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REVIEW

Role of the endothelin axis and its antagonists in the treatment of cancer

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The endothelins (ET) are a group of proteins that act through G-protein coupled receptors. Endothelin-1 (ET-1) was initially identified as a potent vasoconstrictor and dysregulation of the ET axis contributes to pathological processes responsible for cardiovascular disease states. More recently, the ET axis, in particular ET-1 acting through the endothelin A receptor (ET_A), has been implicated in the development of several cancers through activation of pathways involved in cell proliferation, migration, invasion, epithelial-mesenchymal transition, osteogenesis and angiogenesis. The endothelin B receptor (ET_B) may counter tumour progression by promoting apoptosis and clearing ET-1; however, it has recently been implicated in the development of some tumour types including melanomas and oligodendrogliomas. Here, we review emerging preclinical and clinical data outlining the role of the ET axis in cancer, and its antagonism as an attractive and challenging approach to improve clinical cancer management. Clinical data of ET_A antagonists in patients with prostate cancer are encouraging and provide promise for new ET_A antagonist-based treatment strategies. Given the unexpected opportunities to affect pleiotrophic tumorigenic signals by targeting ET_A-mediated pathways in a number of cancers, the evaluation of ET-targeted therapy in cancer warrants further investigation.

Abbreviations

CHF, chronic heart failure; CRPC, castration-resistant prostate cancer; ECE, endothelin-converting enzyme; ET, endothelin; ET_A, endothelin A receptor; ET_B, endothelin B receptor; GPCR, G-protein coupled receptor; ITT, intent to treat; MTD, maximum tolerated dose; NEP, neutral endopeptidase; NSCLC, non-small-cell lung cancer; OS, overall survival; PFS, progression-free survival; PPC-1, prostate cancer cell line; PSA, prostate-specific antigen; TTP, time to tumour progression

Introduction

The endothelin (ET) axis consists of three 21-amino acid peptides, ET-1, ET-2 and ET-3, two distinct rhodopsin-like G-protein coupled receptor (GPCR) subtypes, endothelin A and endothelin B (ET_A and ET_B respectively) and the endothelin-converting enzymes (ECEs), which catalyze the generation of biologically active ET (Rubanyi and Polokoff, 1994). The first major role that was identified for ET-1 was as a potent vasoconstrictor (Rubanyi and Polokoff, 1994). The ET axis has also been shown to play a key role in a number of tissues and systems including somatosensory, respiratory, circulatory, endocrine, urogenital, visual, digestive and the central nervous system (CNS) (Rubanyi and Polokoff, 1994). Dysregulation of the ET axis was initially shown to contribute to the pathological processes responsible for cardiovascular disease states including systemic and pulmonary hypertension, and congestive heart failure (Rubanyi and Polokoff, 1994; Goldie, 1999). More recently, the ET axis has been implicated in a number of other cell signalling pathways such as apoptosis and cell growth (Bagnato and Natali, 2004), which are also common with other GPCR-based signalling pathways such as those induced by angiotensin II (Leung, 2004). The identification of the ET axis in such signalling pathways led to the investigation of its role in the development and progression of cancer. The ET axis was shown to have a relevant role in various cancer cells and stromal cells leading to autocrine/paracrine feedback loops, which promote the development and progression of tumours. Such processes include cell proliferation, escape from apoptosis, angiogenesis, invasion and metastatic dissemination, aberrant osteogenesis and modification of nociceptive stimuli (Nelson *et al.*, 2003). This review will discuss the role of the ET axis in cancer, the pharmacological processes of ET receptor antagonism and

recent advances in the development of ET-targeted treatments for patients with cancer.

Endothelin function in normal physiology and pathology

ET-1 is primarily secreted by endothelial cells through both constitutive and regulated (or rapid release) pathways (Russell and Davenport, 1999). In addition, ET-1 is secreted to a lesser extent by a range of other cell types including macrophages, leukocytes, fibroblasts, vascular smooth muscle cells (VSMC), cardiomyocytes, tubular epithelial cells, mesangial cells and podocytes (Nunez *et al.*, 1990; Resink *et al.*, 1990; Firth and Ratcliffe, 1992; Kohan, 1997). ET-2 is secreted in the kidney and intestine, and ET-3 in the brain, intestine, lung and kidney. Two pathways for the clearance of ET-1 have been identified. The first of these is ET_B-mediated uptake followed by lysosomal degradation (Bremnes *et al.*, 2000) and the second is through the catabolism of ET-1 by extracellular neutral endopeptidase 24.11 (NEP, neprilysin) (Abassi *et al.*, 1992).

The downstream effects of ET-1 are mediated by two distinct receptor subtypes, ET_A and ET_B (Rubanyi and Polokoff, 1994). These receptors display different ligand selectivity; however, both receptors bind to ET-1 with equal affinity (Davenport, 2002). The intracytoplasmic C termini of the two ET receptor types differ, which results in their divergent intracellular effects following activation by ET-1 (Nussdorfer *et al.*, 1999). ET_A and ET_B are differentially expressed in a variety of normal tissues including the vasculature (VSMC, heart, cardiomyocytes, fibroblasts), CNS (trigeminal nerve, brain cells, small diameter sensory neurons), renal epithelial and endothelial cells, prostate, breast and female reproductive tissues (Molenaar *et al.*, 1993; Karet and Davenport, 1996; Kuc and Davenport, 2004; Schinelli, 2006; Smollich and Wülfing, 2008; Khodorova *et al.*, 2009; Chichorro *et al.*, 2010). This differential tissue expression of the ET receptor subtypes is dynamic and contributes to the different actions of the three ETs (Levin, 1995; Nelson *et al.*, 1996).

Extracellular binding of ET-1 to ET_A activates a non-linear, highly interconnected signalling network. Many processes activated by this network are involved in normal cell function and in the development and progression of cancer including cell proliferation, apoptosis, cell invasion and metastasis, angiogenesis, osteogenesis and nociception (Figure 1) (Smollich and Wülfing, 2007; Bagnato and Rosanò, 2008; Nelson, 2009). Dysregulation of ET_A activation can therefore promote tumour development and progression. Moreover, many cancerous cell types, including prostate, ovarian, renal, pulmonary, colorectal, cervical, breast, bladder and melanoma, have been reported to secrete ET-1, and the ET axis has been implicated in a range of tumours suggesting that it is a very attractive target for cancer therapy (Asham *et al.*, 2001; Nelson, 2003; Bagnato *et al.*, 2005; Bagnato and Rosanò, 2008). Indeed, ET-1 plasma levels were increased in patients with colorectal cancer as well as in a syngeneic rat model of colorectal cancer in which inhibition of the ET_A with a selective antagonist (BQ123) significantly reduced tumour weight of metastatic lesions to the liver (Asham *et al.*,

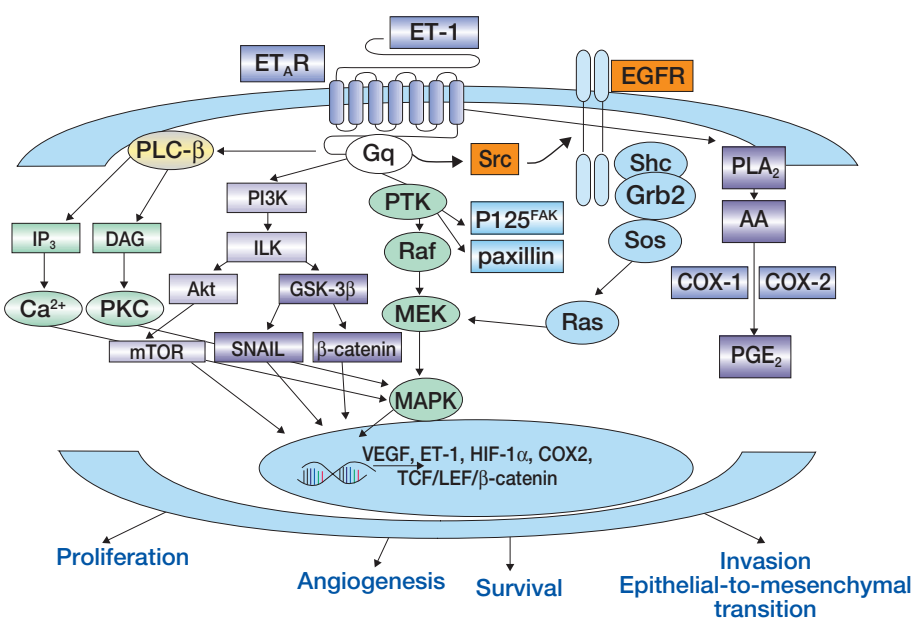


Figure 1

Endothelin-1 (ET-1) signalling through the endothelin A receptor (ET_A) in cancer cells (Bagnato and Rosanò, 2008). Reprinted with permission from Elsevier. AA, arachidonic acid; COX-1, cyclo-oxygenase 1; COX-2, cyclo-oxygenase 2; DAG, diacylglycerol; EGFR, epidermal growth factor receptor; ET_AR, endothelin A receptor; Gq, G protein q; GSK-3, glycogen synthase kinase-3; HIF-1α, hypoxia-inducible factor 1-α; ILK, integrin-linked kinase; IP3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MEK, mitogenactivated protein kinase/extracellular signal-regulated kinase kinase; mTOR, mammalian target of rapamycin; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PTK, protein-tyrosine kinase; VEGF, vascular endothelial growth factor.

2001). Alterations in the relative ratios of ET_A to ET_B can incite the progression of cells from a normal phenotype to a more malignant phenotype (Nelson *et al.*, 1996; 1997; Bagnato *et al.*, 1999; Godara *et al.*, 2005). In fact, down-regulation of ET_B expression has been reported in many tumour types including prostate cancer (Nelson *et al.*, 1996; Bagnato and Rosanò, 2008). In addition, the ET_B gene is frequently hypermethylated leading to reduced or absent receptor expression (Nelson *et al.*, 1997; Pao *et al.*, 2001; Jerónimo *et al.*, 2003). Increased ET_A expression was observed with advancing tumour stage and grade in patients with local and metastatic prostate cancer (Nelson, 2003). Furthermore, the ET-1 clearance pathway is disrupted in prostate cancer; ET_B expression and NEP protein levels are reduced resulting in increased local ET-1 levels (Nelson, 2003; Bagnato and Rosanò, 2008). ET-1 and ET_A are also overexpressed in primary and metastatic ovarian tumours and dysregulation of this autocrine signalling pathway is believed to be a driver of disease progression (Salani *et al.*, 2000; Bagnato *et al.*, 1995; 2005).

In normal cells and a number of tumour types, activation of ET_B by ET-1 has been shown to affect processes involved in the inhibition of cancer; inducing cell death by apoptosis and promoting ET-1 clearance (Okazawa *et al.*, 1998; Dupuis *et al.*, 2000). Recently, ET_B activation has been implicated in a range of cancers. ET_B is overexpressed in melanomas (Lahav, 2005) and oligodendrogliomas (Anguelova *et al.*, 2005), and has been shown to correlate with malignant melanoma development and progression (Lahav, 2005). In addition, gene expression profiling and immunohistochemical analysis of human melanoma biopsies and cell lines indicated the ET_B as a tumour progression marker associated with an aggressive phenotype (Bittner *et al.*, 2000; Demunter *et al.*, 2001). However, reports of the overexpression of ET_B in some tumour types, such as lung cancer, have been conflicting which may be reflective of the methodological variations used to detect ET_B (Ahmed *et al.*, 2000; Knight *et al.*, 2009). Activation of ET_B by ET-1 or ET-3 has been shown to trigger downstream pathways involved in the progression of cutaneous melanoma and blockade of ET_B inhibited human melanoma xenograft growth (Bagnato *et al.*, 2004). Furthermore, inhibition of ET_B has been shown to block glioma cell proliferation and induce apoptosis (Paolillo *et al.*, 2010).

Increased vasodilation in tumours can occur through ET_B, which may aid in the delivery of anticancer treatments to the tumour. Indeed, the selective ET_B agonist SPI-1620 (IRL-1620) was shown to selectively and transiently increase tumour blood flow, allowing increased delivery of chemotherapy agents to tumours in a breast cancer model in rats and a solid tumour model in dogs (Rai *et al.*, 2006; Selting *et al.*, 2008).

The role of ET in immune response was initially established in dendritic cells which were shown to produce large amounts of ET-1 and significantly increase the expression of ET receptors upon maturation (Guruli *et al.*, 2004). Selective blockade of ET_A significantly reduced expression of the mature dendritic marker CD83, down-regulated dendritic cell ability to stimulate T cells and promoted dendritic cell apoptosis, whereas selective ET_B blockade resulted in increased expression of CD83 and improved dendritic cell survival. ET_B has also been shown to have a potential role in immune responses associated with cancer (Guruli *et al.*, 2004; Juergens *et al.*, 2008; Kandalaft *et al.*, 2009; Verri *et al.*, 2009; Wang *et al.*, 2010). Overexpression of ET_B in tumour endothelium has been observed in ovarian tumours, and activation of these receptors by ET-1 was reported to suppress T-cell adhesion and homing to tumours (Kandalaft *et al.*, 2009). The success of immune therapy depends on the ability of effector T cells to infiltrate tumours. ET_B blockade with the selective ET_B antagonist BQ788 increased T-cell homing to tumours, which resulted in the tumour responding to otherwise ineffective immunotherapy *in vivo* without changes to the systemic antitumour immune response (Buckanovich *et al.*, 2008). It has therefore been suggested that ET_B antagonists warrant clinical testing in combination with passive or adaptive immunotherapy. This approach may help enable selective up-regulation of immunotherapy directly to the tumour compartment via the ET_B (Kandalaft *et al.*, 2009). In addition, this therapy combination may inhibit the significant autoimmune toxicities observed with current immunomodulatory approaches (Kandalaft *et al.*, 2009).

There is a growing body of evidence implicating ET-2 in the progression of cancer. Up-regulation of ET-2 mRNA expression was observed in several human breast cancer tumour cell lines and incubation of these cell lines with ET-2 induced chemotaxis (Grimshaw *et al.*, 2004). More recently, ET-2 mRNA was reported to be overexpressed in basal cell carcinoma compared with normal skin, an effect controlled by the Hedgehog signalling pathway (Tanese *et al.*, 2010).

The first study investigating the role of ET-3 in cancer has recently been reported. In this study, ET-3 mRNA expression and protein levels were attenuated in breast cancer tissues compared with normal tissue, an effect caused by hypermethylation of the ET-3 promoter and subsequent gene silencing (Wiesmann *et al.*, 2009). These preliminary data suggest that unlike ET-1 and ET-2, ET-3 may act as a natural tumour suppressor in breast cancer.

ECE-1 expression is significantly elevated in tumours and ECE-1 has been reported to be increased in primary malignant stromal cells compared with benign cells (Dawson *et al.*, 2004). Inhibition of stromal ECE-1 reduced PC-3 (prostate cancer) cell invasion in co-culture and the inclusion of ET-1 to such cultures only partially recovered this effect, suggesting a role for ECE-1 independent of ET-1 activation. A recent investigation of ECE-1 isoforms demonstrated that ECE-1c overexpression increased PC-3 invasion through Matrigel™, whereas ECE-1a overexpression suppressed invasion (Lambert *et al.*, 2008). The ECE-1 isoforms may therefore be relevant targets for the treatment of cancers including castration-resistant prostate cancer (CRPC); however, in terms of 'drugability', the approach of ECE inhibition is something that has not met with much success in the clinic. Theoretical limitations that have potentially had an impact on the success of ECE inhibition have been proposed, including redundancy of the ET-1 generating pathways, non-ECE generating big-ET-1 processing enzymes, potential to reduce beneficial effects of the ligand at respective ET receptors and 'drug-killing'/development-limiting side effects that this approach has brought (Kirkby *et al.*, 2008).

Targeting the ET axis as an anticancer approach

It is currently unknown whether increased plasma ET-1 levels are the cause or consequence of cancers. However, in some cancers such as CRPC, elevated plasma levels have been reported in patients with advanced metastatic disease relative to those with earlier stage disease (Nelson *et al.*, 1995; 2003). In colorectal adenomas, increased expression of pre-pro ET-1 and ECE mRNA was observed compared with normal colon (Egidy *et al.*, 2000). In breast carcinoma, ET-1 immunoreactivity and mRNA levels were greater in breast ductal carcinoma *in situ* specimens compared with normal breast tissue (Alanen *et al.*, 2000). These data suggest that higher ET-1 levels in plasma correlate with disease severity and that the modulation of the ET system is an early event in tumorigenesis. Moreover, there is evidence that in cancers, including CRPC, the gene regulating the ET_B (which would normally promote apoptosis and clearance of ET-1) has undergone hypermethylation (Knight *et al.*, 2009) which could lead to increased ET-1 and provide a basis for increased cell survival. Indeed, in a recent study the ET_B promoter was significantly hypermethylated in nearly 50% of non-small-cell lung cancer (NSCLC) tumour samples investigated. Furthermore, these samples also had reduced ET_B mRNA levels compared with unmethylated samples. These data suggest an involvement of ET_B epigenetic deregulation in the development and progression of lung cancer and highlight this gene as a promising biomarker for response to regimens modulating the ET axis (Knight *et al.*, 2009). There is also evidence of a similar hypermethylation and de-activation of the ET_B in prostate cancer (Nelson *et al.*, 1997). Somatic methylation of CpG island sequences in the ET_B in 5/5 human prostate cancer cell lines, 15/21 primary prostate cancer tissues and 8/14 prostate cancer metastases (70% of samples overall) was observed. Normal tissues contained only unmethylated ET_B. Treatment of human prostatic carcinoma cell line cultures with 5-azacytidine induced ET_B mRNA expression, suggesting that CpG island methylation changes might accompany the apparent transcriptional silencing of ET_B *in vivo*.

Whether the reduction in ET_B functionality *per se* leads to increased binding of the ligand to the ET_A remains to be elucidated. The loss of ET_B functionality will permit ET-1 to reside and accumulate which may drive increased receptor expression to accommodate increasing concentrations of ligand. ET_A/ET-1 expression analyses in tumour samples from patients tend to support that up-regulation or increase in ligand and receptor expression do occur and these phenomena support increased binding of the ligand to ET_A.

Inhibition of the ET axis using specific, selective and dual competitive ET receptor antagonists represents an attractive targeted approach for the treatment of cancer. There are currently over 15 ET_A and/or ET_B antagonists being evaluated in clinical trials for a variety of indications, including cardiovascular disease and cancer. The selective ET_A and ET_B antagonists BQ123 and BQ788 have been valuable tools for the assessment of ET receptor antagonism and have been used extensively in preclinical models. However, as these agents are both peptides their utility in the clinical setting has been limited. Although no data in humans with BQ788 have been published, a number of studies have been performed using BQ123, generally in a setting in which access to small molecule non-peptide agents has not been possible (Spratt *et al.*, 2001). To date, the dual ET_A/ET_B antagonist bosentan, the selective ET_A antagonists atrasentan and YM-598, and the specific ET_A antagonist zibotentan are the only ET receptor antagonists that have been evaluated in both the preclinical and clinical oncology settings (Table 1).

BQ123 and BQ788 (Banyu Pharmaceutical Co; Merck)

BQ123 is a highly soluble, potent, and selective ET_A antagonist with IC_{50} values of 8.3 nM and 61 μM for human ET_A and ET_B, respectively (Ishikawa *et al.*, 1992), and in isolated porcine coronary arteries, BQ123 had an antivasoconstriction pA_2 value of 7.4 (Fukami *et al.*, 1995). BQ123 was the first ET_A antagonist to be developed and its use in preclinical models has had great value in demonstrating a role for the ET_A in cancer cell growth, proliferation, survival, migration and invasion, and pain (Rosanò *et al.*, 2001; Del Bufalo *et al.*, 2002; Grant *et al.*, 2007; Schmidt *et al.*, 2007; Zhang *et al.*, 2008). BQ123 remains widely used in preclinical studies defining the physiology of the ET axis (Battistini *et al.*, 2006); however, due to the cost of its development and the need for parenteral administration (the peptide is hydrolyzed by peptidases in the systemic circulation and gastrointestinal tract) (Motte *et al.*, 2006), the use of BQ123 has been limited to small-scale clinical trials.

BQ788 is a potent and selective ET_B antagonist with IC_{50} values of 1300 nM and 1.2 nM for human ET_A and ET_B respectively (Ishikawa *et al.*, 1994). In isolated rabbit pulmonary arteries rich in ET_B, BQ788 potently antagonized vasoconstriction produced by an ET_B selective agonist with a pA_2 value of 8.4 (Ishikawa *et al.*, 1994). Preclinical studies with BQ788 have reported a role for ET_B in the survival, growth and metastasis of melanoma and glioma cells (Lahav *et al.*, 1999; Lahav, 2005; Paolillo *et al.*, 2010). Furthermore, BQ788 inhibited several pathways mediated by ET-1, including bronchoconstriction and cell proliferation, and was also shown to inhibit clearance of perfused ET-1 (Okada and Nishikibe, 2002). BQ788 has also been used to identify the role of ET_B in control of vascular tone in vessels that supply tumours, implying that ET-1 via ET_B dilates vessels supplying breast tumours in the rat (Gulati and Rai, 2004) and that arteries supplying human colorectal tumours were more sensitive to ET-1 due to increased ET_B responsiveness (Ferrero *et al.*, 2008). More recently, BQ788 has been used to identify the role of ET_B in the growth and invasion of lymphatic endothelial cells and vessels (Spinella *et al.*, 2009) and mediation of the endothelial barrier in T-cell homing to tumours (Buckanovich *et al.*, 2008). As with BQ123, the use of this antagonist in clinical trials has been limited because of the cost of development and the systemic method of its administration (Battistini *et al.*, 2006).

Bosentan (Actelion Pharmaceuticals Ltd)

Bosentan is a dual competitive ET_A and ET_B antagonist, which competitively inhibited the specific binding of ^{125}I-ET-1 on ET_A rich human smooth muscle cells with an inhibitor constant (Ki) of 4.7 nM, and on ET_B rich human placenta with a Ki of 95 nM. In addition, ET-1-induced contractions in

Table 1

Clinical evaluation of endothelin antagonists in cancer*

Compound (target receptor)	Study population and intervention	Trial status	Results and conclusions
Bosentan (Dual competitive ET_A/ET_B)	Phase II, metastatic melanoma (monotherapy; $n = 35$) (Kefford *et al.*, 2007)	Completed	• Stable disease seen in six patients at 12 weeks • No treatment responses
	Phase II, metastatic melanoma (in combination with dacarbazine, placebo-controlled; $n = 80$) (Kefford *et al.*, 2010)	Completed	• No difference in time to progression seen at 12 months
YM598 (Selective ET_A)	Phase II, prostate cancer (monotherapy)	Terminated	
	Phase II, prostate cancer (in combination with mitoxantrone and prednisone)	Terminated	
Atrasentan (Selective ET_A)	Phase III, metastatic prostate cancer (placebo-controlled, monotherapy; $n = 809$) (Carducci *et al.*, 2007)	Completed	• No reduction in disease progression • Study design and prior assumption of progression rates may have limited the ability to define clinical benefit
	Phase III, non-metastatic prostate cancer (placebo-controlled, monotherapy; $n = 941$) (Nelson *et al.*, 2008)	Completed	• No statistically significant difference in time to progression • Large regional differences in TTP suggest trial conduct may have influenced results
	Phase III, prostate cancer with bone metastases (in combination with docetaxel and prednisone)	Ongoing	(data not yet available)
	Phase II, hormone-refractory prostate cancer (double-blind, randomized monotherapy; $n = 288$) (Carducci *et al.*, 2003)	Completed	• Trend towards prolongation of disease • Statistically significant delay in PSA
	Phase II, hormone-naive prostate cancer (monotherapy)	Completed	(data not yet available)
	Phase II, prostate cancer with bone metastases (in combination with zoledronic acid; $n = 44$) (Michaelson *et al.*, 2006)	Completed	• No evidence of additive or synergistic effects of combination therapy
	Phase I-II, metastatic prostate cancer (in combination with docetaxel; $n = 31$) (Armstrong *et al.*, 2008)	Completed	• Survival comparable to that seen with docetaxel and prednisone, but rate of PSA decline lower than expected
	Phase I-II, NSCLC (in combination with paclitaxel and carboplatin; $n = 44$) (Chiappori *et al.*, 2008)	Completed	• Lack of positive response data may reflect deficiencies in clinical trial design and low dose
	Phase II, renal carcinoma (monotherapy; $n = 94$) (Manola *et al.*, 2007)	Completed	• 6-month progression-free rates did not support use as first-line monotherapy
	Phase I, malignant glioma (monotherapy; $n = 25$) (Phuphanich *et al.*, 2008)	Completed	• Primarily a safety study, but two partial responses observed
Zibotentan (specific ET_A)	Phase III, prostate cancer with bone metastases (monotherapy)	Completed	(data not yet available)
	Phase III, non-metastatic prostate cancer (monotherapy)	Recruiting	(data not yet available)
	Phase III, prostate cancer, metastatic (in combination with docetaxel)	Ongoing	(data not yet available)
	Phase II, prostate cancer with bone metastases (placebo-controlled, monotherapy; $n = 312$) (James *et al.*, 2010)	Complete	• No statistically significant improvement in TTP, but overall survival extended versus placebo
	Phase II, prostate cancer, metastatic, patients previously treated with chemotherapy (monotherapy; $n = 24$)	Ongoing	(data not yet available)
	Phase II, NSCLC (in combination with pemetrexed)	Completed	(data not yet available)
	Phase I, metastatic prostate cancer (in combination with docetaxel; $n = 31$) (Trump *et al.*, 2010)	Completed	• Activity observed with the combination, meriting further evaluation
	Phase I, advanced solid malignancies in elderly Chinese patients (monotherapy)	Recruiting	(data not yet available)

*Not including planned clinical trials. ET_A, endothelin A receptor; ET_B, endothelin B receptor; NSCLC, non-small-cell lung cancer; PSA, prostate-specific antigen; TTP, time to tumour progression.

isolated rat aorta, and contractions induced by the selective ET_B agonist sarafotoxin S6C in rat trachea, were competitively antagonized by bosentan with pA_2 values of 7.2 and 6.0, respectively (Clozel *et al.*, 1994).

In preclinical studies of human melanoma cell lines, bosentan was observed to inhibit proliferation, decrease cell viability and DNA synthesis and induce apoptosis (Sekulic *et al.*, 2002; Berger *et al.*, 2006). These preclinical studies provided a rationale to investigate this agent in the clinical cancer setting. A single-arm, Phase II uncontrolled study indicated that bosentan monotherapy may be of benefit to patients with stage IV metastatic melanoma, achieving disease stabilization in 19% of patients at week 6, with confirmation at week 12; five patients still had stable disease after 24 weeks and two remained stable after more than 2 years on study treatment (Kefford *et al.*, 2007). Following these positive results, a Phase II randomized, double-blind, placebo-controlled proof-of-concept study in a similar patient population reported no beneficial effect on time to tumour progression (TTP) or other efficacy parameters [progression-free survival (PFS) and overall survival (OS)] when bosentan was combined with first-line dacarbazine chemotherapy (Kefford *et al.*, 2010). The authors of this study suggested that the failure of this trial may be due to the abnormally high TTP observed in the placebo group, the stringent selection criteria of patients in this study and/or the 50% risk reduction rate selected for efficacy (Kefford *et al.*, 2010).

Bosentan has been extensively investigated in the cardiovascular setting and it is licensed for the treatment of pulmonary arterial hypertension and reduction of digital ulcer formation in patients with systemic sclerosis. There are currently no ongoing trials of bosentan in the cancer setting.

YM-598 [Astellas Pharma (formerly Yamanouchi)]

YM-598 is a potent selective ET_A antagonist developed through the modification of bosentan. This agent inhibited ^{125}I-ET-1 binding to cloned human ET_A and ET_B with a Ki of 0.697 nM and 569 nM, respectively, and antagonized ET-1-induced vasoconstriction in isolated rat aorta with a pA_2 value of 7.6 (Yuyama *et al.*, 2003).

ET_A inhibition with YM-598 significantly reduced tumour growth and liver metastasis in an *in vivo* model of gastric cancer (Fukui *et al.*, 2007). In addition, YM-598 significantly inhibited ET-1-induced potentiation of nociception in murine models of cancer pain (Yuyama *et al.*, 2004a,b). The beneficial effect of YM-598 in preclinical models of cancer pain led to the initiation of two randomized Phase II clinical trials to investigate the impact of this agent on pain in patients. The first of these studies evaluated YM-598 monotherapy in patients with localized prostate cancer and the other investigated YM-598 combined with mitoxantrone and prednisone in patients with metastatic prostate cancer. Both trials were terminated due to a lack of pain reduction (Battistini *et al.*, 2006). Previous studies have demonstrated the difficulty in translating preclinical studies of pain perception into the clinical setting, particularly where comparison with opiate analgesia is concerned (Bell *et al.*, 2006), which may, in part explain the failure of these trials. The clinical development of YM-598 has been discontinued.

Atrasentan (Abbott Laboratories)

Atrasentan (ABT-627) is a selective ET_A antagonist, which competitively inhibited ^{125}I-ET-1 binding to cloned human ET_A and ET_B with Ki values of 69 pM and 139 nM, respectively, and antagonized ET-1-induced vasoconstriction in isolated rat aorta with a pA_2 value of 9.2 (Opgenorth *et al.*, 1996). Atrasentan decreased the binding affinity of ET-1 without affecting the receptor density, indicating that it is a competitive inhibitor of ET-1 binding, with 800- to 1800-fold selectivity for ET_A compared with ET_B (Wu-Wong *et al.*, 2002).

A range of preclinical investigations of atrasentan in the cancer setting have demonstrated its potential anticancer activity. In brief, atrasentan dose dependently inhibited ET-1-driven prostate cancer cell line (PPC-1) proliferation (Nelson *et al.*, 1996), inhibited neoangiogenesis in a cervical cancer xenograft model (Bagnato *et al.*, 2002) and reduced osteoblastic bone metastases in mice inoculated with the ZR-75-1 breast cancer line (Guise *et al.*, 2003). When atrasentan was combined with paclitaxel or docetaxel, additive antitumour, pro-apoptotic and antiangiogenic effects were observed in ovarian cancer cells and prostate cancer cells, respectively (Rosanò *et al.*, 2003; Banerjee *et al.*, 2007).

As shown in Table 1, a number of Phase II and Phase III clinical trials of atrasentan have been completed. Several of these were performed in men with CRPC. The largest Phase II trial enrolled 288 men with asymptomatic CRPC and radiographic evidence of metastatic disease. Patients were randomized to receive once-daily atrasentan 2.5 mg, 10 mg or placebo. The primary endpoint of TTP was significantly increased in a subset of evaluable patients [defined before unmasking of the study by excluding patients who did not meet study-defined prostate-specific antigen (PSA) or antiandrogen withdrawal inclusion criteria, were taking excluded medications, received less than 50% of scheduled doses or fewer than 20 total doses, or initiated excluded medications during the study; $n = 244$], from 129 days in the placebo group to 196 days in the atrasentan 10 mg group ($P = 0.021$). However, median TTP was not significant in the intent to treat (ITT) population (median 183, 178 and 137 days, for atrasentan 10 mg, 2.5 mg and placebo respectively; $P = 0.13$ and $P = 0.29$ for comparisons of atrasentan 10 mg and 2.5 mg with placebo, respectively). The secondary endpoint of time to PSA progression was significant in the ITT population with a median time of 155 days for atrasentan 10 mg, 141 days for atrasentan 2.5 mg and 71 days for placebo ($P = 0.002$ and $P = 0.055$ compared with placebo, respectively). In the evaluable population, median time to PSA progression was also significantly longer in the atrasentan 10 mg group compared with placebo ($P = 0.002$). A favourable tolerability profile of atrasentan was also observed in this patient population (Carducci *et al.*, 2003). This potential to delay the progression of CRPC along with the favourable tolerability profile led to the initiation of two Phase III studies of atrasentan in this disease setting. These Phase III, randomized, double-blind, placebo-controlled trials of once-daily atrasentan 10 mg in the metastatic and non-metastatic CRPC setting failed to meet their primary endpoint of TTP or their secondary endpoint of time to PSA progression (Carducci *et al.*, 2007; Nelson *et al.*, 2008). In the first of these studies in 809 patients with metastatic CRPC, atrasentan did not reduce the risk of disease progression

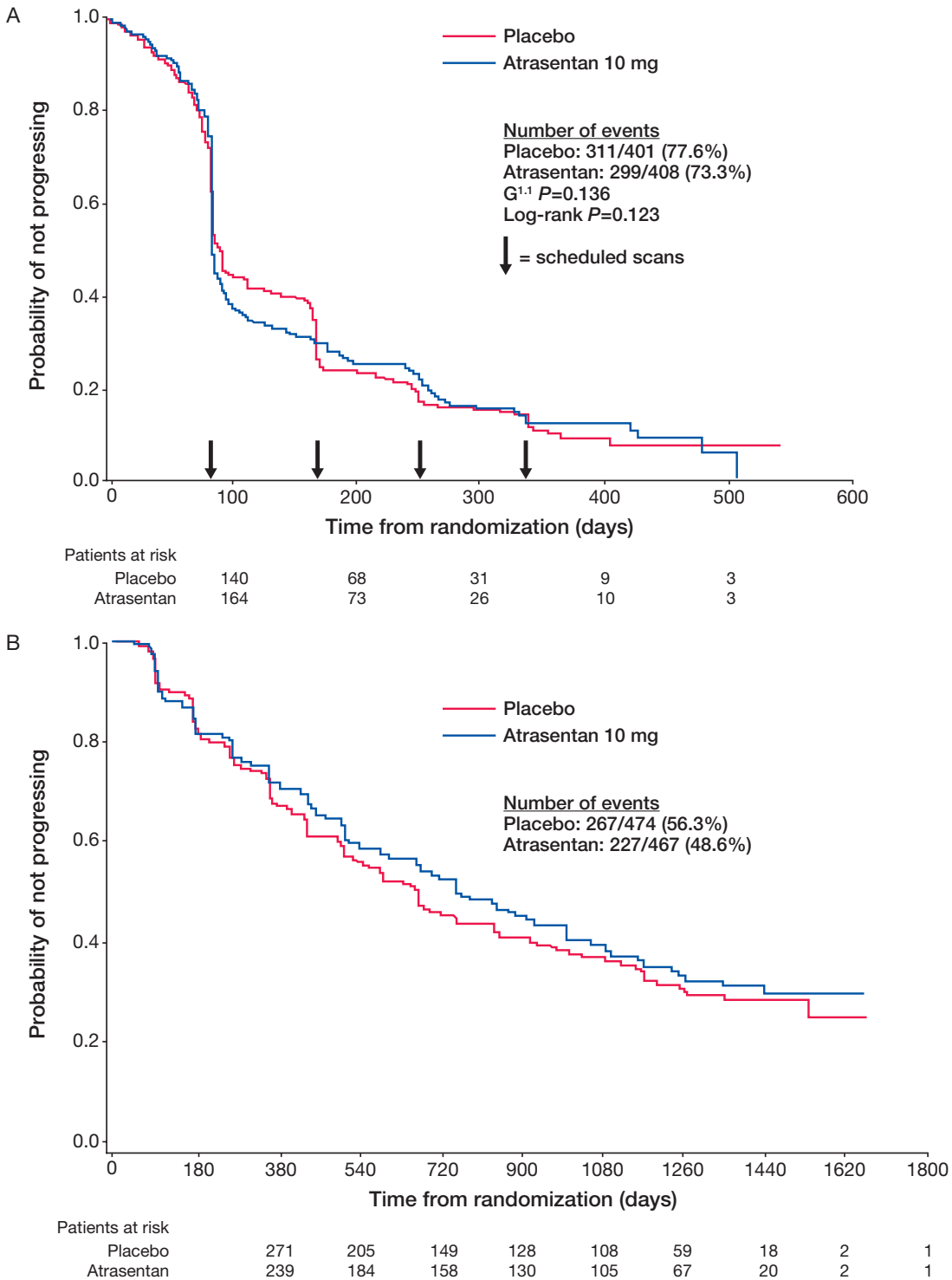


Figure 2

Time to disease progression following treatment with atrasentan in men with (A) metastatic and (B) non-metastatic castration-resistant prostate cancer (Carducci *et al.*, 2007; Nelson *et al.*, 2008). Reprinted with permission from John Wiley and Sons.

relative to placebo [hazard ratio (HR), 0.89; 95% confidence interval (CI) 0.76, 1.04; P = 0.136; Figure 2A]. Most patients progressed radiographically at the first 12 week bone scan without concomitant clinical progression (Carducci *et al.*, 2007). In the second study of 941 patients with non-metastatic CRPC, there was a 93 day delay in the median TTP (defined as the onset of metastases) following treatment with atrasentan 10 mg; however, this was not statistically significant when

compared with placebo (HR, 0.92; 95% CI 0.77, 1.09; $P = 0.288$; Figure 2B). There were, however, large geographic differences observed between the US and non-US sites in this second study. The difference in TTP was 81 and 180 days longer in the US and non-US sites, respectively, following atrasentan treatment compared with placebo treatment. It is thought that the failure of these two Phase III studies of atrasentan may be due to the design (method of assessment of disease progression in the metastatic CRPC trial) and conduct (geographical differences observed in the non-metastatic CRPC trial) of the trials. Atrasentan was generally well tolerated in these studies. The most common adverse events associated with treatment were headache, rhinitis and peripheral oedema, which reflect the vasodilatory and fluid-retention properties of ET_A antagonism (Carducci *et al.*, 2007; Nelson *et al.*, 2008).

Atrasentan has also been investigated in combination with docetaxel in a Phase I-II study of patients with metastatic CRPC to determine the maximum tolerated dose (MTD) of docetaxel in this combination and to investigate preliminary efficacy. The MTD dose of docetaxel once every 3 weeks in combination with once-daily atrasentan 10 mg was 70 to 75 $mg \cdot m^{-2}$ and PFS and OS were comparable to that seen with docetaxel and prednisone (Armstrong *et al.*, 2008). This combination regimen is currently being investigated in a Phase III trial.

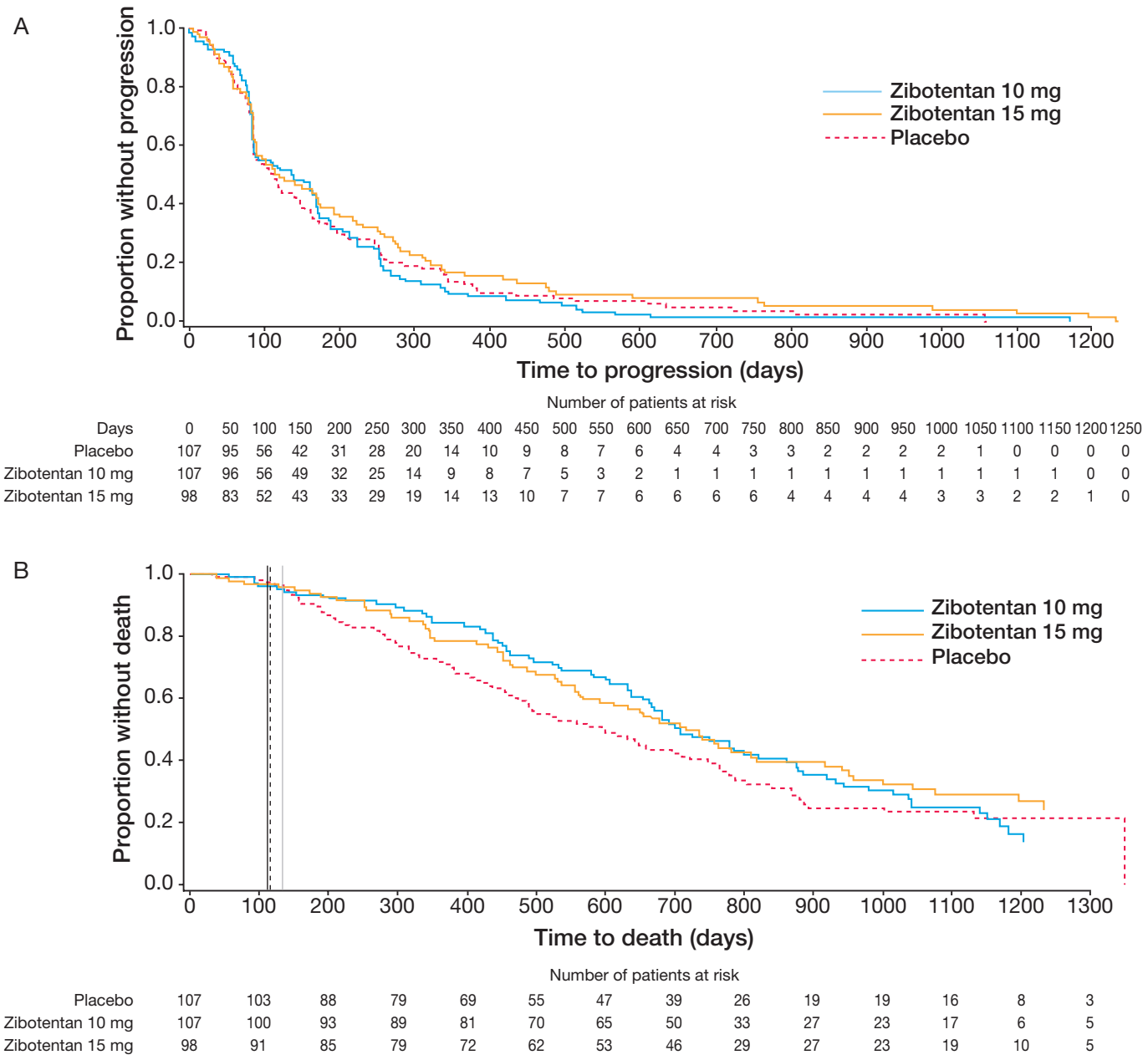
A Phase I-II study of atrasentan in combination with paclitaxel and carboplatin in chemotherapy-naïve patients with stage IIIB and IV NSCLC reported that this combination was well tolerated, and efficacy (Response Evaluation Criteria In Solid Tumors) and median survival were comparable with chemotherapy alone (Chiappori *et al.*, 2008). Atrasentan has also been investigated in a Phase I safety study in patients with progressive or recurrent malignant glioma (Phuphanich *et al.*, 2008), and in a Phase II trial of patients with locally recurrent or metastatic kidney cancer, data from which did not support further investigation as monotherapy in this patient population (Manola *et al.*, 2007).

Zibotentan (AstraZeneca)

Zibotentan (ZD4054) is a specific ET_A antagonist, which potently inhibited ^{125}I-ET-1 binding to cloned human ET_A expressed in mouse erythroleukaemic cells and membranes with an IC_{50} value of 21 nM at ET_A and an undetectable IC_{50} value at ET_B at concentrations up to 100 μM (Bradbury *et al.*, 1997; Morris *et al.*, 2005b). *In vitro* competitive binding assays suggest that zibotentan is a less potent inhibitor of ET_A activity compared with other selective ET_A receptor antagonists (Battistini *et al.*, 2006). However, the relative potency *in vitro* may not necessarily equate with potency *in vivo*. In addition, unlike other agents which have activity at both ET_A and ET_B, the absence of any effect of zibotentan at ET_B may be more clinically important than its relative potency versus ET_A. Indeed, in clinical trials with zibotentan, population pharmacokinetic data have revealed that steady state plasma concentrations of zibotentan following daily oral administration of 10 mg were well in excess of the non-clinical cellular IC_{50} values and therefore should achieve sufficient ET_A antagonism. In a clinical trial that evaluated the effect of zibotentan on ET-1-induced forearm blood flow in healthy volunteers, it was shown that zibotentan 10 mg and 30 mg antagonized the vasoconstrictor effect of infused ET-1, providing evidence that zibotentan was pharmacologically active at these doses (Morris *et al.*, 2005a). Moreover, no evidence of zibotentan-induced ET_B inhibition was detected following administration of zibotentan at doses of 2.5–240 mg, demonstrated by mean plasma levels of ET-1 being within the placebo range at 4 and 24 h post dose (Morris *et al.*, 2005b). In addition, adverse events of headache (a vasodilatory consequence of ET_A antagonism) have been reported following treatment with zibotentan 10 mg (James *et al.*, 2010). These data confirmed that zibotentan is the first specific rather than selective ET_A antagonist (Bradbury *et al.*, 1997; Morris *et al.*, 2005b). Because of the activities exhibited through ET_B, such as induction of apoptosis and clearance of ET-1, specific antagonism of ET_A, with no inhibition of ET_B, offers the promise of a differentiated anticancer therapy.

A range of preclinical studies have been undertaken with zibotentan, the majority of which were in models of prostate and ovarian cancer. These studies have demonstrated that complete blockade of ET_A with zibotentan reversed ET-1-induced inhibition of apoptosis while allowing pro-apoptotic signalling via ET_B (Rosanò *et al.*, 2007a). Zibotentan in combination with paclitaxel or docetaxel was also shown to enhance chemotherapy-induced apoptosis compared with either agent alone (Growcott, 2009). A role for zibotentan in the inhibition of cell proliferation and invasion has been reported; zibotentan was observed to inhibit proliferation of human immature pre-osteoblast cells (Growcott, 2009), and dose-dependently inhibit ET-1-mediated changes in cellular invasiveness in human ovarian cancer cells (Rosanò *et al.*, 2007b). Moreover, in human breast cancer cell lines, zibotentan combined with aromatase inhibitors or fulvestrant produced at least an additive inhibition of cell migration and invasion (Growcott, 2009). Zibotentan in combination with pamidronate completely blocked the development of bone metastases and reduced brain and lung metastases in severe combined immune deficient mice inoculated with a human bladder cancer cell line (Growcott, 2009). In addition, inhibition of tumour angiogenesis in prostate, colorectal and ovarian tumour xenografts has been demonstrated following treatment with zibotentan (Curwen *et al.*, 2007; Rosanò *et al.*, 2007b). Furthermore, in a murine xenograft of ovarian carcinoma, tumour growth and metastasis were reduced following treatment with zibotentan. The tumour growth inhibition was enhanced with the addition of a cytotoxic drug (paclitaxel) or a molecular inhibitor (gefitinib) (Rosanò *et al.*, 2007a,b, 2009). These and other preclinical findings provided a rationale for the use of zibotentan in clinical studies.

The efficacy and safety of zibotentan in patients with metastatic CRPC who were pain free or mildly symptomatic for pain was studied in a double-blind, placebo-controlled Phase II clinical trial (James *et al.*, 2009; 2010). Patients (n = 312) were randomized to receive once-daily zibotentan 10 mg, 15 mg or placebo. The primary endpoint was TTP, defined as time from randomization to clinical progression, objective progression of soft tissue metastasis on CT scan, or death in the absence of progression. Secondary endpoints included OS and time to PSA progression. Three analyses were conducted; at the primary interim analysis there was no difference between the zibotentan groups and placebo for TTP (4.0, 3.8 and 3.6 months for zibotentan 10 mg, 15 mg and placebo respectively; zibotentan 10 mg vs. placebo, HR 0.88;



Vertical lines indicate when treatment was halted; 113 days, 136 days and 112 days for zibotentan 15 mg, 10 mg and placebo respectively.

Figure 3

(A) Time to progression and (B) overall survival of patients with castration-resistant prostate cancer treated with zibotentan or placebo (final analysis) (James *et al.*, 2010). Reprinted with permission from John Wiley and Sons.

80% CI 0.71, 1.09; zibotentan 15 mg vs. placebo, HR 0.83; 80% CI, 0.66, 1.03). However, a signal for prolonged OS was observed in the zibotentan treatment groups versus placebo. At the second interim analysis, TTP was not significantly increased following treatment with zibotentan 10 mg or 15 mg when compared with placebo (4.6, 3.8 and 3.7 months respectively; zibotentan 10 mg vs. placebo, HR 1.09; 80% CI 0.91, 1.31; $P = 0.553$; zibotentan 15 mg vs. placebo, HR 0.94; 80% CI 0.78, 1.14; $P = 0.702$). However, the secondary endpoint of OS was significantly increased from 17.3 months to 24.5 months in patients receiving zibotentan 10 mg compared with patients receiving placebo (HR 0.55; 80% CI 0.41, 0.73; $P = 0.008$). Compared with placebo, patients receiving zibotentan 15 mg also had an improvement in OS with a median survival of 23.5 months (HR 0.65; 80% CI 0.49, 0.86; $P = 0.052$) (James *et al.*, 2009). At the final analysis (Figure 3), the difference in OS was still evident, although it had decreased in patients receiving zibotentan 10 mg and 15 mg when compared with placebo (median OS 23.5, 23.9 and 19.9 months, respectively) (James *et al.*, 2010). However, consis-

tent with previous analyses, HRs of less than one were sustained for both zibotentan 10 mg (HR 0.83, 80% CI 0.67, 1.02, $P = 0.254$) and 15 mg (HR 0.76, 80% CI 0.61, 0.94, $P = 0.103$). Plasma ET-1 levels were measured at baseline and at 4 weeks and 8 weeks following randomization. A small increase from baseline was observed in those patients treated with zibotentan whereas little change in plasma ET-1 levels was observed in placebo treated patients. Zibotentan was well tolerated, with the most commonly reported adverse events considered to be related to zibotentan treatment being peripheral oedema, headache and nasal congestion.

Zibotentan 10 mg once daily was investigated in combination with docetaxel 75 mg·m^{-2} once every 3 weeks in a Phase I clinical trial of patients with CRPC to evaluate the tolerability and preliminary efficacy of this combination. This treatment was demonstrated to be well tolerated with no safety concerns and preliminary efficacy was noted (Trump *et al.*, 2010).

A large Phase III clinical trial programme [ENdoTHelin A USE (ENTHUSE)] is further evaluating the therapeutic potential of zibotentan in men with CRPC. Clinical studies to date of zibotentan in prostate cancer, along with preclinical evidence of an anticancer effect of zibotentan in ovarian cancer cells, provide a rationale to investigate this agent in clinical trials of patients with ovarian cancer. As such, a Phase II trial of zibotentan plus carboplatin and paclitaxel, or placebo plus carboplatin and paclitaxel in patients with advanced ovarian cancer sensitive to platinum-based chemotherapy is ongoing. Other zibotentan clinical trials which have either recently been completed, or are ongoing or planned, include a Phase I trial of zibotentan in male, elderly Chinese patients with advanced solid malignancies, and a Phase II trial of zibotentan in combination with pemetrexed in patients with NSCLC.

In general, clinical trials should aim to collect sufficient events and maturity of data to have confidence in the results. There is currently a great deal of discussion regarding the appropriate endpoints for use in clinical trials of patients with cancer. Biomarker-based assessment and PFS are not confounded by subsequent therapy; however, OS is an unambiguous demonstration of clinical benefit. Surrogacy between biomarkers, PFS and OS endpoints has not been formally proved for prostate cancer. The required duration of trials will be dependent on the primary endpoint. In CRPC, trials can be relatively short for agents which are anticipated to have an effect on PSA, circulating tumour cells or PFS which will subsequently translate into OS benefits. However, for targeted agents such as zibotentan which primarily have a cytostatic action and therefore do not affect intermediate endpoints, OS will be the primary endpoint and trials will need to be longer. The zibotentan Phase II study reported a significant survival advantage at the interim analysis which was not present at the final analysis. Treatment was halted at 113 days, 136 days and 112 days for zibotentan 15 mg, 10 mg and placebo, respectively. The Kaplan–Meier curves for each arm were parallel after treatment was stopped which suggests that patients should remain on treatment provided they are deriving benefit and follow-up periods of trials of ET_A antagonists should be of sufficient duration to allow the full effect of a treatment to be observed (James *et al.*, 2010). In addition, the survival of placebo-treated patients in prostate cancer trials conducted over the last few years appears to have improved. For example, differences have been observed between the Phase II trial of zibotentan (James *et al.*, 2010) and the recent Sipuleucel-T trial (Kantoff *et al.*, 2010). However, as these studies had different inclusion criteria and were conducted in different geographical areas it is not possible to know whether this reflects true changes in life expectancy for metastatic CRPC over time or if this simply reflects the different inclusion criteria of these prospective randomized studies.

Targeting the ET axis in other diseases

The ET_A antagonists sitaxsentan and ambrisentan have been approved for the treatment of pulmonary arterial hypertension; however, these compounds are not currently being evaluated in the oncology setting.

Studies have utilized ET_A antagonists, with little improvement in morbidity and mortality, for the treatment of diseases such as chronic heart failure (CHF), where ET-1 and the ET_A are substantially elevated and correlate with the severity of disease (Kelland and Webb, 2006). Several reasons for this lack of efficacy have been proposed. It is thought that the benefits of ET blockade in CHF may derive from a truly ET_A selective approach. Patients with CHF who had selective blockade of the ET_B with BQ-788 experienced systemic vasoconstriction and elevation of plasma ET-1 concentrations. Endothelin dysfunction was improved after ET_A blockade, but not combined ET_A/ET_B blockade in the coronary microcirculation of patients with ischaemic heart disease. Treatment with high doses of selective ET_A antagonists can result in ET_B blockade, causing increased ET-1 concentrations resulting in chronic systemic vasoconstriction (Kelland and Webb, 2006). However, in trials of patients with CHF, many patients benefit from long-term treatment with ET_A antagonists even though there are some poorly understood negative outcomes after initial acute dosing (Kelland and Webb, 2006).

Conclusions

GPCRs have been linked to many processes involved in normal cell function and dysfunction of these receptors is associated with the development and progression of a range of diseases including cancer. Indeed, the ET-1/ET_A axis is associated with the development and progression of several types of cancer and is therefore an interesting target for the treatment of tumours. Although many preclinical and clinical studies have suggested that the ET axis contributes towards the development and progression of cancers, the specific mechanisms by which this occurs are still to be elucidated.

A range of specific and selective ET_A antagonists and dual ET receptor antagonists have undergone preclinical and clinical studies showing variable efficacy in the cancer setting. Several Phase II clinical trials have reported positive results, such as the promising OS signal observed in the trial of zibotentan in patients with metastatic CRPC. In addition to their potential as monotherapies, experimental and preclinical evidence suggests that ET_A antagonists may be able to potentiate the therapeutic efficacy of conventional cytotoxic drugs, offering a rationale for clinical evaluation of this

approach. Another novel and promising cancer treatment strategy is the incorporation of ET_A blockade with other molecular-targeted drugs to therapeutically overcome compensatory mechanisms of escape. Careful study design and learning from the experience of studies completed to date will be the key to fully evaluating the potential of these approaches. This will be supported by improved preclinical models, including three-dimensional tumour cultures and co-culture models, which more accurately reflect the disease in man. While such models are in development, the use of existing models (largely validated using cytotoxic agents) should take into account the different mechanisms of action between ET antagonists and cytotoxic agents.

A role for ET_B in other cancer types such as melanoma and glioma is also emerging. Furthermore, the potential role of ET_B in tumour-related immune responses is intriguing and warrants further investigation. Agonism of ET_B has also been proposed as an alternative approach to block the effects of ET_A. SP-1620 (IRL-1620) has been investigated in this context (Takai *et al.*, 1992) and the first Phase I clinical trial of this agent, currently recruiting, is being undertaken in patients with recurrent or progressive carcinoma.

A more personalized approach to the treatment of cancers will allow targeted agents such as ET_A antagonists to be used to treat those patients who will derive the greatest benefit. However, appropriate patient selection will require clinically validated markers of disease and response to treatment. Suitable markers that are reliable and easy to detect are needed to help identify those patients who are most likely to respond to ET_A antagonism.

To date, specific inhibition of ET_A with antagonists such as zibotentan has shown the most promise as a therapeutic approach for the treatment of cancer providing the rational basis for the design of more focused clinical trials. The results of the clinical trials of zibotentan as a monotherapy and in combination with cytotoxic drugs are awaited to unravel the opportunities to interfere with critical tumorigenic signals by targeting ET_A-mediated pathways.

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Conflicts of interest

Anna Bagnato has no conflicts of interest to declare. Marilena Loizidou received research consumables from AstraZeneca to investigate the efficacy of zibotentan in colorectal cancer models. Beth Pflug has received funding support from AstraZeneca for basic science/translational studies on the ET axis. Jon Curwen and Jim Growcott are employees of AstraZeneca, and Jim Growcott holds AstraZeneca stock options.

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