B agonists: IRL1620 (PMZ-1620, SPI-1620) in stroke, Alzheimer's disease and cancer

High densities of ET_B receptors are expressed in human brain (~90 % in areas such as cerebral cortex) (Harland *et al.* 1998). IRL1620 [N-Succinyl-[Glu⁹, Ala^{11,15}] endothelin-1] was synthesized as an analogue of ET-1 and found to be highly selective for the ET_B receptor (Takai *et al.* 1992). Although the N-terminus incorporates an N-Succinyl modification to reduce metabolism by non-specific peptidases, plasma half-life is short (only a few minutes) and as a modified peptide it is not orally active and requires administration by injection. Despite this unpromising profile, this compound, known as PMZ-1620, is being explored in a number of diseases associated with the CNS in animal models and in clinical studies (Gulati 2016, Gulati *et al.* 2017, Joshi *et al.* 2016, Briyal *et al.* 2015). The therapeutic strategy is to exploit the IRL-1620 induced vasodilatation and neuroprotection mediated by the ET_B receptor. For example in a rat model of focal ischemic stroke (permanent middle cerebral artery occlusion), intravenous injections of IRL1620 post occlusion reduced infarct volume, reduced oxidative stress and apoptosis but at the same time enhanced neuro-regeneration *via* neurogenic and angiogenic growth factors (Leonard *et al.* 2011, Leonard *et al.* 2012, Leonard *et al.* 2013). Similarly in a rat model of Alzheimer's disease (amyloid peptide Aβ1-40 administered into the intracerebral vessels), IRL1620 improved learning and memory (Briyal *et al.* 2014). The molecular mechanism is unclear but ECE-1 is thought to degrade amyloid β-peptides. Reduction in ECE activity leads to intracellular accumulation of amyloid β-peptide, linked to neurotoxicity in the early progression of Alzheimer's disease (Eckman *et al.* 2001, Eckman *et al.* 2003, Eckman *et al.* 2006, Pacheco-Quinto and Eckman 2013).

In cancer, animal studies suggested that IRL1620 improved the efficacy of cancer agents such as doxorubicin and 5-fluorouracil by causing vasodilatation thus increasing the amount of drug in tumors and therefore improving efficacy for a given dose (Maguire

and Davenport 2014). Thus far however, these promising results have not been replicated in the clinic. In a multicenter, open-label Phase 2 study of a combination of IRL1620 (called SPI-1620) with docetaxel as second-line advanced biliary tract cancer for patients with advanced biliary tract cancer the pre-specified primary end point of progression free survival of 5 months or longer was not met (Kim *et al.* 2017).

Repurposing the macitentan metabolite, ACT-132577

Macitentan is metabolized to an active metabolite, ACT-132577. Although it has a lower potency than the parent compound, ACT-132577 reaches a higher plasma concentration after 30 h and has a longer half-life (~48 h) than macitentan (Iglarz *et al.* 2008, Sidharta *et al.* 2011). ACT-132577 is being tested in a Phase2 clinical trial for the treatment of hypertension.

Dual AT₁/ET_A receptor antagonist, sparsentan

Preclinical studies have shown that combination of antagonists blocking both angiotensin AT₁ and ET_A receptors caused a greater reduction in blood pressure in animal models of hypertension than blocking AT₁ receptors alone. These results suggested a new therapeutic strategy of combining both actions in one molecule. Sparsentan (BMS-346567) is a first-in-class, orally active, antagonist that combines key AT₁ and ET_A receptor blocking moieties in the same compound. AT₁ and ET_A receptor interactions share a number of structural and functional similarities. It was developed by elegantly merging the structural elements present in an AT₁ receptor antagonist irbesartan with structural elements in a biphenylsulfonamide ET_A receptor antagonist.

Sparsentan has similar high affinity at both receptors: 0.8 nM for AT₁ and 9.3 nM at ET_A. In preclinical studies in rats, the compound reduced blood pressure elevations caused by intravenous infusion of angiotensin II or big ET-1 to a greater extent and with longer duration than AT₁ and ET_A receptor antagonists alone. Using telemetrized spontaneously hypertensive rats, sparsentan was more effective than irbesartan. Sparsentan is being evaluated in Phase 2 trials for the treatment of focal segmental glomerulosclerosis, a cause of nephrotic syndrome in children and a major cause of adult renal failure. The pre-clinical studies suggested that

sparsentan is effectively blocking the direct vascular actions of two potent constrictors peptides but it is not yet clear whether there are also effects on the downstream signaling pathways and on cell proliferation (Murugesan *et al.* 2005). Clinically, a further benefit of sparsentan is that patient compliance may be improved. However, it is not clear whether sparsentan has the same or reduced side effects associated with ET_A antagonists (Komers *et al.* 2017).

Combination therapy with ET_A antagonists and PDE5 inhibitors

ET is continuously released from endothelial cells *via* the constitutive pathway and at low physiological concentrations interacts with endothelial ET_B receptors in an autocrine or paracrine manner to

released vasodilators such as nitric oxide, although prostacyclin or endothelium derive hyperpolarizing factor may also be released depending on the vascular bed. As the concentration increases, ET-1 interacts with mainly ET_A receptors on smooth muscle cells to cause vasoconstriction. In addition, endothelial ET_B activation internalizes the ligand-receptor complex and removes ET-1 from the circulation (Gasic *et al.* 1992, Fukuroda *et al.* 1994, Johnstrom *et al.* 2005). As a result, combined ET_A/ET_B blockade results in a significant rise in circulating ET-1 whereas no increase occurs with selective ET_A antagonism (Plumpton *et al.* 1996). Currently, it is not known what happens to plasma ET levels after suddenly withdrawing bosentan where both receptors are blocked whether this leads to a 'rebound' effect.

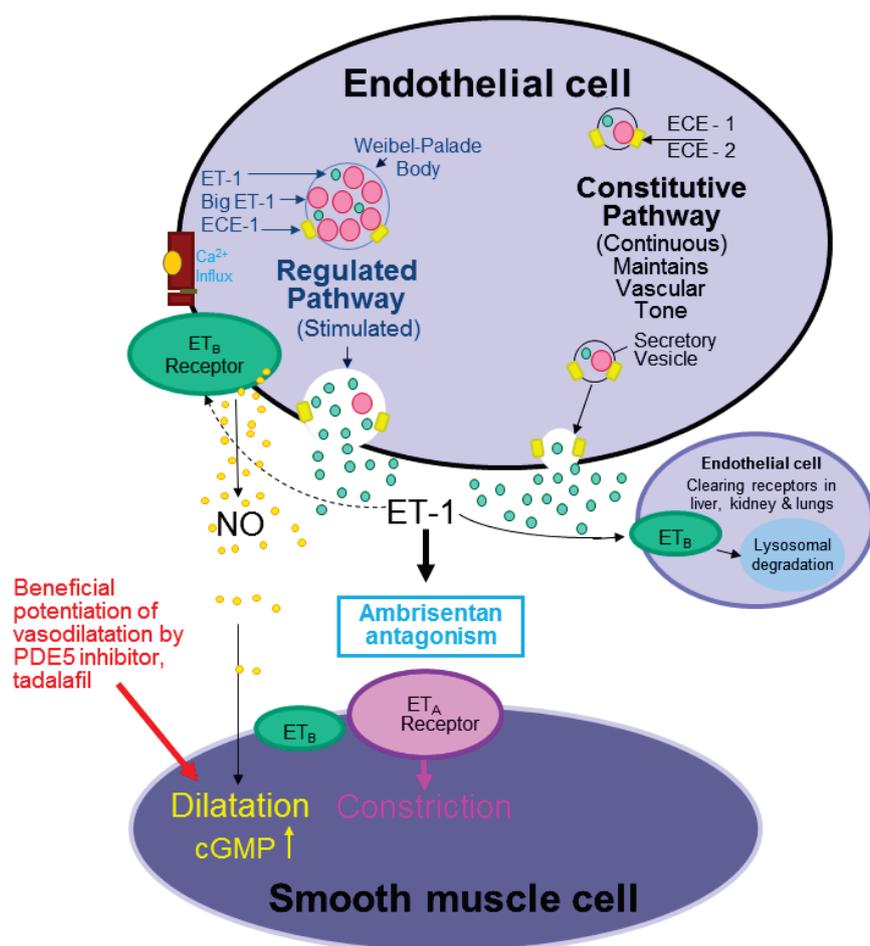


Fig. 4. Schematic diagram of the modulation of the ET-1 pathway in the vasculature of the lungs by combining ambrisentan with tadalafil. ET-1 is synthesized within the secretory vesicles of the constitutive pathway and is continuously released towards ET_A receptors expressed by smooth muscle cells. ET-1 may also be released from the regulated pathway and released in response to external stimuli (Russell *et al.* 1998, Russell *et al.* 1999). ET-1 is increased in PAH and ambrisentan blocks increased signaling at ET_A receptors but spares the beneficial ET_B clearing receptors that are expressed on endothelial cells and remove increased circulating peptide, particularly in the liver, kidney and lungs. It is hypothesized that tadalafil inhibits the breakdown of cGMP in smooth muscle to enhance the action of ET-1 acting in an autocrine/paracrine manner to release endothelium derived vasodilator, nitric oxide (NO) *via* endothelial ET_B activation.

To date monotherapy using ET receptor antagonists or PDE5 inhibitors has been the initial treatment regime for patients with PAH (Kuntz *et al.* 2016). A number of preclinical studies suggested that

combinations of the two compound would be beneficial. For example, Liang *et al.* (2012) found that the ET_A selective antagonist ambrisentan and the PDE5 inhibitor tadalafil acted synergistically to relax ET-1

constricted isolated pulmonary arteries from rats. Crucially, denudation of the endothelium from vessel rings abolished the vasodilator response to tadalafil and the synergistic vasorelaxant effect of tadalafil with ambrisentan. This synergism was not seen with mixed antagonists bosentan and macitentan, suggesting ET_B receptors in the endothelium are necessary to enable a synergistic vasorelaxant effect of the drug combination. The potential molecular mechanism is shown in Figure 4. These results have translated into the clinic. A major study enrolled 500 newly diagnosed, treatment-naive patients with 253 assigned to the combination therapy and 126 and 121, respectively, to the ambrisentan and tadalafil monotherapy groups (Galie *et al.* 2015, Hooper *et al.* 2016). Combination therapy resulted in a significantly lower risk of clinical-failure events (including hospitalization, disease progression and mortality) than the risk with ambrisentan or tadalafil. As might be expected, unwanted side-effect (headache and nasal congestion) thought to be associated with activation of ET_B receptors occurred more frequently in combination-therapy. A retrospective analysis in a small number of patients supported these finding (Mercurio *et al.* 2018).

Pepducins (cell penetrating peptides) directed against the ET_B receptor

Pepducins are synthetic peptides usually based on amino acid sequences of the three intracellular loops or the intracellular C-terminal tail of the GPCR (Carr and Benovic 2016, Tressel *et al.* 2011). The N-terminal is lipidated to aid transversing the cell membrane and anchor the peptide. Unlike small molecule ligands that tend to interact with extracellular loops and N-terminus that form the binding pocket, pepducins stabilize the target receptor in conformations that may stimulate or inhibit intracellular signaling. The main advantages of pepducins as drugs are likely to be similar to other ET peptide ligands of high specificity and selectivity but also the same disadvantages of not having oral bioavailability and potentially causing an immune response.

To date, pepducins have been used as tool compounds functioning as agonist or antagonists against a number of GPCRs but recently the first human clinical studies have been carried out using a pepducin targeting protease-activated receptor-1 and was found to have the anticipated antiplatelet action with no reported adverse

action (Gurbel *et al.* 2016).

A cell penetrating peptide has been identified, IC2B, that was generated as the name suggest to the amino acid sequences of the ET_B second intracellular loop. In a rat model of hypoxia induced pulmonary hypertension, IC2B reduced pulmonary arterial pressure, right ventricular hypertrophy and pulmonary vascular remodeling without an effect on blood pressure, demonstrating efficacy *in vivo*. Interestingly, there was no evidence of hepatic toxicity that is a feature of the mixed antagonist bosentan. In acute studies, after 24 h of hypoxia, pulmonary arteries treatment with IC2B resulted in marked reductions in Akt and ERK activation, key kinases in vascular hypertrophy that are upregulated in this model. Immunofluorescent studies showed that IC2B mainly penetrated cells in the vascular media (where this sub-type, at least in rats, may mediate cell proliferation), implying little or no interaction with the endothelium but the explanation for this selectivity is not yet known. The authors suggested a mechanism of action whereby selective blockade of smooth muscle ET_B receptor mediated vasoconstriction and proliferation *via* Akt and ERK signaling that contribute to disease progression, leaving endothelial ET_B mediated vasodilatation, for example, unaffected. This speculation has not been confirmed and further studies have not been reported (Green *et al.* 2013). However, the study provides some preliminary evidence for the efficacy of pepducins to modulate the action of ET_B receptors that appears effective *in vivo*.

X-ray structures of endothelin ET_B receptor and allosteric modulators

To date, less than 20 structures of Family A GPCRs have been solved experimentally and for small peptide ligands these are limited to receptors activated by shorter peptides, for example the thirteen amino acid peptide neurotensin. For the ET_B receptor, the authors reported the structure in both ligand-free form and in complex with ET-1 (Shihoya *et al.* 2016). Interestingly, the larger twenty one amino acid peptide demonstrates interaction over a substantial portion of the molecule. A thermostabilized receptor was used containing five mutations in order to achieve crystallization. Importantly, the receptor still retained binding affinities for [¹²⁵I]-ET-1 measured by saturation binding, comparable to values obtained in a range of native human receptors (Davenport 2002). Native ET_B receptors display the same

affinity for ET-1 and ET-3. However, the authors found that the combination of these mutations resulted in an order of magnitude decrease in affinity for ET-3 (in a competitive binding assay with [125 I]ET-1), but retained high affinity for ET-1. One of the mutations was Lys¹⁸², that had previously been shown by Lee *et al.* (1994) to not alter ET-1 binding, but unexpectedly affinities for ET-2, ET-3 and the ET_B agonist sarafotoxin 6C were between one and three orders of magnitude lower than their corresponding wild-type values. These results suggest this residue is particularly important for ET-1 binding over the other isoforms. The crystal structure supports the hypothesis that tight associations of ETs with ET_B receptors are mainly mediated through the α -helical and C-terminal regions, whereas the N-terminal region which varies by two (ET-2) and four amino acids (ET-3) modulates selectivity. The N-terminal region stabilizes the overall peptide architecture and thereby facilitates interactions with the receptor, the stabilizing effect of the N-terminal region is weaker in ET-3 owing to the bulky residues.

Over 20 amino acids in the ET_B receptor were identified and proposed to interact with specific residues of ET-1. All of the residues except for one, K²⁷⁰, are conserved in both ET_A and ET_B receptors. This suggests that mutations of these residues can alter ET-1 and ET-3 binding to different degrees and the slightly different binding properties of the residues within the peptide ligands results in the lower affinity for the ET-3 at the thermostabilized receptor compared with the wild type. The authors proposed a model whereby transmembrane helices one, two, six and seven move and envelop the entire endothelin peptide, to form a lid-like architecture that covers the orthosteric pocket, predicted to form a very stable complex. This provides one structural explanation for the unusual property of ET-1 in causing long lasting responses and with a slow dissociation rate from the receptor (Shihoya *et al.* 2016).

Shihoya *et al.* (2017) extended these studies reporting the crystal structure of human ET_B receptor bound to bosentan, the first ET antagonist to be approved for clinical use although it has a comparatively low affinity for native human receptors (Maguire *et al.* 2012). A high affinity ET_B-selective antagonist K-8794 (Sawaki *et al.* 2000) was also co-crystallized. Bosentan is similar to K-8794 except for an ethylene glycol at the 6-position of the central pyrimidine template. Both compounds superimposed well to the receptor structure and the deduced binding was similar. The results were intriguing.

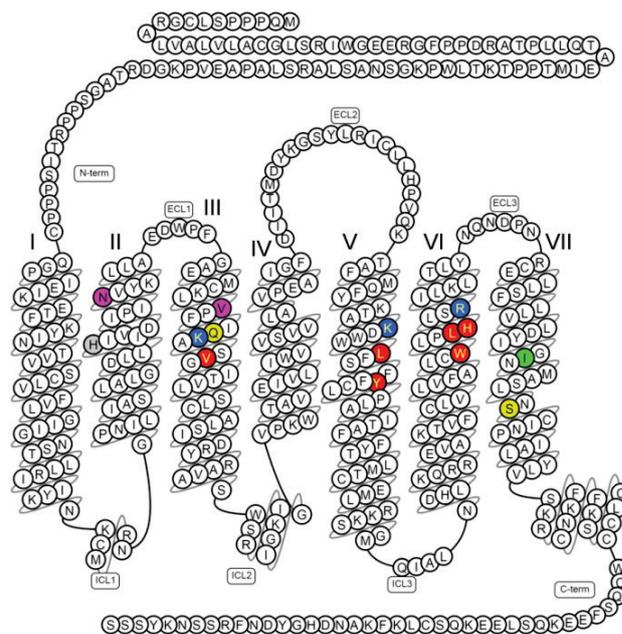


Fig. 5. Amino acids predicted from the crystal structure of the ET_B receptor to interact with the ET_B antagonist K-8794. Roman numerals indicate the transmembrane spanning domains. Ionic interaction of the sulfonamide of K-8794 with amino acids are shown in blue; the 4-t-butyl phenyl group linked to the sulfonamide forms hydrophobic contacts shown in turquoise; the methoxyphenoxy group of K-8794 fits within the hydrophobic pocket formed by amino acids in red; the pyrimidine at the 2-position of the central pyrimidine forms a hydrophobic contact with Ile³⁷² (green). The dimethylphenyl group makes van der Waals interactions with the side chains of Asn¹⁵⁸ and Val¹⁷⁷ (purple). The carbonyl oxygen of the amide bond forms a hydrogen bond with His¹⁵⁰ (grey) and water-mediated hydrogen bonds with Gln¹⁸¹ and Ser³⁷⁹ (yellow) (Pándy-Szekeres *et al.* 2018).

K-8794 interactions were observed between its four substituents and seventeen amino acids in the receptor (Fig. 5). Seven were amino acids associated with mutations: His¹⁵⁰ (TM2), Gln¹⁸¹, Val¹⁸⁵ (TM3), Leu²⁷⁷ (TM5), Trp³³⁶, Leu³³⁹, Arg³⁴³ (TM6).

Bosentan occupied the bottom of the orthosteric site of the ET_B receptor; although bosentan was discovered by screening rather than by rational design based on peptide structure, this corresponded to the binding site for the C-terminal tripeptide of ET-1 (Ile¹⁹, Ile²⁰, and Trp²¹). This suggests ionic and hydrophobic interactions between bosentan and C-terminal tripeptides of ET-1 within receptor are similar and are critical for both agonist and antagonist binding. The authors proposed that despite this similarity bosentan sterically prevents the inward movement of transmembrane helix six, blocking the action of ET-1. Since bosentan displays virtually the same affinity for the ET_A receptor, it is likely this mechanism of action is also conserved in this sub-type. In contrast to ET-1, where

binding induces a lid-like architecture that covers the orthosteric pocket to reduce dissociation, the bosentan binding site is open toward the extracellular solvent in agreement with the fast dissociation kinetics. The clinical use of bosentan, a surmountable reversible antagonist, is being superseded by macitentan, designed to have improved efficacy and optimized for potency and selectivity in structure-activity studies. Macitentan is an insurmountable antagonist and an order of magnitude more potent than bosentan as a result of longer receptor occupancy (seventeen minutes versus seventy seconds for bosentan measured by *in vitro* assays) (Iglarz *et al.* 2008, Sidharta *et al.* 2014, Clozel 2016). It is interesting to speculate whether the slower dissociation of macitentan is the result of better mimicry of the C-terminal tripeptide of ET-1 inducing the lid-like structure of the native peptide. The X-ray structure should foster a better understanding of structure activity with macitentan and the allosteric modulators (Talbot *et al.* 2000).

Why have no ET_A selective agonists been discovered?

The final missing reagent in the pharmacologist's armamentarium is an ET_A selective agonist. The conundrum between the relative merits of blocking only ET_A versus blocking both subtypes continues but it has been generally accepted that most of the effects of ET_A activation in pathophysiological conditions to cause, for example, vasoconstriction are deleterious. Therefore there has been no compelling evidence for activating the ET-1/ET_A pathway. However, the identification of the SNP associated with vascular diseases where higher levels of ET-1 may be beneficial such as migraine headache may provide the stimulus to search for tool compounds.

Biased signaling in the ET pathway

Biased agonism (also known as biased signaling, ligand bias) is an emerging pharmacological paradigm with the potential to dramatically increase the therapeutic targeting of ET receptors. In addition to activating the G-protein pathway, ligands such as ET-1 activate the β -arrestin pathway which can lead to desensitization, receptor internalization and 'silencing' of the pathway. In other GPCR families it is clear that ligands can be designed to selectively activate the G-protein pathway over β -arrestin or vice versa.

In the CNS, TRV130, an agonist biased toward the G-protein pathway at the μ -opioid receptor was found to display desirable analgesic actions but fewer of the unwanted side effects in the gastrointestinal tract and respiratory suppression compared to morphine (Singla *et al.* 2017). In the periphery, apelin receptor agonists biased to the G-protein pathway produce the desirable vasodilatation and inotropic action with expected reduced desensitization in volunteers and animal models (Brame *et al.* 2015, Reed *et al.* 2016). Apelin receptor ligands are downregulated in human pulmonary arterial hypertension and animal models (Yang *et al.* 2017a). Exogenous application of biased ligands to replace the missing peptides attenuates the development of the disease (Yang *et al.* 2017b). Conversely, the β -agonist carvedilol has been proposed as a β -arrestin biased ligand, beneficially and selectively activating this pathway compared to a balanced compound (Wang *et al.* 2017), although it should be noted there may also be a significant contribution from α 1-adrenoreceptor antagonism. While both ET receptors are internalized by β -arrestin and dynamin/clathrin-dependent mechanisms, ET_A are recycled to the plasma membrane for further signaling while ET_B are targeted to lysosomes and degraded (Bremnes *et al.* 2000). Interestingly, increasing evidence shows that ET receptor/ β -arrestin signaling is dysregulated in ovarian cancer (Rosanò and Bagnato 2016a,b, Rosanò *et al.* 2014, Maguire and Davenport 2015) β -arrestin is required for ET-1-induced NF- κ B activation (Cianfrocca *et al.* 2014). These studies suggest that the development of antagonists selectively blocking the ET-1/ β -arrestin pathway could be a new strategy. Although bosentan was the first mixed antagonist to enter the clinic to treat PAH, it displays little selectivity for ET_A over ET_B receptors in radioligand binding (Maguire and Davenport 2014) and G-protein functional assays and has surprisingly low potency in vasoconstrictor assays ($K_B=1-3 \mu\text{M}$, Maguire *et al.* 2012). However, it was found to be a highly effective inhibitor of β -arrestin recruitment mediated by ET_A compared to the ET_B receptor (Maguire *et al.* 2012, Maguire 2016). Since some of the deleterious action of ET-1, at least in ovarian cancer, may be mediated by the β -arrestin pathway, similar biased antagonists may block the cancerous action of ET-1 but preserve beneficial G-protein mediated actions.

Prospects for personalized precision medicine in endothelin

The goal of personalized medicine is to customize drug treatment for an individual patient. This can be achieved by linking variation in the patients genetics, such as a single nucleotide polymorphism (SNP, where a DNA sequence variation occurring when a single nucleotide in the genome differs between individuals) with a drug target such as a transmitter system suspected of causing the disease. Ideally such SNPs will occur in a large population which have very high genome wide association with a specific disease. These SNPs are common and replicated in a number of populations of different ethnicities. Recently Gupta *et al.* (2017) have identified rs9349379, a common A/G SNP in the third intron of the Phosphatase And Actin Regulator 1 (PHACTR1) gene, that has a key role in tubule formation and in endothelial cell survival but also regulates expression of the gene encoding ET-1, EDN1, located

600 kb upstream. The authors found rs9349379 had the strongest association with coronary artery disease and coronary calcification for a single variant, and this far exceeded the association for the next most highly associated SNP, rs78145402. A high genome wide significant correlation with linked vascular conditions ischemic or coronary artery disease leading to atherosclerosis and angina (causing chest pain as a result of constricting coronary arteries) was also found in UK Biobank (~400,000 individuals, Fig. 6, Sudlow *et al.* 2015).

Targeted deletion of a small region of the apparent regulatory DNA at rs9349379 element increased ET-1 gene and protein expression in endothelial cells and to a lesser extent in vascular smooth muscle cells. Gupta *et al.* (2017) tested the hypothesis that big ET-1 levels would be higher in individuals with the minor allele (G/G), intermediate levels in those with A/G and lowest level in those with A/A at rs9349379. In thirty three

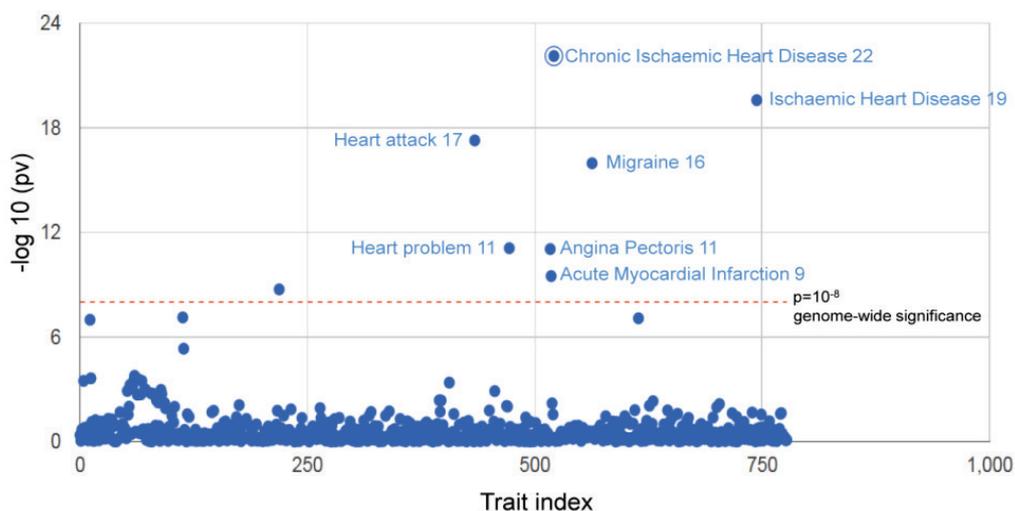


Fig. 6. Graph showing a high genome-wide significant correlation ($P < 10^{-8}$, indicated by dashed line) between the SNP rs9349379 and vascular conditions including chronic and ischemic heart disease and heart attack. Numbers adjacent to the trait indicate the significance. Vertical axis shows $-\log_{10}$ probability value (pv) versus the trait index. Data are from UK Biobank (Sudlow *et al.* 2015).

healthy individuals there was significant association between G genotype with, as predicted, plasma levels 20 % higher. Importantly this minor G/G allele is common, with a 38 % frequency in the 1000 Genomes Project Consortium database (Auton *et al.* 2015). The genome studies suggest individuals with this minor allele SNP G/G would be predicted to have a lifetime exposure to significantly elevated levels of ET-1 and implying that in coronary artery disease leading to atherosclerosis and calcification, the ET signaling pathway would be

upregulated. New imaging techniques (Irvine *et al.* 2015) that identify active unstable atherosclerosis using positron emission tomography provide the mechanism for testing the efficacy of ET receptor antagonism in patients with the genotype for the G/G allele compared with A/A.

In patients with atherosclerosis, a number of studies show a consistent pattern of increased plasma levels of ET (Davenport and Maguire 2006). Tissue levels of ET-1 mRNA (Winkles *et al.* 1993) as well as both the mature peptide and big ET-1 were significantly

increased within the wall of human vessels containing atherosclerotic lesions (raised plaque, Fig. 7A, Bacon *et al.* 1996). Gupta *et al.* (2017) measured elevated levels of big ET-1 rather than the mature peptide. About one in four big ET-1 molecules synthesized within endothelial cells escapes conversion within the endothelium and is released to circulate in the plasma. In humans, infusion of big ET-1 into volunteers causes pronounced vasoconstriction by local conversion to ET-1, by a phosphoramidon sensitive ECE localized to the smooth muscle (Plumpton *et al.* 1996). In human coronary arteries with atherosclerotic lesion target receptors expressed by contractile smooth muscle remain unaltered although proliferating smooth muscle cells within the lesion display few receptors (Fig. 7B). In diseased coronary arteries denuded of endothelium, smooth muscle ECE activity is upregulated, increasing the amount of ET-1 synthesized from big ET-1 at the site of the lesion (Fig. 7C). In agreement with the increased contractile response to big ET-1, there was a corresponding increase in mature ET formed in the bathing medium (Fig. 7D, compared with non-diseased arteries) (Maguire and

Davenport 1998). Animal models can provide insight into the long-term exposure to ET-1. In mice, endothelial ET-1 overexpression (by an order of magnitude) caused sustained blood pressure elevation and vascular and renal injury *via* ET_A receptors (Coelho *et al.* 2018).

The study by Gupta *et al.* (2017) combined with the results described above showing ET-1 is a powerful, long lasting constrictor of human coronary arteries through the ET_A receptor, contributing to vasospasm and angina in patients with coronary artery disease support the suggestion that those with the G/G allele would have higher ET-1 levels and therefore enhanced vasoconstriction. Patients with this G/G allele are predicted to be more responsive to ET_A antagonism than those with A/A or A/G. Conversely, Gupta *et al.* (2017) identified four vascular disease where the minor allele G/G and elevated big ET-1 is alternatively associated with a reduced risk of cervical dissection, migraine headache, fibromuscular dysplasia (three diseases that co-occur epidemiologically), and hypertension and in which a lifetime of elevated ET-1 levels may be beneficial.

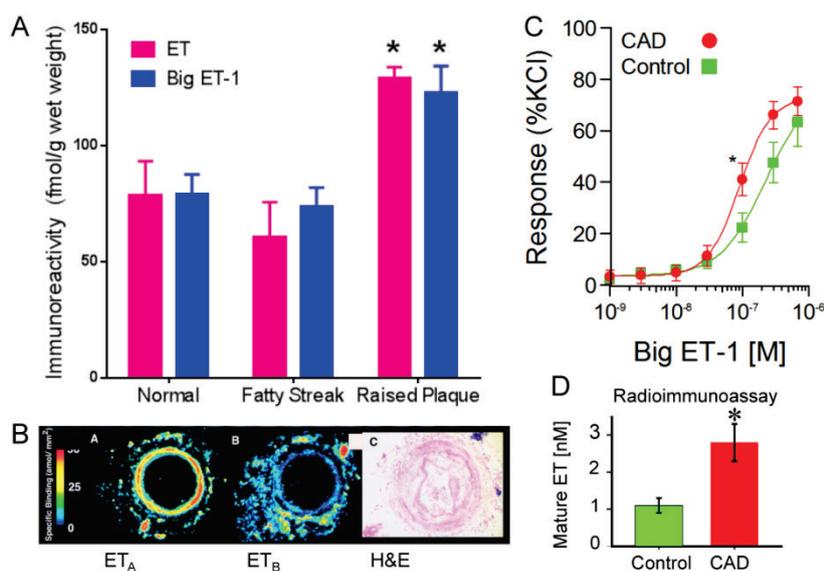


Fig. 7. Upregulation of the ET-1 pathway in human atherosclerosis. (A) Tissue levels of both ET-1 and big ET-1 were increased in coronary arteries with developed atherosclerosis (raised plaque) compared with healthy vessels or those with fatty streaks (early atherosclerosis). (B) Target receptors expressed by contractile smooth muscle remain unaltered although proliferating smooth muscle cells within the lesion display few receptors. (C) In human coronary arteries with atherosclerotic lesion denuded of endothelium, smooth muscle ECE activity was upregulated resulting in increased responses to big ET-1 with (D) a corresponding increase in mature ET formed in the bathing medium compared with non-diseased arteries. Data from Maguire *et al.* (1997), Bacon *et al.* (1996).

The causes of migraine headache continues to be debated as it is a complex, multisystem disorder, and changes in vascular function, particularly unwanted vasodilatation, as a cause has been challenged (Brennan and Charles 2017). However, reduced levels of ET-1 to oppose vasodilatation is a plausible hypothesis to test in patients with the G/G allele. Pial arteries, the targets for meningeal vasodilatation causing headache, for example, are exquisitely sensitive to ET-1 (Pierre and Davenport 1999). A link to ET-1 and the pathophysiology of

cervical dissection or fibromuscular dysplasia has not been established but this new direction for research should be explored. The association of the G/G allele with lower systolic blood pressure is counter intuitive but in agreement a negative correlation between systolic blood pressure and plasma ET levels has previously been reported in patients with hypertension (Davenport *et al.* 1990). This is consistent with ET-1 acting in an autocrine/paracrine manner on endothelial cell ET_B receptors to release vasodilators to reduce constrictor

ET_A responses (Fig. 4) and is a possible mechanism for synergy between ET_A selective ligands and PDE5 inhibitors.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by the Wellcome Trust 107715/Z/15/Z, Medical Research Council MC_PC_14116 and in part, by the National Institute for

Health Research Cambridge Biomedical Research Center and the Pulmonary Hypertension Association UK.

Abbreviations

ECE, endothelin converting enzyme; ERKs, extracellular signal-regulated kinases; ET-1, endothelin-1; ET-2, endothelin-2; ET-3, endothelin-3; MABs, monoclonal antibodies; MAPK, MAP-kinase; PAH, pulmonary arterial hypertension; PDE, phosphodiesterase; SNP, single nucleotide polymorphism.

References

- ALLARD B, WIJKHUISEN A, BORRULL A, DESHAYES F, PRIAM F, LAMOURETTE P, DUCANCEL F, BOQUET D, COURAUD JY: Generation and characterization of rendomab-B1, a monoclonal antibody displaying potent and specific antagonism of the human endothelin B receptor. *MAbs* **5**: 56-69, 2013.
- ASUNDI J, REED C, ARCA J, McCUTCHEON K, FERRANDO R, CLARK S, LUIS E, TIEN J, FIRESTEIN R, POLAKIS P: An antibody-drug conjugate targeting the endothelin B receptor for the treatment of melanoma. *Clin Cancer Res* **17**: 965-975, 2011.
- AUTON A, BROOKS LD, DURBIN RM, GARRISON EP, KANG HM, KORBEL JO, MARCHINI JL, MCCARTHY S, McVEAN GA, ABECASIS GR: A global reference for human genetic variation. *Nature* **526**: 68-74, 2015.
- BACON CR, CARY NR, DAVENPORT AP: Endothelin peptide and receptors in human atherosclerotic coronary artery and aorta. *Circ Res* **79**: 794-801, 1996.
- BAGNATO A, ROSANÒ L, SPINELLA F, Di CASTRO V, TECCE R, NATALI PG: Endothelin B receptor blockade inhibits dynamics of cell interactions and communications in melanoma cell progression. *Cancer Res* **64**: 1436-1443, 2004.
- BITTNER M, MELTZER P, CHEN Y, JIANG Y, SEFTOR E, HENDRIX M, RADMACHER M, SIMON R, YAKHINI Z, BEN-DOR A, SAMPAS N, DOUGHERTY E, WANG E, MARINCOLA F, GOODEN C, LUEDERS J, GLATFELTER A, POLLOCK P, CARPTEN J, GILLANDERS E, LEJA D, DIETRICH K, BEAUDRY C, BERENS M, ALBERTS D, SONDAK V: Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* **406**: 536-540, 2000.
- BLANDIN V, VIGNE P, BREITTMAYER JP, FRELIN C: Allosteric inhibition of endothelin ETA receptors by 3, 5-dibromosalicylic acid. *Mol Pharmacol* **58**: 1461-1469, 2000.
- BORRULL A, ALLARD B, WIJKHUISEN A, HERBET A, LAMOURETTE P, BIROUK W, LEIBER D, TANFIN Z, DUCANCEL F, BOQUET D, COURAUD JY, ROBIN P: Rendomab B4, a monoclonal antibody that discriminates the human endothelin B receptor of melanoma cells and inhibits their migration. *MAbs* **8**: 1371-1385, 2016.
- BROME AL, MAGUIRE JJ, YANG P, DYSON A, TORELLA R, CHERIYAN J, SINGER M, GLEN RC, WILKINSON IB, DAVENPORT AP: Design, characterization, and first-in-human study of the vascular actions of a novel biased apelin receptor agonist. *Hypertension* **65**: 834-840, 2015.
- BREMNES T, PAASCHE JD, MEHLUM A, SANDBERG C, BREMNES B, ATTRAMADAL H: Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem* **275**: 17596-17604, 2000.
- BRENNAN KC, CHARLES A: An update on the blood vessel in migraine. *Curr Opin Neurol* **23**: 266-274, 2010.
- BRIYAL S, SHEPARD C, GULATI A: Endothelin receptor type B agonist, IRL-1620, prevents beta amyloid (A β) induced oxidative stress and cognitive impairment in normal and diabetic rats. *Pharmacol Biochem Behav* **120**: 65-72, 2014.

- BRIYAL S, NGUYEN C, LEONARD M, GULATI A: Stimulation of endothelin B receptors by IRL-1620 decreases the progression of Alzheimer's disease. *Neuroscience* **301**: 1-11, 2015.
- CARR R 3RD, BENOVIC JL: From biased signalling to polypharmacology: unlocking unique intracellular signalling using pepducins. *Biochem Soc Trans* **44**: 555-561, 2016.
- CIANFROCCA R, TOCCI P, SEMPRUCCI E, SPINELLA F, DI CASTRO V, BAGNATO A, ROSANÒ L: beta-arrestin 1 is required for endothelin-1-induced NF-kappaB activation in ovarian cancer cells. *Life Sci* **118**: 179-184, 2014.
- CLOZEL M: Endothelin research and the discovery of macitentan for the treatment of pulmonary arterial hypertension. *Am J Physiol Regul Integr Comp Physiol* **311**: R721-R726, 2016.
- COELHO SC, BERILLO O, CAILLON A, OUERD S, FRAULOB-AQUINO JC, BARHOUMI T, OFFERMANN S, PARADIS P, SCHIFFRIN EL: Three-month endothelial human endothelin-1 overexpression causes blood pressure elevation and vascular and kidney injury. *Hypertension* **71**: 208-216, 2018.
- CZOPEK A, MOORHOUSE R, WEBB DJ, DHAUN N: Therapeutic potential of endothelin receptor antagonism in kidney disease. *Am J Physiol Regul Integr Comp Physiol* **310**: R388-R397, 2016.
- DAVENPORT AP: International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. *Pharmacol Rev* **54**: 219-226, 2002.
- DAVENPORT AP, MAGUIRE JJ: Endothelin. *Handb Exp Pharmacol* **176**: 295-329, 2006.
- DAVENPORT AP, ASHBY MJ, EASTON P, ELLA S, BEDFORD J, DICKERSON C, NUNEZ DJ, CAPPER SJ, BROWN MJ: A sensitive radioimmunoassay measuring endothelin-like immunoreactivity in human plasma: comparison of levels in patients with essential hypertension and normotensive control subjects. *Clin Sci (Lond)* **78**: 261-264, 1990.
- DAVENPORT AP, HYNDMAN KA, DHAUN N, SOUTHAN C, KOHAN DE, POLLOCK JS, POLLOCK DM, WEBB DJ, MAGUIRE JJ: Endothelin. *Pharmacol Rev* **68**: 357-418, 2016.
- ECKMAN EA, REED DK, ECKMAN CB: Degradation of the Alzheimer's amyloid beta peptide by endothelin-converting enzyme. *J Biol Chem* **276**: 24540-24548, 2001.
- ECKMAN EA, WATSON M, MARLOW L, SAMBAMURTI K, ECKMAN CB: Alzheimer's disease beta-amyloid peptide is increased in mice deficient in endothelin-converting enzyme. *J Biol Chem* **278**: 2081-2084, 2003.
- ECKMAN EA, ADAMS SK, TROENDLE FJ, STODOLA BA, KAHN MA, FAUQ AH, XIAO HD, BERNSTEIN KE, ECKMAN CB: Regulation of steady-state beta-amyloid levels in the brain by neprilysin and endothelin-converting enzyme but not angiotensin-converting enzyme. *J Biol Chem* **281**: 30471-30478, 2006.
- FUKURODA T, FUJIKAWA T, OZAKI S, ISHIKAWA K, YANO M, NISHIKIBE M: Clearance of circulating endothelin-1 by ETB receptors in rats. *Biochem Biophys Res Commun* **199**: 1461-1465, 1994.
- GALIE N, BARBERA JA, FROST AE, GHOFRANI HA, HOEPER MM, McLAUGHLIN VV, PEACOCK AJ, SIMONNEAU G, VACHIERY JL, GRUNIG E, OUDIZ RJ, VONK-NOORDEGRAAF A, WHITE RJ, BLAIR C, GILLIES H, MILLER KL, HARRIS JH, LANGLEY J, RUBIN LJ: Initial use of ambrisentan plus tadalafil in pulmonary arterial hypertension. *N Engl J Med* **373**: 834-844, 2015.
- GASIC S, WAGNER OF, VIERHAPPER H, NOWOTNY P, WALDHAUSL W: Regional hemodynamic effects and clearance of endothelin-1 in humans: renal and peripheral tissues may contribute to the overall disposal of the peptide. *J Cardiovasc Pharmacol* **19**: 176-180, 1992.
- GREEN DS, RUPASINGHE C, WARBURTON R, WILSON JL, SALLUM CO, TAYLOR L, YATAWARA A, MIERKE D, POLGAR P, HILL N: A cell permeable peptide targeting the intracellular loop 2 of endothelin B receptor reduces pulmonary hypertension in a hypoxic rat model. *PLoS One* **8**: e81309, 2013.
- GULATI A: Endothelin receptors, mitochondria and neurogenesis in cerebral ischemia. *Curr Neuropharmacol* **14**: 619-626, 2016.
- GULATI S, BRIYAL S, JONES S, BHALLA S, GULATI A: Attenuation of opioid tolerance by ETB receptor agonist, IRL-1620, is independent of an accompanied decrease in nerve growth factor in mice. *Heliyon* **3**: e00317, 2017.

- GUPTA RM, HADAYA J, TREHAN A, ZEKAVAT SM, ROSELLI C, KLARIN D, EMDIN CA, HILVERING CRE, BIANCHI V, MUELLER C, KHERA AV, RYAN RJH, ENGREITZ JM, ISSNER R, SHORESH N, EPSTEIN CB, DE LAAT W, BROWN JD, SCHNABEL RB, BERNSTEIN BE, KATHIRESAN S: A genetic variant associated with five vascular diseases is a distal regulator of endothelin-1 gene expression. *Cell* **170**: 522-533.e515, 2017.
- GURBEL PA, BLIDEN KP, TURNER SE, TANTRY US, GESHEFF MG, BARR TP, COVIC L, KULIOPULOS A: Cell-penetrating pepducin therapy targeting PAR1 in subjects with coronary artery disease. *Arterioscler Thromb Vasc Biol* **36**: 189-197, 2016.
- HARLAND SP, KUC RE, PICKARD JD, DAVENPORT AP: Expression of endothelin(A) receptors in human gliomas and meningiomas, with high affinity for the selective antagonist PD156707. *Neurosurgery* **43**: 890-898, 1998.
- HERBET A, COSTA N, LEVENTOUX N, MABONDZO1 A, BORRULL A, HUGNOT J-P, BOQUET D: Antibodies targeting human endothelin-1 receptors reveal different conformational states in cancer cells. *Physiol Res* **67** (Suppl 1): S257-S264, 2018.
- HOEPER MM, McLAUGHLIN VV, BARBERA JA, FROST AE, GHOFrani HA, PEACOCK AJ, SIMONNEAU G, ROSENKRANZ S, OUDIZ RJ, WHITE RJ, MILLER KL, LANGLEY J, HARRIS JHN, BLAIR C, RUBIN LJ, VACHIERY JL: Initial combination therapy with ambrisentan and tadalafil and mortality in patients with pulmonary arterial hypertension: a secondary analysis of the results from the randomised, controlled AMBITION study. *Lancet Respir Med* **4**: 894-901, 2016.
- HOUDE M, DESBIENS L, D'ORLEANS-JUSTE P: Endothelin-1: biosynthesis, signaling and vasoreactivity. *Adv Pharmacol* **77**: 143-175, 2016.
- HUTCHINGS CJ, KOGLIN M, OLSON WC, MARSHALL FH: Opportunities for therapeutic antibodies directed at G-protein-coupled receptors. *Nat Rev Drug Discov* **16**: 787-810, 2017.
- IGLARZ M, BINKERT C, MORRISON K, FISCHLI W, GATFIELD J, TREIBER A, WELLER T, BOLLI MH, BOSS C, BUCHMANN S, CAPELETO B, HESS P, QIU C, CLOZEL M: Pharmacology of macitentan, an orally active tissue-targeting dual endothelin receptor antagonist. *J Pharmacol Exp Ther* **327**: 736-745, 2008.
- IRKLE A, VESEY AT, LEWIS DY, SKEPPER JN, BIRD JL, DWECK MR, JOSHI FR, GALLAGHER FA, WARBURTON EA, BENNETT MR, BRINDLE KM, NEWBY DE, RUDD JH, DAVENPORT AP: Identifying active vascular microcalcification by ¹⁸F-sodium fluoride positron emission tomography. *Nat Commun* **6**: 7495, 2015.
- JOHNSTROM P, FRYER TD, RICHARDS HK, HARRIS NG, BARRET O, CLARK JC, PICKARD JD, DAVENPORT AP: Positron emission tomography using ¹⁸F-labelled endothelin-1 reveals prevention of binding to cardiac receptors owing to tissue-specific clearance by ET_B receptors in vivo. *Br J Pharmacol* **144**: 115-122, 2005.
- JOSHI MD, OESTERLING BM, WU C, GWIZDZ N, PAIS G, BRIYAL S, GULATI A: Evaluation of liposomal nanocarriers loaded with ETB receptor agonist, IRL-1620, using cell-based assays. *Neuroscience* **312**: 141-152, 2016.
- KIM R, CHIOREAN EG, AMIN M, ROCHA-LIMA CMS, GANDHI J, HARRIS WP, SONG T, PORTNOY D: Phase 2 study of combination SPI-1620 with docetaxel as second-line advanced biliary tract cancer treatment. *Br J Cancer* **117**: 189-194, 2017.
- KOMERS R, GIPSON DS, NELSON P, ADLER S, SRIVASTAVA T, DEREBAIL VK, MEYERS KE, PERGOLA P, MACNALLY ME, HUNT JL, SHIH A, TRACHTMAN H: Efficacy and safety of sparsentan compared with irbesartan in patients with primary focal segmental glomerulosclerosis: randomized, controlled trial design (DUET). *Kidney Int Rep* **2**: 654-664, 2017.
- KUC RE, DAVENPORT AP: Comparison of ET_A and ET_B receptor distribution visualised by radioligand binding versus immunocytochemical localisation using sub-type selective antisera. *J Cardiovasc Pharmacol* **44** (Suppl 1): S224-S226, 2004.
- KUNTZ M, LEIVA-JUAREZ MM, LUTHRA S: Systematic review of randomized controlled trials of endothelin receptor antagonists for pulmonary arterial hypertension. *Lung* **194**: 723-732, 2016.

- LAHAV R: Endothelin receptor B is required for the expansion of melanocyte precursors and malignant melanoma. *Int J Dev Biol* **49**: 173-180, 2005.
- LEE JA, ELLIOTT JD, SUTIPHONG JA, FRIESEN WJ, OHLSTEIN EH, STADEL JM, GLEASON JG, PEISHOFF CE: Tyr-129 is important to the peptide ligand affinity and selectivity of human endothelin type A receptor. *Proc Natl Acad Sci U S A* **91**: 7164-7168, 1994.
- LEONARD MG, GULATI A: Endothelin B receptor agonist, IRL-1620, enhances angiogenesis and neurogenesis following cerebral ischemia in rats. *Brain Res* **1528**: 28-41, 2013.
- LEONARD MG, BRIYAL S, GULATI A: Endothelin B receptor agonist, IRL-1620, reduces neurological damage following permanent middle cerebral artery occlusion in rats. *Brain Res* **1420**: 48-58, 2011.
- LEONARD MG, BRIYAL S, GULATI A: Endothelin B receptor agonist, IRL-1620, provides long-term neuroprotection in cerebral ischemia in rats. *Brain Res* **1464**: 14-23, 2012.
- LIANG F, YANG S, YAO L, BELARDINELLI L, SHRYOCK J: Ambrisentan and tadalafil synergistically relax endothelin-induced contraction of rat pulmonary arteries. *Hypertension* **59**: 705-711, 2012.
- MAGUIRE JJ: Evidence for biased agonists and antagonists at the endothelin receptors. *Life Sci* **159**: 30-33, 2016.
- MAGUIRE JJ, DAVENPORT AP: Increased response to big endothelin-1 in atherosclerotic human coronary artery: functional evidence for up-regulation of endothelin-converting enzyme activity in disease. *Br J Pharmacol* **125**: 238-240, 1998.
- MAGUIRE JJ, DAVENPORT AP: Endothelin@25 - new agonists, antagonists, inhibitors and emerging research frontiers: IUPHAR Review 12. *Br J Pharmacol* **171**: 5555-5572, 2014.
- MAGUIRE JJ, KUC RE, ROUS BA, DAVENPORT AP: Failure of BQ123, a more potent antagonist of sarafotoxin 6b than of endothelin-1, to distinguish between these agonists in binding experiments. *Br J Pharmacol* **118**: 335-342, 1996.
- MAGUIRE JJ, KUC RE, DAVENPORT AP: Defining the affinity and receptor sub-type selectivity of four classes of endothelin antagonists in clinically relevant human cardiovascular tissues. *Life Sci* **91**: 681-686, 2012.
- MERCURIO V, MUKHERJEE M, TEDFORD RJ, ZAMANIAN RT, KHAIR RM, SATO T, MINAI OA, TORRES F, GIRGIS RE, CHIN K, DAMICO R, KOLB TM, MATHAI SC, HASSOUN PM: improvement in right ventricular strain with ambrisentan and tadalafil upfront therapy in scleroderma pulmonary arterial hypertension. *Am J Respir Crit Care Med* **197**: 388-391, 2018.
- MURUGESAN N, GU Z, FADNIS L, TELLEW JE, BASKA RA, YANG Y, BEYER SM, MONSHIZADEGAN H, DICKINSON KE, VALENTINE MT, HUMPHREYS WG, LAN SJ, EWING WR, CARLSON KE, KOWALA MC, ZAHLER R, MACOR JE: Dual angiotensin II and endothelin A receptor antagonists: synthesis of 2'-substituted N-3-isoxazolyl biphenylsulfonamides with improved potency and pharmacokinetics. *J Med Chem* **48**: 171-179, 2005.
- PACHECO-QUINTO J, ECKMAN EA: Endothelin-converting enzymes degrade intracellular beta-amyloid produced within the endosomal/lysosomal pathway and autophagosomes. *J Biol Chem* **288**: 5606-5615, 2013.
- PÁNDY-SZEKERES G, MUNK C, TSONKOV TM, MORDALSKI S, HARPSØE K, HAUSER AS, BOJARSKI AJ, GLORIAM DE: GPCrdB in 2018: adding GPCR structure models and ligands. *Nucleic Acids Res* **46** (D1): D440-D446, 2018.
- PIERRE LN, DAVENPORT AP: Blockade and reversal of endothelin-induced constriction in pial arteries from human brain. *Stroke* **30**: 638-643, 1999.
- PLUMPTON C, FERRO CJ, HAYNES WG, WEBB DJ, DAVENPORT AP: The increase in human plasma immunoreactive endothelin but not big endothelin-1 or its C-terminal fragment induced by systemic administration of the endothelin antagonist TAK-044. *Br J Pharmacol* **119**: 311-314, 1996.
- READ C, FITZPATRICK CM, YANG P, KUC RE, MAGUIRE JJ, GLEN RC, FOSTER RE, DAVENPORT AP: Cardiac action of the first G protein biased small molecule apelin agonist. *Biochem Pharmacol* **116**: 63-72, 2016.
- ROSANÒ L, BAGNATO A: beta-arrestin1 at the cross-road of endothelin-1 signaling in cancer. *J Exp Clin Cancer Res* **35**: 121, 2016a.

- ROSANÒ L, BAGNATO A: Endothelin therapeutics in cancer: Where are we? *Am J Physiol Regul Integr Comp Physiol* **310**: R469-R475, 2016b.
- ROSANÒ L, CIANFROCCA R, TOCCI P, SPINELLA F, DI CASTRO V, CAPRARA V, SEMPRUCCI E, FERRANDINA G, NATALI PG, BAGNATO A: Endothelin A receptor/beta-arrestin signaling to the Wnt pathway renders ovarian cancer cells resistant to chemotherapy. *Cancer Res* **74**: 7453-7464, 2014.
- RUSSELL FD, DAVENPORT AP: Evidence for intracellular endothelin-converting enzyme-2 expression in cultured human vascular endothelial cells. *Circ Res* **84**: 891-896, 1999.
- RUSSELL FD, SKEPPER JN, DAVENPORT AP: Human endothelial cell storage granules: a novel intracellular site for isoforms of the endothelin-converting enzyme. *Circ Res* **83**: 314-321, 1998.
- SAWAKI M, WADA A, TSUTAMOTO T, OHNISHI M, FUJII M, MATSUMOTO T, KINOSHITA M: Chronic effects of an orally active selective endothelin-B-receptor antagonist in experimental congestive heart failure. *J Cardiovasc Pharmacol* **36** (Suppl 1): S323-S326, 2000.
- SHIHOYA W, NISHIZAWA T, OKUTA A, TANI K, DOHMAE N, FUJIYOSHI Y, NUREKI O, DOI T: Activation mechanism of endothelin ETB receptor by endothelin-1. *Nature* **537**: 363-368, 2016.
- SHIHOYA W, NISHIZAWA T, YAMASHITA K, INOUE A, HIRATA K, KADJI FMN, OKUTA A, TANI K, AOKI J, FUJIYOSHI Y, DOI T, NUREKI O: X-ray structures of endothelin ETB receptor bound to clinical antagonist bosentan and its analog. *Nat Struct Mol Biol* **24**: 758-764, 2017.
- SIDHARTA PN, VAN GIERSBERGEN PL, HALABI A, DINGEMANSE J: Macitentan: entry-into-humans study with a new endothelin receptor antagonist. *Eur J Clin Pharmacol* **67**: 977-984, 2011.
- SIDHARTA PN, KRAHENBUHL S, DINGEMANSE J: Pharmacokinetic and pharmacodynamic evaluation of macitentan, a novel endothelin receptor antagonist for the treatment of pulmonary arterial hypertension. *Expert Opin Drug Metab Toxicol* **11**: 437-449, 2015.
- SINGLA N, MINKOWITZ HS, SOERGEL DG, BURT DA, SUBACH RA, SALAMEA MY, FOSSLER MJ, SKOBIERANDA F: A randomized, Phase IIb study investigating oliceridine (TRV130), a novel micro-receptor G-protein pathway selective (μ -GPS) modulator, for the management of moderate to severe acute pain following abdominoplasty. *J Pain Res* **10**: 2413-2424, 2017.
- SUDLOW C, GALLACHER J, ALLEN N, BERAL V, BURTON P, DANESH J, DOWNEY P, ELLIOTT P, GREEN J, LANDRAY M, LIU B, MATTHEWS P, ONG G, PELL J, SILMAN A, YOUNG A, SPROSEN T, PEAKMAN T, COLLINS R: UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age. *PLoS Med* **12**: e1001779, 2015.
- TAKAI M, UMEMURA I, YAMASAKI K, WATAKABE T, FUJITANI Y, ODA K, URADE Y, INUI T, YAMAMURA T, OKADA T: A potent and specific agonist, Suc-[Glu⁹,Ala^{11,15}]-endothelin-1(8-21), IRL 1620, for the ETB receptor. *Biochem Biophys Res Commun* **184**: 953-959, 1992.
- TALBODEC A, BERKANE N, BLANDIN V, BREITTMAYER JP, FERRARI E, FRELIN C, VIGNE P: Aspirin and sodium salicylate inhibit endothelin ETA receptors by an allosteric type of mechanism. *Mol Pharmacol* **57**: 797-804, 2000.
- TRESSEL SL, KOUKOS G, TCHERNYCHEV B, JACQUES SL, COVIC L, KULIOPULOS A: Pharmacology, biodistribution, and efficacy of GPCR-based pepducins in disease models. *Methods Mol Biol* **683**: 259-275, 2011.
- WANG J, HANADA K, STAUS DP, MAKARA MA, DAHAL GR, CHEN Q, AHLES A, ENGELHARDT S, ROCKMAN HA: Galphai is required for carvedilol-induced beta1 adrenergic receptor beta-arrestin biased signaling. *Nat Commun* **8**: 1706, 2017.
- WARNER TD, ALLCOCK GH, VANE JR: Reversal of established responses to endothelin-1 in vivo and in vitro by the endothelin receptor antagonists, BQ-123 and PD 145065. *Br J Pharmacol* **112**: 207-213, 1994.
- WINKLES JA, ALBERTS GF, BROGI E, LIBBY P: Endothelin-1 and endothelin receptor mRNA expression in normal and atherosclerotic human arteries. *Biochem Biophys Res Commun* **191**: 1081-1088, 1993.

-
- WOUTERS J, HUNGER RE, GARROD T, DUBUIS B, HUNZIKER T, VAN DEN OORD JJ, LAHAV-LE COUTRE R: First-in-human proof-of-concept study: intralesional administration of BQ788, an endothelin receptor B antagonist, to melanoma skin metastases. *Oncologist* **20**: 1121-1122, 2015.
- YANG P, READ C, KUC RE, BUONINCONTRI G, SOUTHWOOD M, TORELLA R, UPTON PD, CROSBY A, SAWIAK SJ, CARPENTER TA, GLEN RC, MORRELL NW, MAGUIRE JJ, DAVENPORT AP: Elabela/toddler is an endogenous agonist of the apelin APJ receptor in the adult cardiovascular system, and exogenous administration of the peptide compensates for the downregulation of its expression in pulmonary arterial hypertension. *Circulation* **135**: 1160-1173, 2017a.
- YANG P, CROSBY A, SOUTHWOOD M, MAGUIRE JJ, BUONINCONTRI G, READ C, UPTON PD, KUC RE, SAWIAK SJ, MORRELL NW, DAVENPORT AP: The novel biased apelin receptor agonist MM07 attenuates pulmonary vascular remodeling in monocrotaline-induced pulmonary arterial hypertension. *Circulation* **136**: A17882, 2017b.
-