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Endothelin@25 – new agonists, antagonists, inhibitors and emerging research frontiers: IUPHAR Review XX

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Summary

Since the discovery of endothelin-1 in 1988, the main components of the signalling pathway have become established comprising three structurally similar endogenous twenty-one amino acids peptides, ET-1, ET-2 and ET-3, that activate two G-protein coupled receptors, ET_A and $ET_{B.}$. Our aim in this review is to highlight the recent progress in endothelin research. The endothelin-like domain peptide, corresponding to prepro-$ET\text{-}1_{93\text{-}166}$, has been proposed to be co-synthesised and released with ET-1, to modulate the actions of the peptide. ET-1 remains the most potent vasoconstrictor in the human cardiovascular system with a particularly long lasting action. To date, the major therapeutic strategy to block the unwanted actions of ET in disease, principally in pulmonary arterial hypertension, has been to use antagonists that are selective for the ET_A receptor (ambrisentan) or that block both receptor subtypes (bosentan). Macitentan represents the next generation of antagonists, being more potent than bosentan, with longer receptor occupancy and it is converted to an active metabolite; properties contributing to greater pharmacodynamic and pharmacokinetic efficacy. A second strategy is now being more widely tested in clinical trials and uses combined inhibitors of endothelin converting enzyme and neutral endopeptidase such as SLV306 (daglutril). A third strategy based on activating the ET_B receptor, has led to the renaissance of the modified peptide agonist IRL1620 as a clinical candidate in delivering anti-tumour drugs and as a pharmacological tool to investigate experimental pathophysiological conditions. Finally we discuss biased signalling, epigenetic regulation and targeting with monoclonal antibodies as prospective new areas for endothelin research.

Key words (10 MAX): Biased signalling; daglutril; Endothelin-1, ET-1; ET_A; ET_B; Endothelin converting enzymes, ECE-1; endothelin-like domain peptide; macitentan, pulmonary arterial hypertension; SLV306.

Non standard abbreviations: ET-1, endothelin-1; ET-2, endothelin-2; ET-3, endothelin-3; ECE-1, endothelin converting enzyme-1; ECE-2, endothelin converting enzyme-2; ELDP, endothelin-like domain peptide; HIF-1α, hypoxia inducible factor-1α; NEP, neutral endopeptidase; PAH, pulmonary arterial hypertension.

Introduction

Since the discovery of endothelin-1 (ET-1) in 1988 (Yanagisawa, *et al.*, 1988, Inoue *et al.*, 1989) the components of the ET signalling pathway have become established, comprising three structurally similar endogenous twenty-one amino acids peptides, ET-1, ET-2 and ET-3, that activate two G protein-coupled receptors, ET_A (Arai *et al.*, 1990) and ET_B (Sakuri, *et al.*, 1990). In humans ET-2 differs from ET-1 by only two amino acids, whereas ET-3 differs by six amino acids representing more substantial changes. ET-3 is the only isoform that can distinguish between the two receptor sub-types, having a similar potency at the ET_A receptor as ET-1 and ET-2, but much lower affinity than these isoforms for the ET_B sub-type (Figure 1). Structurally, ETs are unusual amongst the mammalian peptides in possessing two disulphide bridges. This feature is shared by the sarafotoxins, a family of peptides that were isolated from snake venom in the same year as the discovery of ET-1 (Takasaki *et al.*, 1988) and that provided the first selective agonist at the ET_B receptor, sarafotoxin S6C (Williams *et al.*, 1991).

A number of features of the ET signalling pathway are unusual compared with other peptidergic systems and these continue to intrigue investigators, with over a thousand ET-related papers still published each year. ET-1 is the most abundant isoform in the human cardiovascular system, predominantly released from endothelial cells to cause potent and unusually long lasting vasoconstriction that may persist for many hours. ET-1 is a key mediator in regulating vascular function in the majority of organs systems balanced by opposing vasodilators, particularly nitric oxide, prostacyclin and endothelium derived hyperpolarising factor. Endothelial cell dysfunction occurs in pathophysiological conditions such as pulmonary arterial hypertension (PAH) and is associated with loss of these dilators and increased synthesis of ET. The consequence of this is vasoconstriction, proliferation of multiple cell types particularly vascular smooth muscle, fibrosis and inflammation; processes

associated with vascular remodelling. In disease the deleterious actions of ET in the vasculature are mainly mediated by the ET_A receptor, whereas ET_B activation results in many of the beneficial effects of the peptide that frequently act as a regulatory counterbalance (Davenport and Maguire, 2006). The formation of the disulphide bridge in the ET peptides blocks the N-terminal amino acid conferring resistance to enzymatic degradation in plasma: the internalization by ET_B scavenging receptors is therefore particularly important for termination of the ET signal in health and disease.

The major therapeutic strategy (Figure 1) to block the unwanted actions of ET in disease has been to use antagonists of ET_A or both receptor sub-types (Palmer, 2009) with the first clinical application being bosentan in PAH (Rubin *et al.,* 2002). More recently, a second strategy has started to be more widely tested in clinical trials using inhibitors of endothelin converting enzymes, ECE-1 (Xu *et al.*, 1994) and ECE-2 (Emoto and Yanagisawa, 1995), the major synthetic pathway of ET (Figure 1) at least in the human vasculature (Russell and Davenport, 1999a, b). A third emerging strategy based on biosimilar agonists at the ET_B receptor (molecules similar but not identical to the endogenous ligand) has led to the renaissance of IRL1620 as a clinical candidate in delivering anti-tumour drugs and in other pathophysiological conditions such as cerebral ischaemia.

The aim of this focused IUPHAR review is to highlight recent progress and some surprising new discoveries in the pharmacology of the ET system. The following are recommended for more detailed information on specific ET research areas including the heart (Kelland and Webb, 2006; Kirby *et al.,* 2008; Kohan *et al.,* 2012; Ohkita *et al.,* 2012; Vignon-Zellweger *et al.,* 2012; Drawnel *et al.,* 2013), renal (Kohan *et al.,* 2011a, b; Dhaun *et al.,* 2011, 2012; Hyndman and Pollock, 2013), hypertension (Rautureau and Schiffrin, 2012; Speed and Pollock, 2013), PAH (Pernow *et al.,* 2012; Rubin, 2012; Liu *et al.* 2013), cancer (Bagnato *et al.,* 2011; Said and Theodorescu, 2012; Rosanò *et al.*, 2013b),

atherosclerosis and diabetes (Pernow *et al.*, 2012).

ET Peptides

Evidence for a new ET peptide: the endothelin-like domain peptide (ELDP)

The ELDP has been recently identified as a peptide corresponding to prepro-ET-$1_{93\text{-}166}$ (Yuzgulen *et al.*, 2013) immediately adjacent to the gene sequence encoding big ET-1. The 74 amino acid peptide has been detected by high performance liquid chromatography and specific double recognition site immunoassays in conditioned media from two cell lines, endothelial (EA.hy 926) and epithelial (A549), as well as from primary cell cultures of human aortic endothelial cells that are known to secrete ET-1. In the aortic endothelial cells, the peptide was co-synthesised and co-released with ET-1. Plasma levels in untreated patients were 6.5 pmol l^{-1} which compares with typical basal levels of immunoreactive ET-1 of 5 pmol l^{-1} (Davenport *et al.* 1990). Levels of ELDP were significantly elevated in heart failure patients suggesting a potential use as a bio-marker. While no effect was observed on blood pressure in the anaesthetised rat, intriguingly ELDP significantly increased the duration of the pressor response of ET-1 (0.3 nmol kg^{-1}, likely to be a sub-maximal dose). Pre-treatment of rat mesenteric arteries with 10 nM ELDP also potentiated a sub-maximal response of ET-1 by five fold (Yuzgulen *et al.*, 2013). It is not unexpected that a second peptide sharing a cleavage site with ET-1 would also be co-released, but it is intriguing that the peptide was able to potentiate ET-1 responses *in vitro* and *in vivo.* It is not yet reported using saturation or competition binding experiments whether ELDP binds directly to ET receptors, binds to an allosteric site or whether the peptide modulates ET responses by other mechanisms. Intriguingly, ELDP encompasses the sequence of the putative 'endothelin-like peptide' corresponding to prepro-ET-$1_{109\text{-}123}$ proposed in the original Nature paper by Yangisawa *et al.* (1988). Eight out of fifteen residues in the corresponding sequence in ET-1 are identical and the four Cys residues are perfectly conserved and flanked by dibasic pairs

that are recognised by endopeptidase processing enzymes, to yield a cleaved peptide. However, a synthetic peptide corresponding to this sequence was devoid of agonist or antagonist activity against ET-1, in vascular preparations (Cade *et al.*, 1990).

Global knock-out of ET-2 gene reveals distinct phenotype compared with ET-1/ET_A and ET-3/ET_B gene deletions

ET-1-deficient homozygous mice die at birth of respiratory failure which is secondary to severe craniofacial and cardiovascular abnormalities. ET_A receptor and ECE-1 knockout mice have similar morphological abnormalities (Kurihara *et al*. 1994; Clouthier *et al.* 1998; Yanagisawa *et al.* 1998,). The phenotype is similar to a spectrum of human conditions, CATCH 22 (**C**ardiac anomaly, **A**bnormal face, **T**hymic hypoplasia, **C**left palate, **H**ypocalcemia, chromosome 22 deletions) and established the importance of the ET_A/ET-1 signalling system for cardiovascular and craniofacial development. ET-3 and ET_B gene deletions exhibit a different and non-overlapping phenotype to ET-1/ET_A-deficient animals. They are viable at birth and survive for up to eight weeks, but display aganglionic megacolon, as a result of absence of ganglion neurons, together with a pigmentary disorder in their coats (Baynash *et al.*, 1994; Hosoda *et al.*, 1994). In these mice, enteric nervous system precursors and neural crest-derived epidermal melanoblasts fail to colonise the intestine and skin. This phenotype resembles Hirschsprung's disease in man.

Deleting genes encoding all the key molecules, ET-1, ET-3, ET_A, ET_B, ECE-1 and ECE-2 has been accomplished in mice generating important information about their effect on phenotype. The deleting the gene for ET-2 has now been reported. The physiological role of ET-2 has been puzzling. It had been assumed that the actions of ET-2 would be similar, if released, to the more widely distributed and abundant ET-1. Current antagonists block both ET-2 and ET-1 with the same potency and are not yet able to distinguish the actions of these

peptides.

A key advance in the field was the generation by Chang *et al.* (2013) of a global ET-2 gene knock-out mouse, which surprisingly exhibited a distinct phenotype to global ET-1 or ET-3 gene deletions. These mice showed severe growth retardation, internal starvation characterised by hypoglycemia, ketonemia, and increased levels of starvation-induced genes. Mice were profoundly hypothermic and the median lifespan could be significantly extended by housing in a warm environment. The intestine was morphologically and functionally normal, which was unexpected as murine ET-2 (see Ling *et al,*. 2013), also known as vasoactive intestinal contractor or VIC, is present throughout the gastrointestinal tract suggesting, in this tissue at least, in the absence of ET-2, ET-1 continues to mediate signalling. In agreement, intestinal epithelium-specific ET-2 knockout mice showed no abnormalities in growth and survival. In marked contrast, dramatic changes were observed in lung morphology and function. Mice had breathing difficulties after the first week exhibiting enlarged air spaces with substantial simplification of lung alveolar structure, larger lung capacities leading to abnormally elevated carbon dioxide (hypercapnia) and deficiency of oxygen (hypoxaemia) in the blood. Hypothermia and lung dysfunction might not be specific but may be due to a secondary effect of internal starvation due to ET-2 deficiency. However, it is possible that these studies identify an important function for ET-2 in the pulmonary system. The authors showed that mRNA encoding ET-2 was only present in epithelial cells whereas receptor mRNA was mainly present in mesenchyme, consistent with a paracrine function for ET-2 in the lung.

Pharmacological significance: Is ET-2 the inducible isoform?

The dramatic effects on the lung suggest a crucial role for ET-2 at birth, at least in mice. The lungs, and potentially the heart, remain major therapeutic targets for ET antagonists in

humans in the treatment of PAH. In rodents ET-2 was less widely distributed that ET-1, mainly found in heart, lung, ovary, stomach and all regions of the intestine (De La Monte *et al.*, 1995; Takizawa *et al.*, 2005). ET-2 expression in human tissue was similar, being present in human heart (Plumpton *et al.*, 1996b), lung (Marciniak *et al.*, 1992), kidney (Karet and Davenport, 1996), vasculature, (Howard *et al.*, 1992) intestine and ovaries (Palanisamy *et al.*, 2006) but has not been investigated in pathophysiological tissue. In humans alternatively spliced mRNA variants encoding ET-2 have been detected with a specific pattern of distribution in various tissues. Some of these variants contain sites for the post-transcriptional processing of preproET-2 into mature ET-2 which may be altered in a tissue specific manner (O'Reilly *et al.*, 1993). The most well established model of spatial and temporal ET-2 signalling is in the ovary, a highly vascular tissue, which undergoes cyclic changes as follicles grow, rupture and transform into corpora lutea and eggs are periodically released (Ko *et al.*, 2012). In rats, low levels of ET-1 are constitutively expressed throughout the ovulatory cycle, whereas ET-2 is induced transiently at much higher concentrations during the period of ovulation to luteal phases (Ko *et al.*, 2006). ET-2 is expressed in the granulosa cells of periovulatory follicles but not during other stages of follicular development. In mice, induced superovulation results in a dramatic increase in ET-2 mRNA expression (Palanisamy *et al.*, 2006). ET-2 expression surged in response to gonadotropin and quickly declined by 13 h, which coincided with the time of follicular rupture. Crucially, both ET receptor sub-types are present and their ratio does not seem to change. Thus the ET-2 gene appears to be switched on only when increased levels of ET are required, with ET-2-mediated contraction being the final signal facilitating ovulation (Ling *et al.*, 2013).

ET-1 signalling is well established in neural crest migration. In the developing mouse retina constitutive over-expression of ET-2 affects vascular development by inhibiting endothelial cell migration across the retinal surface and subsequent endothelial cell invasion

into the retina, an action mediated by ET_A receptors. Interestingly, over-expression is spatially localised as it has no obvious action on vascular structures in brain or skin (Rattner *et al.,* 2013). Constitutive over-expression of ET-2 signalling also protected photoreceptors from light damage (Braunger *et al.,* 2013). Similarly, Bramall *et al, (*2013) found expression of ET-2 mRNA was greatly increased in the photoreceptors of mouse models of inherited photoreceptor degeneration and using the global ET-2 knock-out mice showed increased ET-2 expression is protective of the mutant photoreceptors.

The case for re-evaluating the role of ET-2

Targeting the ET-2 gene in mice provides compelling evidence that while both ET-1 and ET-2 can co-exist in the same tissue compartments, there is a critical but distinct ET-2 pathway. A key role has now been established for ET-2 in ovarian physiology. This may be accomplished at the level of gene expression, but differences may also exist in peptide synthesis by ECEs and chymase, which may allow the two ET peptide pathways to be distinguished pharmacologically and become separate drug targets. Additionally, pharmacological differences have been identified, for example ET-2 dissociates from receptors much more rapidly than ET-1 and higher affinity has been reported for example in brain (Ling *et al.,* 2013). Detailed studies comparing rat mesenteric resistance and basilar arteries demonstrated that ET-1 and ET-2 initiate and maintain vasoconstriction by different downstream mechanisms raising the prospect of 'biased signalling' mediated by two structurally different agonists activating the same receptor (Compeer *et al.,* 2013).

Potential new therapeutic strategies exploiting ET_B receptor agonists

The pharmacological rationale for this strategy is that ET-1 topically released from endothelial cells also interacts with endothelial cell ET_B receptors. The importance of this

counter regulatory pathway has been underestimated to date. Endothelial cells line the vasculature of every organ and tissue in the body that receives a blood supply. Although the cells represent ~1% of the weight of the vessel wall, they have a combined mass comparable to some endocrine glands. Crucially, ET-1 feeding back onto endothelial receptors to release nitric oxide not only limits ET_A mediated vasoconstriction by stimulation of vascular cyclic GMP, but also limits further ET-1 release. Thus in the vasculature, nitric oxide and other dilators are crucial in balancing the ET system, but these may be reduced or absent in pathophysiological conditions.

ET-$1^{+/-}$ heterozygous mice developed *elevated* blood pressure and mild hypertension, rather than the fall in blood pressure that might have been expected. Partial deletion of the gene allows survival and produced lower levels of ET-1 in plasma and lung tissue than wild-type (Kurihara *et al.,* 1994). These results suggest that ET-1 has an essential physiological role in cardiovascular homeostasis. Low levels promote vasodilatation whereas higher and pathophysiological concentrations of ET-1 increase blood pressure and total peripheral vascular resistance. While ET_A selective antagonists such as BQ123 (Ihara *et al.,* 1992) cause the expected vasodilatation in humans (Haynes and Webb, 1994), the ET_B selective antagonist BQ788 (Ishikawa *et al.,* 1994) caused systemic *vasoconstriction* in healthy volunteers, showing that the main consequence of activation of endothelial ET_B receptors by tonically secreted ET-1 was the physiological basal release of nitric oxide (Love *et al.* 2000). In agreement, initial vasodilatation can be detected in the human forearm vascular bed following infusion of low concentrations of ET-1 whereas higher doses caused sustained vasoconstriction (Kiowski *et al,* 1991). A contribution to vasoconstriction may also be the result of ET-1 occupancy of the clearance ET_B receptors causing an ET_A-mediated vasoconstriction.

ET_B agonists in chemotherapy: IRL1620

ET-1 acting on ET_A receptors has been proposed to stimulate cell proliferation, migration, invasion, osteogenesis and angiogenesis in several cancers. New vessels forming in tumours are characterised by high densities of ET_A receptors in smooth muscle, for example in glioblastoma multiforme in the brain (Harland *et al.,* 1998). Conversely, ET_B receptors may oppose tumour progression by promoting apoptosis and clearing ET-1 (Bagnato *et al.*, 2011; Rosanò *et al.*, 2013b). The strategy of stimulating ET_B receptors to cause transient vasodilatation is being developed to increase the penetration of cytotoxic anti-tumour agents into tumours and to minimise the concentration in healthy tissue.

IRL1620 was originally developed as a tool compound (Takai *et al.,* 1992). The N-terminus has an N-sucinyl modification, likely to beneficially reduce metabolism by non-specific peptidases, but it is not orally active and requires injection. Despite these unpromising pharmacokinetic features it is being used *in vivo* and has emerged as a possible clinical candidate in improving the delivery of drugs to tumours. IRL1620 infused into rats improved the efficacy of doxorubicin and 5-flurouracil by significantly increasing the amount of drug in tumours in rat models of prostrate and breast cancer. In addition, radiation induced reduction in tumour volume was enhanced, suggesting IRL1620 can significantly increase the efficacy of radiotherapy in the treatment of solid tumors. The results suggest that for a given dose of drug the efficacy in reducing the tumour could be improved (Gulati *et al.*, 2004, 2012; Rajeshkumar *et al.*, 2005a, b; Lenaz *et al.*, 2006; Rai *et al.*, 2006). A Phase 1 trial to determine the safety, tolerability, pharmacokinetics and pharmacodynamics of IRL1620 (known as SPI-1620 licensed by Spectrum Pharmaceuticals) in patients with recurrent progressive carcinoma has been successfully completed and shown to selectively and transiently increase tumour blood flow (Gulati *et al.,* 2012; www.cancer.gov/clinicaltrials) . A phase II trial was initiated in 2013 to determine the effectiveness of SPI-1620 in

combination with docetaxel in patients with advanced biliary cancer (http://clinicaltrials.gov/ct2/show/NCT01773785) and in combination with docetaxel compared with docetaxel alone for patients with non small-cell lung cancer after failure of platinum-based chemotherapy (http://clinicaltrials.gov/show/NCT01741155).

ET_B agonists in neuroprotection

The human brain contains the highest density of ET receptors, with the ET_B sub-type comprising about 90%, in areas such as cerebral cortex (Harland *et al.* 1998). Binding and functional studies have demonstrated glia mainly express ET_B whereas ET_A are localised mainly on neurones (Morton and Davenport, 1992). Smooth muscle cells from large arteries and small intracerebral cerebral only express ET_A receptors (Adner *et al.* 1994; Harland *et al.* 1995; Pierre and Davenport, 1999) with endothelial cell ET_B receptors mediating relaxation (Lucas *et al.* 1996). The small pial arteries and arterioles penetrating into the brain play a major role in the maintenance of cerebral blood flow (autoregulation). These vessels are particularly sensitive to ET-1 compared to peripheral vessels and the peptide has been a long standing candidate in the genesis or maintenance of cerebrovascular disorders such as delayed vasospasm associated with subarachnoid haemorrhage or stroke. ET-1 does not cross the blood brain barrier from the plasma, but may do so when compromised by subarachnoid haemorrhage, stroke or head injury. Strategies for targeting cerebrovascular disease have focused previously on the use of ET receptor antagonists, firstly to block vascular receptors mediating cerebrovasospasm that may be responsible for delayed cerebral ischaemia seen after aneurysmal subarachnoid haemorrhage and could contribute to ischaemic core volume in stroke. Secondly, to block neural receptors that mediate increases in intracellular free calcium (Morton and Davenport, 1992) and initiate the pathophysiological processes leading to neuronal death.

A new emerging strategy is to use ET_B agonists such as IRL1620 to provide vasodilatation and neuroprotection. The peptide reduced neurological damage following permanent middle cerebral artery occlusion in rats, a model of focal ischaemic stroke. Animals received intravenous injections of IRL1620 post occlusion which dramatically reduced infarct volume (by more than 80% in the acute and 70% in the chronic study), prevented cerebral oedema, reduced oxidative stress markers and improved all neurological and motor function for up to seven days (Leonard *et al.,* 2011, 2012, 2013). Rats treated with the amyloid peptide $A\beta_{1\text{-}40}$ administered into the intracerebral vessels increased markers of oxidative stress in the brain. IRL1620 significantly reduced oxidative stress and importantly the cognitive impairment (Briyal *et al.,* 2014). As discussed below, a reduction in ECE activity is associated with accumulation of amyloid β-peptide and neurotoxicity early in progression of Alzheimer's disease (Eckman *et al.*, 2001, 2003, 2006, Pacheco-Quinto and Eckman, 2013). These results are limited to disease models in a single species and it is unclear whether the molecular mechanisms would translate to humans, but taken together they suggest that an ET_B agonist might offer a new therapeutic strategy in Alzheimer's disease and provide neuroprotection following cerebral ischemia in conditions such as stroke.

No evidence for further ET_B receptor sub-types

Previous studies have suggested that ET_B receptors could be further sub-divided into ET_{B1} present on endothelial cells and ET_{B2} on smooth muscle cells. Studies continue to be published with this misleading nomenclature but current evidence only supports the existence of two sub-types, ET_A and ET_B, according to NC-IUPHAR nomenclature (Davenport 2002; Alexander *et al.,* 2013). Firstly, Mizuguchi *et al.* (1997) demonstrated unequivocally that in ET_B receptor knockout mice both the direct constrictor and indirect vasodilator responses to the ET_B agonist sarafotoxin S6C were abolished. Selective deletion of endothelial ET_B

receptors in mice (demonstrated by autoradiography to leave unaltered ET_B receptors expressed by other cell types) impaired, as expected, the clearance of an intravenous bolus of labelled ET-1 compared with controls (Bagnall *et al.*, 2006; Kelland *et al.*, 2010). Secondly, Flynn *et al.* (1998) were unable to distinguish pharmacologically, in extensive competition binding experiments, between ET_B receptors expressed by human isolated endothelial compared with smooth muscle cells in culture. In concordance, saturation binding assays in human tissue always found ET_B radiolabelled ligands bound with a single affinity and Hill slopes close to unity with no suggestion of further sub-types (Molenaar *et al.*, 1992, 1993; Nambi *et al.*, 1994) or in competition binding versus radiolabelled ET-1 in human native (Peter and Davenport 1995, 1996; Russell and Davenport, 1996) or recombinant ET_B receptors (Nambi *et al*, 1994; Reynolds *et al.*, 1994). Clozel and Gray (1997) showed that endothelial and smooth muscle ET_B receptors cannot be distinguished functionally.

Do ETs interact with any other G protein-coupled receptors?

The virtually complete sequencing of the human genome has allowed the identification of all of the human gene sequences that could potentially encode G protein-coupled receptors that are currently classified as 'orphan' to indicate their endogenous ligand is not yet known (Foord *et al., 2005;* Davenport *et al.*, 2013). In this catalogue, the most closely related to the ET_A and ET_B sub-types are the orphan receptors GPR37 (also know as endothelin receptor type B-like or Parkin-associated endothelin receptor-like receptor) and its related receptor GPR37L1. A recent high throughput screen tested ~10,000 biologically active compounds for binding to 82 remaining orphan GPCRs. None of the ~ 20 ET peptides tested at high concentration (including all three mature isoform and their corresponding big ET precursors, C-terminal metabolites, BQ123 and the ET_B agonist BQ3020) activated any of the expressed receptors, including GPR37 or GPR37L, supporting the established concept of ETs binding to

only two receptor sub-types. Two orphan neuropeptides, prosaptide and prosaposin have recently been proposed as cognate ligands for GPR37 and GPR37L (Meyer *et al.,* 2013).

Clinical application of ET antagonists

Bosentan, ambrisentan and withdrawal of sitaxentan

PAH is a progressive condition with no cure and a major impact on the ability to lead a normal life. It is an orphan disease (~100,000 patients in US and Europe). PAH involves constriction of pulmonary arteries and is characterised by high blood pressure in the lungs, ultimately leading to right heart failure and death. A number of pathways have been implicated in the development of PAH including bone morphogenetic proteins, prostacyclin and ET-1. Restoring the imbalance between constriction and vasodilatation of blood vessels is the basis for current medical therapies, although the cause of death is right heart failure. Although ET_A receptors are significantly increased in the right ventricle of patients with PAH (Kuc *et al.,* 2014) and in the left ventricle of patients with heart failure (Zolk *et al.*, 1999), surprisingly ET antagonists have clinical efficacy in the former but not the latter group (Kohan *et al.* 2012).

Bosentan (Tracleer, Ro47-0203) was the first ET antagonist to be introduced into the clinic for the treatment of PAH (Rubin *et al.,* 2002) and as an orally acting agent, at the time represented a major advance over existing therapies such as prostacyclin analogues. Bosentan is classified as a mixed ET_A/ET_B antagonist blocking both receptors (Figure 2 and 3). The second antagonist to enter the clinic in 2007 was ambrisentan (Letairis, Volibris, LU208075, Figure 2 and 3) reported to display some ET_A selectivity (Vatter *et al.,* 2006) followed by the most highly selective ET_A selective antagonist sitaxentan (Thelin, TBC11251) (Barst *et al.* 2004). While hepatotoxicity is a known side effect of ET

antagonists it is usually reversible and related to dose. Unfortunately, cases of idiosyncratic hepatitis resulting in acute liver failure leading to death have been reported with sitaxentan and the compound was withdrawn in 2010 (Don *et al.*, 2012).

Next generation of ET antagonists: macitentan

Despite the current use of ET receptor antagonists and drugs targeting the two other principal pathways nitric oxide (phosphodiesterase-5 (PDE5) inhibitors) and prostacyclin (PGI2), meta-analysis of PAH trials shows existing therapies only moderately increased the most widely used objective evaluation of functional exercise capacity (6-Minute Walk Distance) by 11%. The prognosis for PAH patients remains poor with ~15% mortality within one year. There remains an urgent need for new efficacious treatments that has lead to the development of macitentan.

Macitentan (Opsumit, ACT-064992, Figure 2 and 3) represents the next generation of orally active ET antagonist and was developed by modifying the structure of bosentan to improve efficacy and tolerability (Bolli *et al.*, 2013). Macitentan (ACT-064992) is described as a dual antagonist that blocks both ET_A and ET_B receptors. Macitentan (ACT-064992) inhibited [^{125}I]-ET-1 binding to human recombinant ET_A receptors with an IC_{50} of 0.2 nM and 391 nM at ET_B receptors. On the basis of these results, macitentan displays about 800 fold selectivity. A Phase III clinical trial was successfully completed in 2012 (Pluido *et al.*, 2013) and the compound gained approval from the U.S. Food and Drug Administration in 2013 for the treatment of PAH. Macitentan is metabolised by the cytochrome P450 system, predominantly CYP3A4 and to a lesser extent the CYP2C19 iso-enzyme. Unlike other antagonists in current use one of the metabolites of macitentan, ACT-132577 (Figure 2), is pharmacologically active. Although it has a lower potency than the parent compound ACT-132577 reaches higher plasma concentrations, with a longer half-life of about 48 hours

(Iglarz *et al.*, 2008; Sidharta *et al.*, 2011, 2013a, b). These factors are likely to contribute to improved activity of macitentan compared with bosentan. Whilst *in vitro* studies suggested macitentan (ACT-064992) was likely to interact with other drugs (Weiss *et al.*, 2013), other observed pharmacokinetic benefits included fewer interactions with other drugs at clinically used concentrations, no requirement to alter doses in patients with renal or hepatic impairment, improved hepatic safety and reduced oedema/fluid retention compared with bosentan. Key differences were also identified in the pharmacodynamic parameters. For example, in calcium release assays macitentan (ACT-064992) was more potent (K_B= 0.1 nM) than bosentan (K_B =1.1 nM) and had a significantly longer receptor occupancy (17 minutes compared with 70 seconds) (Iglarz, *et al.*, 2008; Bruderer, *et al.*, 2012a, b, 2013; Gatfield *et al.*, 2012). The authors speculated that the macitentan (ACT-064992) binding site is partially different from the bosentan binding site and that this difference in interaction with amino acids in the receptor contributes to the slow receptor dissociation of macitentan (ACT-064992), particularly leading to insurmountable antagonism. A number of clinical trials are actively recruiting (Patel and McKeage, 2014) including the use of macitentan for the treatment of digital ulcers in systemic sclerosis patients, Eisenmenger Syndrome and perhaps the most challenging, in patients with brain tumours (glioblastoma).

Compounds interacting with ET-1 synthesis and metabolism

Members of the neprilysin-like family of zinc metalloendopeptidases play key roles in the ET pathway (Turner *et al.*, 2001; Turner and Murphy, 1996). Neutral endopeptidase (neprilysin, NEP) is a membrane bound thermolysin-like zinc metalloendopeptidase which is particularly abundant in human kidney. The enzyme metabolises a number of peptides including enkephalins, tachykinins, natriuretic peptides as well as ETs (Turner and Tanzawa, 1997). Inactivation of ET-1 is via a two stage process, opening of the Ser^5-Leu^6 bond, followed by

cleavage at the amino side of Ile^{19} resulting in an inactive peptide, which is inhibited by phosphoramidon (Skolovsky *et al.,* 1990). Pharmacological intervention in the pathway is challenging because NEP-like enzymes also include the synthetic enzymes ECE-1, ECE-2 and KELL. The ECEs are also inhibited by phosphoramidon and ECE inhibitors currently in clinical trials have significant NEP inhibitory activity and it seems counter-intuitive to inhibit the degradative pathway. In practice however, inactivation of ET-1 is thought to be mainly via binding and internalization of the ET_B receptor and ET-1 is essentially stable in plasma. Binding to ET_B receptors, particularly in those organs such as the lung expressing high densities of the sub-type, are critical for inactivation of the peptide. After internalization of the ligand-receptor complex to the lysosome ET-1 is thought to be degraded, like other peptides, by cathepsin A. In support, cathepsin A knock-out mice showed reduced ET-1 degradation and significantly increased arterial blood pressure. In humans, genetic defects of cathepsin A include hypertension and cardiomyopathies (Seyrantepe *et al.,* 2008).

ECE-1

It is now well established that ET is synthesised in a three step process, with pre-pro-ET-1 initially cleaved by a signal peptidase to proET-1, which is in turn cleaved by a furin enzyme to an inactive precursor big-ET-1 (Figure 1). Although small molecule inhibitors of furins have been reported, furins cleave a number of other proteins to mature or active forms and therefore are not an easy tractable drug target for selectively reducing ET-1, without altering other pathways. More promising has been to target the ECE enzymes responsible for transformation of big ET-1 to the mature, biologically active ET-1 (Xu *et al.,* 1994; Turner and Murphy, 1996). In humans there are four isoforms, ECE-1a-d, derived from a single gene by the action of alternative promoters. Structurally, they differ only in the amino acid sequence of the extreme N-terminus. ECE-1 localises to the small secretory vesicles of the

constitutive pathway from where ET-1 is continuously released to maintain normal vascular tone. Unusually for vasoactive peptides, ET-1 is also synthesised by ECE-1 and stored in specialized Weibel-Palade bodies within endothelial cells until released following an external physiological or pathophysiological stimulus (the regulated pathway) to produce further vasoconstriction (Russell *et al.*, 1998a, b; Russell and Davenport, 1999b).

In addition to intracellular endothelial cell ECE, the enzyme is also present on vascular smooth muscle, efficiently converts big ET-1 in human vessels *in vitro* and is upregulated in atherosclerosis (Maguire *et al.*, 1997; Maguire and Davenport, 1998). Given the larger volume of the smooth muscle compared with single layer of endothelium, smooth muscle ECE may be a more important source of ET-1 in pathophysiological conditions.

ECE-2

ET-1 is also synthesized by a second membrane-bound metalloprotease, ECE-2 (Emoto and Yanagisawa, 1995; Lorenzo *et al*., 2001, Yanagisawa *et al*., 2000) with ~60% sequence similarity to ECE-1. It is distinguishable from ECE-1 by having an optimum pH of 5.5 for activity. In human endothelial cells ECE-2 was found to be localised to the acidified environment of vesicles of secretory pathway, but unlike ECE-1 it is not found in storage granules (Russell and Davenport, 1999b). Four isoforms exist, differing in their N-terminus: ECE-2a-1 and ECE-2a-2 are expressed predominantly in peripheral tissues and ECE-2b-1 and ECE-2b-2 in the brain, possibly representing the neuronal isoforms (Ikeda *et al.* 2002). The physiological importance of this pathway for ET-1 synthesis remains to be determined (ECE-2 also metabolises other peptides such as bradykinin), but the requirement for an acidic pH suggests a role in pathophysiological conditions associated with low pH such as ischemia. ECE-1/ECE-2 knock-put mice display increased developmental defects compared with deletion of ECE-1 or ECE-2.

Alternative, non-ECE synthetic pathway: chymase

ET-1 can also be synthesised indirectly by chymase, a serine protease present in mast cells. Big ET-1 is converted to ET-$1_{1–31}$ by cleaving the Tyr^{31}–Gly^{32} bond (Figure 1), which in turn is converted to the mature peptide via Trp^{21}–Val^{22} bond (Fecteau, *et al.*, 2005; D'Orleans-Juste *et al*., 2008*).* The existence of an alternative pathway was originally predicted when ET-1 and ET-2 was detected in embryos of the ECE-1/ECE-2 double-knock-out mouse (Yanagisawa *et al.*, 2000).

The importance of this alternative pathway remains unclear but importantly ET-$1_{1–31}$ was equipotent compared with big ET-1 in causing vasoconstriction in human isolated vessels, including coronary arteries, and this was associated with the appearance of measurable levels of ET-1 in the bathing medium. ET-$1_{1–31}$ displayed no selectivity between ET_A and ET_B receptors in human heart and vasoconstriction was fully blocked by ET_A selective antagonists, reflecting the predominance of the ET_A receptor on vascular smooth muscle (Maguire *et al.*, 2001, 2004). The precise physiological role of mast cells within human blood vessels is unclear but following degranulation, which may occur under pathophysiological conditions, the mast cell chymase is associated with interstitial spaces with the potential to convert circulating big ET-1 and provide a further source of ET-1. Mast cell expression is increased in cardiovascular disease, for example in atherosclerotic lesions. It is therefore possible that the contribution of this pathway within the vasculature, leading to over-expression of ET-1, may be underestimated particularly in conditions of endothelial malfunction where opposing levels of endogenous vasodilators may be reduced. It is unclear whether under conditions of NEP/ECE inhibition the rising levels of big ET-1 would favour increased conversion by the serine protease pathway, thus increasing the pressor effect via ET_A receptors or whether excretion of unmetabolised big ET-1 by the kidney would be

sufficient to remove the elevated levels of precursors (Johmstrom *et al.*, 2010).

KELL and ET-3 synthesis

Although big ET-3 is converted by ECE-1 to ET-3, owing to difference in the C-terminus the efficiency is much less than for ET-1. In contrast, big ET-3 is reported to be efficiently converted by Kell (Lee *et al*., 1999). Kell is a membrane bound glycoprotein expressed in human erythrocytes and one of the major antigens; it is also related to mammalian NEP-like enzymes including ECE-1 and ECE-2 (Turner and Tanzawa, 1997). If Kell is the main synthetic pathway for ET-3, a possible benefit of inhibiting ECE would be to increase the ratio of ET-3 to ET-1, which could then differentially produce beneficial vasodilatation via the ET_B receptor, but this speculative hypothesis has not been tested.

Pharmacological inhibition of ECE by research compounds

A combination of phosphoramidon and thiorphan has been widely used to identify ECE activity. This is based on demonstrating that the conversion of big ET-1 to ET-1 is inhibited by phosphoramidon, but not by thiorphan, and has been shown both *in vitro* and *in vivo*. Importantly for evaluating the significance of animal models, both compounds have also been used in clinical studies to characterise big ET-1 conversion (see for example, Webb, 1995; Plumpton, *et al.* 1995; Hand *et al.*, 1999). Small molecule, non-peptide ECE inhibitors have been developed; one that has been widely used *in vitro* and *in vivo* animal models and is commercially available is CGS26303 (De Lombaert *et al.*, 1994). CGS26303 has been shown experimentally to significantly inhibit conversion of all three big-ETs in human isolated vessels but importantly does not interfere with the interaction of mature peptides with ET receptors (Yap *et al.*, 2000). Although primarily an NEP inhibitor, SOL1, a more recent combined NEP/ECE non-peptide inhibitor with modest inhibition of ECE-1 *in vitro*,

was remarkably potent *in vivo,* fully blocking the big-ET-1-induced rise in blood pressure at a dose of 10 μmol kg^{-1} (Nelissen *et al.,* 2012).

A disadvantage of using phosphoramidon is that it there is of course the possibility of significant inhibition of NEP and any pharmacological actions may also be mediated via inhibition of degradation of other peptides such as ANP. An alternative tool compound is PD159790, which inhibits ECE-1 with an IC_{50} value of 3 μM; at this concentration the compound is selective for ECE-1 over NEP (Ahn *et al.,* 1998). PD159790 has been shown experimentally in human umbilical vein endothelial cells to inhibit conversion of big ET-1 at pH 6.9, optimum for ECE-1, but did not affect big ET-1 conversion to the mature peptide at pH 5.4, optimum for ECE-2 (Russell and Davenport, 1999a). The compound did not inhibit the further metabolism of $ET\text{-}1_{1-31}$, the chymase product of big ET-1 (Maguire *et al.* 2001) and can be used to distinguish between the three different pathways for ET synthesis. While the mature peptide is located to intracellular Weibel-Palade bodies or secretory vesicles within endothelial cells and a proportion of big ET-1 is converted to ET-1 intracellularly, it is not reported whether ECE inhibitors can cross the plasma membrane to access these intracellular sites. The main effects of these inhibitors may be on external ECE: in agreement with this proposal, the SLV306 metabolite KC-12615 (see below) effectively prevented conversion of exogenous big ET-1 in human vasculature (Seed *et al.,* 2012).

Emerging NEP/ECE inhibitors

Selective inhibitors of ECE have not progressed into clinical applications. SLV306 (Daglutril, Figure 2) is an orally active, mixed enzyme inhibitor of both ECE and NEP. It is a pro-drug being converted *in vivo* to an active metabolite, KC-12615. This latter molecule has a pharmacological profile similar to phosphoramidon, inhibiting NEP in the nanomolar range but with more modest inhibition in the micromolar range for ECE (Meil *et al.,* 1998;

Jeng *et al.*, 2002) The therapeutic basis is that while inhibition of NEP alone increased plasma concentrations of ANP to cause vasodilatation, NEP inhibitors are ineffective as anti-hypertensives, probably because NEP also degrades vasoconstrictor peptides including ET. A combined ECE/NEP inhibitor would be predicted to reduce the systemic conversion of big ET-1 to the mature peptide and increase dilator peptides such as ANP. SLV306 has been reported to be well tolerated with few or none of the side effects such as increases in liver function, oedema, observed with ET receptor antagonists (Dickstein *et al.,* 2004; Parvanova *et al.* 2013). A potential disadvantage is big ET-1 might still be converted to ET-1 by an alternative pathway such as chymase. However, in animal models with normal renal function, this does not occur: big ET-1 labelled with the positron emitter ^{18}F was rapidly accumulated unchanged in the kidney following inhibition of NEP/ECE, with no evidence of conversion by another pathway (Johnström *et al.*, 2010).

The effect of a combined NEP/ECE inhibitor has been tested in volunteers in a randomised, double blinded trial. Following oral administration of three increasing doses of SLV306 (to reach an average target concentration of 75, 300, 1200 ng ml^{-1} of the active metabolite KC-12615), big ET-1 was infused into thirteen male volunteers at a rate of 8 and 12 pmol $kg^{-1}min^{-1}$ (20 min each). At the two highest concentrations tested, SLV306 dose dependently attenuated the rise in blood pressure after big ET-1 infusion. There was a significant increase in circulating big ET-1 levels compared with placebo, indicating that SLV306 was inhibiting an increasing proportion of endogenous ECE activity. Importantly, plasma ANP concentrations also significantly increased, consistent with systemic NEP inhibition (Seed *et al.,* 2012).

SLV306 in animal models and patients with type 2 diabetes and nephropathy

Diabetes causes activation of the renal endothelin system which leads to progressive renal

damage by cell proliferation and interstitial inflammation. Inhibitors of the renin–angiotensin system are widely used in treatment for hypertensive patients with type 2 diabetes but are less effective in the advanced stages of diabetic renal disease. Studies suggested in an animal model that SLV306 had a similar efficacy to the angiotensin enzyme inhibitor captopril in reducing proteinuria and preventing nephrosclerosis (Thöne-Reinke *et al.* 2004). In this study, rats were treated with streptozotocin for twenty weeks and the effects of SLV306 (30 mg kg^{-1} per day) compared with captopril (10 mg kg^{-1} per day). SLV306 significantly decreased renal interstitial matrix content as well as protein and albumin excretion in diabetic rats, independent of blood pressure and was as effective as captopril. These results suggested SLV306 treatment on top of blocking the rennin-angiotensin system might have additional benefit in reducing blood pressure and improving renal function.

Parvanova *et al.* (2013) tested the efficacy of SLV306 in 45 patients with type 2 diabetes mellitus who had albuminuria, on top of the angiotensin receptor antagonist losartan together with up to two additional anti-hypertensive drugs, in a randomised, crossover, double-blind, placebo controlled trial. Although eight weeks of treatment with SLV306 together with losartan did not significantly alter urinary albumin excretion or renal haemodynamic measures, the authors showed for the first time the combination decreased ambulatory blood pressure (particularly for systolic hypertension) in this patient group that are often resistant to treatment. There was a small but significant increase in plasma big ET-1, consistent with ECE inhibition, but surprisingly not in proANP; increases in the natriuretic peptides was measured in healthy volunteers by Seed et al, (2012). Interestingly, the effect of SLV306 in this study on blood pressure was higher at night (10 versus 12 mm Hg). This is of potential importance since increased hypertension at night is a strong cardiovascular risk factor in this patient population. The molecular mechanism is not yet known, as plasma levels of big ET-1 were not reported separately for day time versus night. The study was comparatively short

and did not reveal significant changes in albumin excretion as predicted from animal studies; long-term trials are required to determine whether the observed lowering of blood pressure by SLV306 will translate into longer term renal and cardio-protection.

SLV306 and congestive heart failure

The effect of three single oral doses of SLV306 was tested in patients with congestive heart failure who underwent right-sided heart catheterization in a randomized, double-blind, placebo-controlled design (Dickstein *et al.,* 2004). Pulmonary pressures and right atrial pressure decreased significantly in all SLV306 dose groups with the maximum decrease occurring at 6 to 8 hours. Despite plasma levels of the drug increasing with dose, there was no clear dose-response relationship which may have been the result of the comparatively small numbers (18-20) in the study.

Insight on NEP/ECE inhibition from animal models

The efficacy of inhibiting NEP/ECE in animal models associated with increases in the ET signalling pathway has provided clues to future clinical applications. The development of nephropathy in diabetes is associated with a poor outcome, eventually leading to end-stage renal disease. In diabetic patients urinary excretion of protein and albumin rises and is associated with increased risk of cardiovascular disease. In diabetic rats, SLV306 decreased renal matrix protein content, protein and albumin excretion. The magnitude of these effects was comparable to angiotensin converting enzyme inhibition and independent of blood pressure (Thöne-Reinke *et al.,* 2004). Currently there are few drugs for the treatment of chronic renal failure. SLV338, a NEP/ECE-inhibitor, abolished renal tissue damage (interstitial fibrosis, glomerulosclerosis, renal arterial remodelling) in a rat models of both acute kidney failure as well as chronic renal failure. The compound preserved kidney

function and reduced mortality (Sharkovoska *et al.*, 2011). In spontaneously hypertensive stroke-prone rats SLV338 significantly improved survival in comparison to the vehicle treated group in a blood pressure-independent manner and could offer a new therapeutic approach for primary stroke prevention and improvement of mortality (Wengenmayer *et al.*, 2011). SLV338 was also tested for cardiac protection in rat model of experimental renovascular hypertension (2-kidney, 1-clip). SLV338 prevented cardiac remodelling to the same extent as losartan, but in a blood pressure-independent manner. This effect is at least partially mediated via suppression of cardiac transforming growth factor-β1 expression (Kalk *et al.*, 2011).

ET has been proposed to be a mediator in toxic liver injury. However, while SLV338 largely prevented the activation of the ET system it did not prevent D-galactosamine-induced acute liver injury in rats. The authors speculated that SLV388 should be tested in a less severe model of liver injury, since very severe intoxication might not be relevantly amenable to pharmacological interventions (Hocher *et al.*, 2011).

ECE-1 and amyloid deposition

The strategy in cardiovascular and renal systems has been to *inhibit* ECE-1 activity. However, evidence is emerging that ECE-1 may function in the brain as a novel enzyme degrading amyloid β-peptides at multiple sites. Deposition of amyloid in the brain in Alzheimer's disease is determined not only by its production but also by its catabolism. ECE-1 inhibition produces, in addition to extracellular accumulation, intracellular amyloid β-peptides accumulation within endosomal/lysosomal and autophagic vesicles and an intracellular pool is partly regulated by ECE activity at the sites of production. Reduction in ECE activity leads to accumulation of amyloid β-peptide which is associated with neurotoxicity early in progression of Alzheimer's disease (Eckman *et al.*, 2001, 2003, 2006;

Pacheco-Quinto and Eckman, 2013). The clearance of $A\beta_{1-40}$ in mice was almost completely inhibited by phosphoramidon as well as insulin indicating that human $A\beta_{1-40}$ was degraded, at least in part, by a phosphoramidon-sensitive pathway, implicating both ECE and NEP (Ito *et al.*, 2013).

To date these investigations have comprised *in vitro* or *in vivo* rodent studies It is not yet clear whether *enhancing* ECE-1 activity is a potential drug target in Alzheimer's rather than inhibiting ECE-1, as in the periphery. ECE-like immunoreactivity has been localised to afferent and efferent fibres of neurones and neuronal cell bodies of mixed morphology in human brain (Giaid *et al.*, 1991). Drugs increasing ECE activity such as enzyme enhancers or recombinant ECE would have to cross the blood brain barrier and it is not clear what effect this would have on the peripheral ET signalling.

What are the new ET drug targets in the future?

Epigentics

Epigenetics can be defined as heritable changes in phenotype through mechanisms other than changes in DNA sequence. Epigenetic changes will therefore be preserved when cells divide and affect normal development and disease progression. Processes mediating epigenetic regulation include DNA methylation and histone modification which involves post-translational covalent modification of histone proteins by a range of writers, erasers and readers. This in turn modulates the ability of associated DNA to be transcribed. The histone code is read by specific families of proteins such as the bromodomains. These are of pharmacological significance because of the recent discovery of small molecule inhibitors, which selectively modulate gene expression (Prinjha *et al*., 2012).

Epigenetic regulation is of particular importance in the ET pathway with transcription being the primary level of ET-1 regulation of the gene EDN1 by histone modifications and

DNA methylation (Welch *et al*., 2012). Silencing of the EDNRB gene by DNA methylation during development of tumours results in the down-regulation of the receptor. As a result, promotion of apoptosis via the ET_B receptor is reduced or lost, suggesting the ET_B receptor could be a target for epigenetic drugs or ET_B agonists where ET may be the cause of some tumour types, including melanomas and oligodendrogliomas (Bagnato *et al*., 2011). Intriguingly, epigenetic inactivation of ET-2 and ET-3 mRNA and protein was found in rat and human colon tumours and cancer cell lines, as a result of hypermethylation of EDN2 and EDN3 genes. Restoring expression of ET-2 and ET-3 in human cells significantly attenuated the migration and invasion of human colon cancer cells (Wang *et al.,* 2013). Since ET-3 displays high affinity for the ET_B receptor, forced expression of ET-3 might antagonise the actions of ET-1 mediated through ET_A receptors. Such a mechanism would be consistent with proposed beneficial effects of IRL-1620, the ET_B agonist in cancer.

Life before birth – is ET a critical pathway?

Maternal malnutrition and uteroplacental vascular insufficiency causes foetal growth restriction or intrauterine growth retardation. Low birth weight is linked to the later development of cardiovascular disease and hypertension. Maternal treatment with dexamethasone increased ET-1 constrictor responses and ET_A receptor expression in placental arteries from the foetus (Kutzler *et al.,* 2003; Docherty *et al.,* 2003). Recently, Xu (*et al.,* 2103) have shown maternal nutrient restriction increased the histone acetylation and hypoxia inducible factor-1α (HIF-1α) binding levels in the ET-1 gene promoter of growth restricted newborn rats, which continued to 6 weeks after birth. The authors speculate that that this intrauterine growth retardation could cause varying degrees of PAH later in life.

Biased signalling in the ET pathway

Pharmacology is undergoing a revolution in understanding the mechanism of 'biased

signalling' via G protein-coupled receptors. It was originally thought that ligands binding to a receptor would equally activate the G protein pathway to produce a physiological response such as vasoconstriction (such as ET-1 acting on an ET_A receptor) as well as activating the β-arrestin pathway which eventually leads to desensitisation, receptor internalization and 'silencing' of the pathway. It is now clear that some ligands are biased to one pathway over the other and secondly, rather than silencing, β-arrestin can activate alternative signalling pathways, some of which may be pathophysiological leading to longer term signalling responses such as migration and proliferation.

Both ET receptor subtypes follow an β-arrestin and dynamin/clathrin-dependent mechanism of internalization, but it has been established that ET_A are recycled to the plasma membrane for further signalling while ET_B are targeted to lysosomes and degraded (Bremnes *et al.*, 2000). In epithelial ovarian cancer, activation of ET-1/ET_A receptor signalling is linked to many tumour promoting processes including proliferation, angiogenesis, invasion and metastasis. NF-κB is an important signalling molecule in immunity, inflammation and cancer and β-arrestin is required for ET-1-induced NF-κB activation (Cianfrocca *et al.*, 2014). ET-1 promoted podocyte migration via ET_A receptors and increased β-arrestin-1, sustaining renal injury, a pathogenetic pathway that can affect podocyte phenotype in proliferative glomerular disorders (Buelli *et al.*, 2014). β-Arrestin-1 has also been found to be a nuclear transcriptional regulator of ET-1-induced β-catenin signalling, an important mechanism for controlling cell division and progression of epithelial ovarian cancer and necessary for epigenetic modification, such as histone acetylation, and gene expression (Rosanò *et al.*, 2013, 2009). In addition these effects are blocked by ET receptor antagonists and support a role for ET_A-mediated/β-arrestin-1 facilitating inter-protein interaction in invasive and metastatic behaviour of ovarian cancer.

Biased ET ligands?

Agonists have been identified that are biased towards β-arrestin signalling for parathyroid hormone and AT1 receptors. G protein pathway-selective agonists have been identified for nicotinic acid and μ opioid receptors (Luttrell, 2014). The race is now to determine whether such strategies can be exploited therapeutically.

Do biased ligands (ligands that binding to the same receptor but activate different signalling pathways) exist for ET receptors? The study by Compeer *et al.* (2013) already mentioned suggested ET-1 and ET-2 initiate and maintain vasoconstriction by different downstream mechanisms. Biased signalling can be identified by comparing the affinities of ligands in β-arrestin recruitment assays with a G protein mediated response such as vasoconstriction (Maguire *et al.*, 2012). In this study the rank order of potency for β-arrestin recruitment at the ET_A (ET-1≥ET-2>>ET-3) and ET_B (ET-1=ET-2=ET-3) receptors was as expected and there was no obvious major differences in potency of ETs when comparing with G protein mediated constrictor assays in human vessels. However, at the ET_A receptor sarafotoxin S6b was a partial agonist in β-arrestin recruitment but a full agonist in causing constriction, suggesting the possibility of biased ligands. Such a bias could have been selected for during evolution by prolonging the effects of envenomation of the mammalian prey. While bosentan displays no selectivity for ET_A over ET receptors in radioligand binding and G protein functional assays, unexpectedly it was significantly more effective an inhibitor of β-arrestin recruitment mediated by ET_A compared to the ET_B receptor (Maguire *et al.*, 2012). The result for bosentan is intriguing since many of the detrimental actions of ET-1, particularly in cancer, may use the β-arrestin pathway and this suggests the potential to block a deleterious pathway whilst preserving activation of a beneficial pathway.

The next twenty-five years –quo vadis?

Since the discovery of ET intense medicinal chemistry programmes have identified receptor antagonists, ET_B receptor agonists and inhibitors of the key synthetic enzyme, ECE-1. Both mixed ET_A/ET_B and ET_A selective antagonists have become established in the treatment of PAH, while NEP/ECE inhibitors such as SLV306 have promise as an alternative to receptor blockage and IRL1620 and other ET_B analogues have potential in improving cancer therapy.

All of these are approaches that have exploited small molecules. Over 50 therapeutic monoclonal antibodies have been approved for clinical use, but none yet against a GPCR target, emphasising the technical challenge. Endomab-B1, a monoclonal antibody has recently been reported to bind with sub-nanomolar affinity for the ET_B receptor, competed with ET-1 binding with greater efficacy than BQ788, and functions as an antagonist to block the ET-1-induced IP_3-calcium signalling pathway (Allard et al., 2013). Whether this antibody has clinical applications remains to be discovered.

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Conflicts of Interest

None

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Figure Legends

Figure 1. Schematic diagram illustrating synthesis of ET peptides and interaction with receptors.

Figure 2. Structures of ET receptor antagonists in clinical use bosentan, ambrisentan and macitentan. The structures of the NEP/ECE inhibitor pro-drug SLV306 and its active metabolite are also shown.

Figure 3. Selectivity of ET receptor antagonists for ET_A versus ET_B receptors shown on the vertical axis as reported by the companies that discovered the compounds. Selectivity was mainly determined by measuring affinity constants in separate competition assays against [^{125}I]-ET-1 using human recombinant ET_A versus ET_B receptors and may not reflect selectivity measured in clinically relevant native tissues. Bosentan, ambrisentan and macitentan are currently approved for clinical use and are highlighted.