

# The Antagonists of Endothelin Receptors: Results and Perspectives

Alessandro Cosenzi\*

*Dipartimento di Medicina Clinica e Neurologia, University of Trieste, Trieste, Italy*

**Abstract:** In 1988 Yanagisawa described endothelins, a new class of vasoconstrictor agents produced by endothelial cells. Further biological effects of these peptides have subsequently been demonstrated, for example, induction of cell proliferation and fibrosis. Two types of endothelin receptors have been described: ET<sub>A</sub> are responsible for endothelin-induced vasoconstriction whereas ET<sub>B</sub> induce endothelial cells to release nitric oxide (NO) and prostacyclin. Many antagonists of endothelin receptors have been synthesized and evaluated in animal models and in humans. Satisfactory results have been obtained in animal models of arterial hypertension, pulmonary hypertension, stroke and heart failure but clinical trials have failed to demonstrate that these drugs have a beneficial effect in the treatment of heart failure and a dose-dependent reversible hepatic toxicity has been observed. However, the efficacy of bosentan, a mixed antagonist of endothelin receptors, in the treatment of primary pulmonary hypertension has been demonstrated and the drug is now marketed worldwide for this condition. Further studies are ongoing to evaluate other clinical applications of these drugs. Recently it has been reported that atrasentan, a selective ET<sub>A</sub> antagonist, delayed the progression of hormone-refractory prostate cancer in humans. This review describes the results of the studies performed in animals and in humans and their potential future clinical applications.

**Keywords:** Endothelins, lung, heart, kidney, brain, liver, cancer.

## INTRODUCTION

Since 2001 it has been possible to treat patients with pulmonary arterial hypertension with the oral endothelin receptor antagonist bosentan. However, this is only one chapter in the history of endothelins, which began in 1988 when Yanagisawa described this novel class of powerful vasoconstrictor peptides produced by endothelial cells [1]. Endothelins are a family of three 21-aminoacid peptides which derive from three distinct genes and are strikingly similar to sarafotoxins, a family of 21 aac peptides isolated from the venom of the snake *Actraspis engadensis*. The most important is endothelin-1 (ET-1) particularly in the cardiovascular system [2, 3, 4]. Endothelin synthesis was first demonstrated in endothelial cells; the peptides are mainly released by endothelium abluminally, suggesting a paracrine role [5, 6]. However, further studies have demonstrated that endothelins are also produced by leukocytes, macrophages, smooth muscle cells, cardiomyocytes and mesangial cells in the kidney [7, 8]. These peptides are synthesized as a pre-pro-protein of about 200 aminoacids, which is subsequently converted to an inactive precursor of about 40 aminoacids called big-endothelin. Big-endothelin is cleaved at the Trp<sup>21</sup>-Val<sup>22</sup> bond to form active endothelin [4, 1]. The enzyme specific for this cleavage, a zinc metallopeptidase, has been identified and called endothelin converting enzyme (ECE). Phosphor-amidon is an inhibitor of this enzyme and new compounds have been synthesized by modifying its structure to obtain a more potent and specific molecule. Indeed ECE-

inhibition is one of the two possible approaches to inhibit the endothelin system, the alternative being receptor antagonism [9]. ECE-2 and chymase can also convert big-endothelin to the active molecule [10]. Further studies have demonstrated that endothelins not only cause a potent and prolonged vasoconstriction but are also known to enhance cell proliferation and to stimulate extracellular matrix accumulation. In addition to these effects, endothelins play a role in prostate growth, carcinogenesis and regulation of bronchial tone, and they are also involved in embryonic development; for this reason endothelin antagonists are contraindicated during pregnancy and in women with child-bearing potential [4,11]. High levels of plasma or tissue endothelins have been demonstrated in patients with heart failure, diffuse atherosclerosis, diabetes, stroke, primary pulmonary hypertension, liver cirrhosis and some tumoral forms [12,13,14,15,16]. Given the effects of endothelins, blocking of their receptors has been indicated as a possible new target for reducing blood pressure and for the treatment of other illnesses. ET receptors belong to the seven-transmembrane-domain spanning, G-protein coupled receptor superfamily. Two types of receptors for endothelins have been identified: ET<sub>A</sub> are located on the smooth muscle cells of the vascular wall and are responsible for the vasoconstriction induced by endothelins; ET<sub>B</sub> are located on the endothelial cells and induce the release of NO and prostacyclin from these cells with subsequent vasodilation and reduction in further production of endothelins; however, a subclass of ET<sub>B</sub> receptors has been demonstrated on the smooth muscle cells, inducing vasoconstriction [17]. Many antagonists of endothelin receptors have been developed and evaluated *in vitro* or in animal models; one of these, bosentan, has recently been approved for the treatment of primary pulmonary hypertension [18].

\*Address correspondence to this author at the Dipartimento di Medicina Clinica e Neurologia, University of Trieste, c/o Ospedale di Cattinara, Strada di Fiume 447, 34100 Trieste, Italy; Tel: 39-40-3994395; Fax: 39-40-912881; Email: a.cosenzi@fmc.units.it

**Table 1. Endothelin Receptor Antagonists Evaluated in Clinical Trials**

DRUG	Antagonism	Condition	Comments
Bosentan	ET <sub>A</sub> , ET <sub>B</sub>	Pulmonary arterial hypertension Heart Failure, Hypertension	Approved for pulmonary arterial hypertension
Enrasentan	ET <sub>A</sub> , ET <sub>B</sub>	Heart failure	
SB-209670	ET <sub>A</sub> , ET <sub>B</sub>	Radiocontrast medium nephrotoxicity	
TAK-044	ET <sub>A</sub> , ET <sub>B</sub>	Subarachnoid hemorrhage	
Tezosentan	ET <sub>A</sub> , ET <sub>B</sub>	Heart failure	Intravenous administration
BQ-123	ET <sub>A</sub>	Chronic renal insufficiency	Cyclic peptide; Only acute effect after intravenous administration
Ambrisentan	ET <sub>A</sub>	Pulmonary arterial hypertension	
Atrasentan	ET <sub>A</sub>	Hormone-refractory prostate carcinoma	
Darusentan	ET <sub>A</sub>	Heart failure, Hypertension	
Sitaxsentan	ET <sub>A</sub>	Pulmonary arterial hypertension, Hypertension	
SLV-306	ECE + NEP	Heart failure	

## CHEMISTRY

As saralasin, the first antagonist of the renin angiotensin aldosterone system, the primordial antagonists of endothelin receptors were modified peptides. These molecules could be administered only intravenously and are used in experimental studies performed in animals. ABT 123, a cyclic pentapeptide, and FR139317, a modified linear peptide, are the most highly selective ET<sub>A</sub> receptor antagonists and ABT 788 is a selective ET<sub>B</sub> receptor antagonist [19,20].

Subsequently, many non-peptidic antagonists of endothelin receptors have been developed. Luescher and Barton report 52 compounds in a review published in 2000 [7]. A classification of these drugs with a few examples is reported below:

- Natural compounds (extracted from cultural medium of *Streptomyces misakiensis* or from the Chinese herb *Cissus assamica*) [21,22].
- Etherocyclic sulfonamides (bosentan) [23].
- Indane carboxylic acid derivatives (enrasentan) [24].
- Dihydropyridinic anidrides (CGS 27830) [25].
- Diarylpyrrolidine carboxylic acids (atrasentan) [26].
- Ethensulfonamide derivatives [27].

## SIDE EFFECTS

The most frequently reported clinical adverse effects in patients treated with antagonists of endothelin receptors are headache, peripheral edema, nausea, nasal congestion and dizziness.

In 213 patients with pulmonary arterial hypertension treated for 16 weeks adverse effects led to premature discontinuation of the medication in 6% of patients receiving

bosentan and in 7% of patients receiving placebo. Asymptomatic liver toxicity was the main cause of drug withdrawal in bosentan group (2%). Headache was reported by 21% of patients, dizziness by 11% flushing by 9% [28]. In 32 patients treated for 1 year with bosentan headache was observed in 29% of patients, nasal congestion in 25%, dizziness in 12%, nausea in 12% and lower-limb edema in 12% [29]. The occurrence of side effects was similar in patients treated with a selective ET<sub>A</sub> antagonist. In a group of 178 patients treated with sitaxsentan headache was reported in 34% of patients, peripheral edema in 17%, nausea in 19%, nasal congestion in 10% and dizziness in 10% but the incidence of serious side effects was similar in patients treated with the drug and with placebo [30]. Similar side effects have been observed also in patients with heart failure and arterial hypertension [31,32].

## Liver Toxicity

Most, if not all, endothelin antagonists predispose to hepatic toxicity as is shown by an increase in transaminases. The REACH-1 study, evaluating the effect of bosentan in patients with chronic heart failure, was interrupted following the recommendations of the Data Safety Monitoring Committee due to concerns about increased levels of hepatic transaminases in 10% of patients [33]. An asymptomatic, reversible and dose dependent increase in transaminases was observed also by Krum in a group of hypertensive patients treated with bosentan at different doses (range 100 to 2000 mg daily) [34].

Liver injury has been observed also with selective ET<sub>A</sub> antagonists. In a pilot study with sitaxsentan in patients with pulmonary arterial hypertension, dosage ranging from 100 to 500 mg daily for 12 weeks, two patients developed severe hepatitis during the extension phase and one patient died despite discontinuation of the drug. Histological examination of the liver revealed acute fulminant hepatitis with central-

to-bridging necrosis [35]. In the subsequent STRIDE-1 study, which evaluated the effect of this drug at different dosages in patients with pulmonary arterial hypertension, the incidence of liver enzyme abnormalities (> 3 times upper limit of normal) were 0% for the sitaxsentan 100 mg group but 10% for the 300 mg group. After 9 months, in the extension phase of the study, the incidence increased to 32% in the 300 mg group and was 8% for 100 mg sitaxsentan [30].

Bosentan and their metabolites are eliminated almost exclusively through biliary excretion and it causes a rise in serum bile salt concentration in patients and in rats. Accordingly, it has been suggested that liver injury might be a consequence of cholestasis [36]. Cholestasis leads to intracellular accumulation of bile salts, which are detergents and can promote hepatocellular death by interfering, for example, with mitochondrial functions. Cyclosporine, rifampin and glyburide can induce cholestasis or cholestatic hepatitis. Drug-induced cholestasis may result from impaired transport of bile constituents through the canalicular membrane of hepatocytes. Bile salt extrusion through the bile salt-export pump (bsep) generates bile salt-dependent bile flow. Glutathione efflux, through the multidrug-resistant protein-2 (mrp-2), and bicarbonate secretion act as the driving force for bile-salt independent bile flow. A study performed by Fattinger *et al.* in rats and *in vitro* shows that bosentan could inhibit bsep, thus reducing taurocholate transport. These authors suggest that increased levels of transaminases can be observed in humans but not in rats because of the distinct composition of bile salt pools in rats and in humans. Indeed, chenodeoxycholate has been shown to induce liver injury and represents 31% of human bile salt pool but only 2.5% in rats. The authors hypothesize that bsep polymorphism might exist in humans and thus explain the different susceptibility to develop liver injury during treatment with bosentan [36]. Fouassier *et al.* have also evaluated the effect of bosentan on the mechanisms of bile flow in rats. The authors have observed a choleric effect after bolus injections of a high dose of drug. Choleresis was combined with a significant increase in both the biliary concentration and output of glutathione, bicarbonate and bilirubin, whereas a reduction in secretion of phospholipids and cholesterol was observed. The authors suggest that bosentan might induce cholestatic liver injury through the inhibition of lipid secretion in bile. Indeed phospholipids normally inactivate the cytotoxicity of bile salts and a reduction in their concentration is followed by liver damage. Moreover, they indicate that the effect of bosentan on mrp-2 appears to be involved in this mechanism [37].

Bosentan is metabolized in the liver by cytochrome P450 isoenzymes 2C9 and 3A4 and its metabolites are excreted into the bile. Only 5% of an oral dose of bosentan is recovered in the urine. Hence an alteration of its pharmacokinetics and metabolism could be expected in patients with liver impairment. Van Giersbergen *et al.* have performed a pharmacokinetic study after single and repeated oral doses of bosentan in 8 patients with mild liver impairment (Child-Pugh class A) and compared the results with those obtained in healthy volunteers. The authors conclude that in patients with mild liver impairment the pharmacokinetics of the drug is unaffected and therefore

dose adaptation is not requested. Moreover, the results of the study indicate that bosentan induces CYP2C9 and/or CYP3A4 in subjects with normal and with mildly impaired liver function [38].

## TERATOGENICITY

Endothelins are considered growth factors during embryonic development. ET-1 and ET<sub>A</sub> receptor null mice die shortly after birth from respiratory failure and cardiac abnormalities; moreover these animals have hypoplasia of the facial bones [39,40]. The effects of enrasentan, a mixed endothelin receptor antagonist, on embryo-fetal development has been evaluated in pregnant rats and rabbits. Embryoletality was seen at 300 mg/kg/day. Craniofacial, large vessels, heart and thyroid were the predominant malformations observed, consistent with abnormalities found in ET null mice [11]. For this reason administration of antagonists of endothelin receptors is not advised during pregnancy and in women with child-bearing potential.

## PULMONARY ARTERIAL HYPERTENSION

ET-1 is overexpressed in the plasma and lung tissue of patients with pulmonary arterial hypertension (either primary or associated with scleroderma) [41]. This peptide not only has a very powerful vasoconstrictor activity in the pulmonary vascular bed but it can also induce smooth muscle proliferation in the vessel wall [16].

Williamson *et al.* first demonstrated in an open label study that the acute intravenously administration of bosentan, a mixed endothelin receptor antagonist, significantly reduced pulmonary resistances and mean pulmonary arterial pressure and slightly improved cardiac index in a dose-dependent fashion in a group of 7 women with pulmonary arterial hypertension primary or secondary to scleroderma [42]. In a subsequent double-blind, placebo controlled study the efficacy of bosentan was compared with that of placebo for 12 weeks in a group of 32 patients with pulmonary arterial hypertension (primary or associated with scleroderma). The primary endpoint was a change in exercise capacity. At the end of the study, the distance walked in 6 min improved by 70 m in the bosentan group and worsened by 6 m in the placebo group. Moreover, the cardiac index was 1.0 l greater and pulmonary arterial resistance significantly lower in patients treated with bosentan than in those with placebo [43]. The double-blind, placebo-controlled BREATHE-1 study was performed in 213 patients with severe (World Health Organization [WHO] class 3-4) pulmonary artery hypertension (primary or associated with connective-tissue disease), who were randomized to receive bosentan (125 or 250 mg twice daily) or placebo for a minimum of 12 weeks. The results demonstrated that bosentan significantly improved the six-minute walking distance in patients with primary pulmonary hypertension and prevented deterioration in the performance in those with scleroderma; the mean difference between the placebo group and combined bosentan groups was 44 m. Moreover, 42% of patients receiving bosentan were in a better WHO functional class at the end of the study and the time span of clinical worsening was significantly longer [28]. Thereafter the drug was approved by the Food and Drug Administration (FDA)

**Table 2. Randomized Clinical Trials Evaluating Endothelin Receptor Antagonists In Pulmonary Arterial Hypertension**

Trial	Drug	Duration	N° Patients	Change in 6MWD	Comments
-	Bosentan 125-250 mg b.i.d	12 weeks	33	+ 70 m	Improved hemodynamic parameters.
BREATHE-1	Bosentan 62.5-125-250 mg b.i.d	16 weeks	213	+ 44 m	Improved composite end point.
BREATHE-2	Epoprostenol + Bosentan 125 mg b.i.d or placebo	16 weeks	30		Trend toward a greater reduction in PVR in patients with active treatment
STRIDE-1	Sitaxsentan 100-300 mg	12 weeks	180	+22m /+20 m	This study enrolled also patients with congenital systemic to pulmonary shunts. Improved functional class, C.I. PVR, and only in 300 mg-group. ppVO <sub>2</sub>
-	Ambrisentan 1-2.5-5-10 mg	12weeks	64	+33.9m /+38.1m	
ARIES-I	Ambrisentan 5-10 mg	12 weeks	186 (expected)		Patients enrolment started in January 2004
ARIES-II	Ambrisentan 2.5-5 mg	12 weeks	186 (expected)		Patients enrolment started in January 2004

Legenda: 6MWD: 6 minute walked distance (functional test); PVR: pulmonary vascular resistance; C.I: cardiac index; ppVO<sub>2</sub> = predicted peak VO<sub>2</sub>

for the treatment of patients with pulmonary hypertension and is now used worldwide.

Studies evaluating the long term effect of bosentan in patients with pulmonary arterial hypertension are now available. Sitbon *et al.* reported a sustained improvement in functional class and pulmonary hemodynamics for at least 1 year in a group of 29 patients; none of them died or had lung transplantation [29]. McLaughlin *et al.* have evaluated the three-year survival in 169 patients treated with bosentan as first-line therapy (monotherapy in 70%); these authors have observed a 86% survival compared to a predicted 48% based on a validated National Institutes of Health (NIH) survival equation [44].

It has been suggested that the addition of bosentan to eprostenol, another widely employed treatment for patients with pulmonary arterial hypertension, might be a potentially attractive approach; a trend toward a greater reduction in total pulmonary resistance versus monotherapy was found in a randomized controlled trial evaluating the effect of epoprostenol-bosentan association [45]. In a further study Hoeper *et al.* evaluated whether bosentan was safe and effective in patients with primary pulmonary hypertension receiving inhaled iloprost or oral beraprost. After three months, 6-min walking distance had increased by 58 m, maximal oxygen consumption was improved and the combination therapy was well tolerated [46]. Galie *et al.* have performed an echocardiographic study to evaluate the effects of 16-week bosentan treatment in 85 patients with primary pulmonary hypertension in WHO class III or IV. These authors have observed that bosentan improved right ventricle systolic function and decreased right ventricle dilation in comparison with placebo [47].

Recently the efficacy of bosentan has been evaluated also in a small group [n=9] of patients with Eisenmenger syndrome. Bosentan 125 mg was administered twice a day; the time of follow-up ranged from 5 to 14 months. At the end of the study 6/9 patients showed an improvement in NYHA class of 1 grades. Oxygen saturation levels increased from 79 ± 5% to 88 ± 6% [48].

The effects of enrasentan, another non-selective endothelin receptor antagonist, on pulmonary hypertension have been evaluated *in vitro* and *in vivo*. *In vitro* enrasentan prevented the contraction of isolated pulmonary artery rings from guinea pigs induced by ET-1. Exposure of these animals to hypoxia (9% O<sub>2</sub>) for 10 days induced a 100% rise in pulmonary artery pressure, right ventricle hypertrophy and a 3-fold increase in plasma endothelin concentration. In animals treated with enrasentan pulmonary artery pressure was halved and right ventricle hypertrophy was significantly attenuated whereas no effect of the drug on the erythropoietic response to hypoxia was observed [49].

Studies evaluating the efficacy of selective ET<sub>A</sub> antagonists in patients with pulmonary hypertension are ongoing. Sitaxsentan is an oral endothelin receptor antagonist with a long duration of action (t<sub>1/2</sub>= 5-7hrs) and is approximately 6500 times more selective as an antagonist for ET<sub>A</sub> compared with ET<sub>B</sub> receptors. In the randomized, double blind, placebo controlled STRIDE-1 trial the efficacy and the safety of this drug was evaluated in 178 patients with pulmonary arterial hypertension - idiopathic, related to connective disease, or related to congenital systemic to pulmonary shunts. Patients received 100 or 300 mg of the drug or placebo once daily for 12 weeks. Compared to placebo both 100mg and 300mg sitaxsentan improved 6 minute walk distance, respectively by 22m and 20 m, functional class, cardiac index and pulmonary vascular resistance but only in the 300 mg-group did the predicted peak VO<sub>2</sub> increase. In addition, no improvement occurred in either sitaxsentan group versus placebo for changes in the other end points that were determined by cardiopulmonary exercise testing. The most frequently reported clinical adverse effects were headache, peripheral edema, nausea, nasal congestion and dizziness. The incidence of liver enzyme abnormalities (> 3 times upper limit of normal) were 0% for the sitaxsentan 100 mg group but 10% for the 300 mg group. After 9 months, in the extension phase of the study, the incidence increased to 32% in the 300 mg group and was 8% for 100mg sitaxsentan. Interaction with warfarin with

prolonged INR has also been observed as a consequence of the inhibition of the CYP2C9 P450 enzyme; accordingly, a reduction in warfarin dosage is suggested in patients treated with sitaxsentan [30].

In theory, sparing the ET<sub>B</sub> receptor on vascular endothelial cells could unmask an indirect vasodilatory effect of endothelins mediated through nitric oxide and prostacyclin release; however, on the basis of this study, sitaxsentan, a selective ET<sub>A</sub> antagonist, does not appear to offer major advantages over bosentan, a mixed endothelin receptor antagonist, in terms of safety or efficacy and is more likely to emerge as an alternative rather than a superior treatment [50].

Ambrisentan (LU-208075, BSF208075) is another oral, selective ET<sub>A</sub> antagonist. In a randomized, double blind, dose ranging Phase II study this drug was evaluated in a group of 64 patients with moderate to severe pulmonary arterial hypertension. The patients were randomly allocated into four dose groups of 1, 2.5, 5 and 10 mg ambrisentan and treated for 12 weeks followed by 12-week open label dose-adjustment period. The primary end-point was a change in six-minute walk distance (6MWD) at week 12 compared to baseline. The improvement in 6MWD in the four groups ranged from 33.9 to 38.1 meters ( $p=0.008$ ). Four patients developed transient rises in serum aminotransferases greater than three times the upper limit of normal range, these changes were reversible. (Data presented at the satellite meeting "Emerging therapies for PAH" at the American Thoracic Society 2004 International Conference). On the basis of the results of this study, two randomized, double-blind, placebo controlled Phase III Trials – ARIES 1 and ARIES 2 – are now ongoing respectively in USA & Canada and in Europe.

## CANCER

Endothelins are usually associated with their vascular effects; however, it has been recognized that endothelin-1 is synthesized by very many normal extravascular cells including epithelial cells (particularly bronchial, endometrial, mammary and prostatic cells). Neoplastic cells often produce bioactive substances, including hormones, cytokines and growth factors that influence their cells of origin, as well as surrounding normal tissues. These tumoral products are capable of exerting autocrine, paracrine and endocrine actions. ET-1 exerts a mitogenic effect for many cell types. It has been demonstrated that ET-1 is produced by many types of epithelial tumors (lung, breast, liver, pancreas, colon, endometrium, larynx, prostate, ovary) and can play a role as autocrine growth factor [51]. It has also been shown that ET-1 stimulates proto-oncogen expression in several cell types [52, 53] and enhances epidermal growth factor (EGF)-induced transformation of rat-1 and NRK49 F fibroblasts [54].

Brain endothelin receptors are mainly of the subtype ET<sub>B</sub>. However, in astrocytomas and in meningiomas ET<sub>A</sub> receptors become the predominant form. Wu-Wang *et al.* has therefore suggested that aberrant ET<sub>A</sub> expression could participate in tumor development and progression [55]. Nelson *et al.* have expressed a similar observation in prostate cancer. In the healthy gland ET<sub>B</sub> are widely expressed but

these receptors disappear in the prostate tumoral tissue and are substituted by ET<sub>A</sub>. [56]. In ovarian carcinoma cells ET-1 causes dose-dependent increases in DNA synthesis and cell proliferation. Selective antagonism of ET<sub>A</sub> receptors prevents <sup>3</sup>H-thymidine incorporation in these cells [57]. The loss of ET<sub>B</sub> receptor has been observed also in other neoplasms including Erwing's sarcoma and neuroblastoma. In these tumors the loss of this receptor is of clear prognostic value since it has been associated with a mean survival time of 26 months compared with 88 months when the receptor was maintained ( $p=0.004$ ). In this study it has also been demonstrated that ET-1 induce proliferation of neoplastic cells through ET<sub>A</sub> receptor [58].

However the role of endothelins in the growth biology of neoplasms is not only limited to their mitogen activity; experimental data have demonstrated that they can also promote cancer growth by enhancing angiogenesis, by reducing apoptosis, by activating osteoblasts and blocking osteoclasts in secondary bone metastasis and they can also play a role in the development of neoplastic pain.

ET-1 modulates various stages of neovascularization, including endothelial cell proliferation, migration, invasion, protease production, tube formation and stimulates neovascularization; both ET<sub>A</sub> and ET<sub>B</sub> receptors are involved in these processes *in vivo* [59]. Since in ovarian carcinoma the degree of intratumoral stromal vascularization correlated with prognosis and the mechanisms of angiogenic events were not clear, Salani *et al.* have evaluated the expression of ET-1 and the degree of vascularization in specimens of ovarian neoplasms. These authors have demonstrated a significant correlation ( $n=60$ ) between microvessel density and ET-1 expression and suggest that the angiogenic effect of ET-1 could be in part direct, in part mediated through the stimulation of the vascular endothelial growth factor (VEGF) [60].

Several antineoplastic agents induce cytotoxicity through activation of apoptosis and the inability of cancer cells to activate apoptosis is one of the mechanisms of resistance to cancer treatment [61]. Del Bufalo *et al.* have evaluated *in vitro* the effect of ET-1 on the response of two ovarian carcinoma cell lines to paclitaxel, one of the most common drugs used for the treatment of this disease. The results of this study have demonstrated that ET-1 confers resistance to paclitaxel-induced apoptosis *via* ET<sub>A</sub> receptors [62], confirming previous results obtained with other cell lines

High endothelin concentrations have been detected in the ascitic fluid of patients with ovarian carcinoma [60]. Many studies have demonstrated that there is a close association between the expression of various members of the matrix metalloproteinase (MMP) family by ovarian tumors and their invasive behavior and metastatic potential [63]. Indeed, tumor progression involves the disruption of anatomical barriers and penetration of tumor cells into normal adjacent tissues; such migratory events are regulated by different proteolytic systems. Rosanò *et al.* have demonstrated that ET-1 upregulates production and activation of MMP2 and MMP9 and that of PAI-1 and PAI-2; this effect was prevented by a selective ET<sub>A</sub> receptor antagonist (BQ-123). These authors conclude that ET-1 is a key component of tumor progression and invasion and that the use of a specific

ET<sub>A</sub> receptor antagonist may provide an additional approach to the treatment of ovarian carcinoma [64].

The algogenic effects of endothelins have been reported in several studies. In 1990 Dahlof observed in humans the painful effect of intraarticular ET-1 injection [65]; in 1998 Davar *et al.* described that exogenous ET-1 elicits pain behaviors when applied locally to the rat sciatic nerve [66]. In 2001 Gokin *et al.* reported that subcutaneous ET-1 injections in Sprague-Dawley rats triggered pain behavior and that this effect was prevented by a selective ET<sub>A</sub> receptor antagonist. In a subsequent experiment the authors recorded the impulse activity of single, physiologically characterized sensory C-, A - and A -fibers after subcutaneous endothelin injection. C and A - fibers were excited by endothelins whereas A -fibers were not. The authors concluded that ET-1 is acting through ET<sub>A</sub> receptors [67]. In 2002 Zhou *et al.* have demonstrated, in dorsal root ganglion of Sprague-Dawley rats, that the dominant effect of ET-1 is an ET<sub>A</sub> receptor-dependent shift in the hyperpolarizing direction for the voltage-dependent activation of tetrodotoxin-resistant Na<sup>+</sup> channels [68]. In 2004 Yuyama *et al.* reported that ET-1 increased cancer pain in mice inoculated with an androgen-independent prostate cancer cell line. This effect was prevented by atrasentan and YM598, selective ET<sub>A</sub> receptor antagonists [69]. Peters *et al.* have evaluated a murine osteolytic sarcoma model of bone cancer pain. These authors have demonstrated that it is possible to reduce pain by blocking ET<sub>A</sub> receptors whereas blocking the ET<sub>B</sub> receptor increases pain. ET-1 was highly expressed by these tumoral cells and ET-1 receptors were demonstrated in subpopulations of sensory neurons and non-myelinating Schwann cells [70]. All these data suggest an important role for endothelins in the pathogenesis of neoplastic pain, one of the most difficult problems to solve in patients with disseminated cancer and offer the opportunity for a new therapeutic approach. Data recorded in patients with advanced prostate cancer appear to confirm this opportunity (see below).

### Osteoblastic Metastases

Osteoblastic metastases occur in most patients with prostate cancer, are frequently observed in patients with breast cancer and are occasionally described in other neoplasms. In osteoblastic metastases there is an overall increase in the bone remodeling process characterized by an imbalance between osteoclastic bone resorption and osteoblastic replacement of bone resorption. The osteoblastic reaction is characterized by increased osteoid surface, osteoid volume and mineralization rate. Newly formed bone is characterized by collagen fibers that are oriented randomly and loosely packed, i.e. a weak bone which is more susceptible to fracture [71].

ET<sub>A</sub> receptors are widely expressed in osteoblasts; ET-1 induces a mitogenic activity and increases the synthesis of collagenous and non collagenous proteins in these cells but reduces the activity of osteoclasts [72, 73]. Le Roy *et al.* have observed *in vitro* that treatment of cultured rat *calvaria* for 24 hours with proteins from normal dog prostate stimulated alkaline phosphatase (AP) activity in a dose-dependent manner. Stimulation could be prevented by

blocking ET receptors. Homogenates of other organs did not modify AP activity [74]. Co-cultures of prostate cancer and bone show that ET-1 production is increased by prostate cancer cells in contact with bone [75]. MDA-MB-231 and ZR-75-1, two lines of human breast cancer cells have been injected into the left ventricle of nude mice. Mice receiving MDA-MB-231 cells developed osteoclastic metastases and rats receiving ZR-75-1 cells developed osteoblastic metastases. In a medium of cultured ZR-75-1 cells ET-1 RNA was found in excess compared with a medium of MDA-MB-231 cells. After *in vitro* experiments the effect of atrasentan (ABT-627), a selective ET<sub>A</sub> receptor antagonist, was evaluated in two groups of mice respectively receiving MDA-MB-231 and ZR-75-1 cells. In the second group the development of osteoblastic metastases was completely prevented whereas the drug had no effect on the osteolytic lesions developed in the first group injected with MDA-MB-231 cells [71]. Although many other factors may be important, these studies demonstrate that endothelins presumably contribute to the formation of bone observed around osteoblastic metastases and that this effect is mediated by ET<sub>A</sub> receptors, thus suggesting a potential therapeutic role for the selective antagonists of this receptor.

### Prostate Cancer

Casey *et al.* have demonstrated that ET-1 is normally found at the highest concentrations of any body fluid in seminal plasma [76]. As previously reported, the ET<sub>B</sub> receptor is the predominant receptor in normal prostatic epithelium whereas ET<sub>A</sub> is more common in the underlying stroma [77]. However, in prostate adenocarcinoma ET<sub>B</sub> expression is lost and that of ET<sub>A</sub> receptors [78] may be increased. Nelson *et al.* have found higher circulatory levels of ET-1 in patients with androgen refractory prostate cancer compared with those with localized disease or healthy individuals [79]. The role of ET-1 in the development of bony metastases of prostate cancer has been presented in the previous paragraph.

On the basis of these observations, the effect of the selective blockade of ET<sub>A</sub> receptors has been evaluated in two preliminary studies performed in patients with advanced prostate cancer. Atrasentan (ABT-627) is an oral, potent (K<sub>i</sub>= 34pM) and selective (ET<sub>A</sub> / ET<sub>B</sub> = 1862/1) antagonist of ET<sub>A</sub> receptors. In a phase I study the safety and pharmacokinetics of the drug was evaluated in male and female patients with advanced neoplasms. The drug was well tolerated at doses up to 60 mg/day; at higher doses severe hypotension and hyponatremia occurred. The most frequent adverse events were headache (60%), rhinitis (49%) and peripheral edema (31%); these effects were mild to moderate and reversible. Mean terminal half life was 26 hrs and no influence of gender on the pharmacokinetic parameters was observed [80]. In subsequent phase I trials, performed by Carducci in USA and by Zonenberg in the Netherlands, 22 patients with hormone refractory prostate cancer were evaluated. In these open-label studies a decline in prostatic specific antigen (PSA) was observed in 68% of patients and pain was decreased in 70% of subjects with symptomatic disease [81].

Carducci *et al.* have evaluated the effect of atrasentan in a double blind, randomized, placebo controlled study performed in 288 asymptomatic patients with metastatic hormone-refractory prostate cancer. Patients were randomly allocated into three groups of treatment: placebo, atrasentan 2.5 mg and atrasentan 10 mg. The primary end point was time to disease progression which was defined as: the development of new metastatic lesions; requirement of opioid analgesic treatment; new disease related symptoms that require therapeutic intervention; death. Secondary end points included time to PSA progression (> 50% over baseline), bone scan changes and changes in bone and tumor markers. Median time to progression was significantly prolonged from 129 to 196 days in patients in 10 mg atrasentan group ( $p=0.021$ ) a significant protective effect was observed also by evaluating the median time to PSA progression (155 days vs. 71 days;  $p=0.002$ ) [82].

Nelson *et al.* report the effects of the drug on bone remodeling in the same group of 288 patients with hormone refractory prostate adenocarcinoma. Patients receiving placebo experienced a 58% increase in total alkaline phosphatase and a 99% increase in bone alkaline phosphatase whereas patients receiving atrasentan 10 mg had stable values of these enzymes. Positive effects were observed also on other parameters including bone scan studies [83].

These studies cannot be considered conclusive but further trials are ongoing and if they confirm the positive results of studies reported so far selective antagonism of ET<sub>A</sub> receptors can be considered a new tool for the treatment of hormone-resistant prostate cancer.

## HEART FAILURE

Many studies have demonstrated elevated plasma levels of endothelins in patients with heart failure. The levels of endothelins are positively correlated with the New York Heart Organization (NYHA) functional class and negatively with the ejection fraction. High levels of plasma endothelins are a negative prognostic marker in patients with heart failure [12]. Moreover, the cardiac content of endothelins has been demonstrated to be higher in failing human hearts [84]. These peptides exert several activities with potential negative effects on the heart: they augment sympathetic tone, increase sodium retention and the activity of the renin angiotensin system, increase the cardiac inotropism, have proarrhythmogenic effects and induce deposition of matrix proteins [85, 86]. All these data suggested that endothelins might play a pathogenetic effect in heart failure. Many studies have therefore been conducted with antagonists of endothelin receptors.

In 1996 Shimoyama *et al.* evaluated the acute effect of bosentan in 11 dogs with chronic heart failure induced by multiple sequential intracoronary microembolisation and with left ventricular ejection fraction =  $25 \pm 2\%$ . Sixty minutes after a bolus injection of 10 mg/kg bosentan no change was observed in blood pressure and heart rate but a significant increase in cardiac output with reduced left ventricle end-diastolic pressure and systemic vascular resistance was detected [87]. In 1997 Mulder *et al.* evaluated the chronic effect of bosentan in rats with heart failure

induced by coronary bindings. The animals were treated for 2 ( $n=22$ ) or 9 ( $n=105$ ) months and received 30 or 100 mg/kg bosentan. After 9 months the higher dose of bosentan (100 mg) markedly increased survival and reduced arterial blood pressure, central venous pressure, left ventricular end-diastolic pressure, left ventricular dilatation and fibrosis. The lower dose of bosentan had no hemodynamic or structural effect and did not increase survival [88]. Pandey *et al.* have evaluated the effect of enrasentan in mice with myocarditis caused by encephalomyocarditis virus. Ten days after the infection, left ventricle function was evaluated *ex vivo* by means of an intraventricular balloon connected with a transducer measuring +dP/dt and -dP/dt. In infected animals treated with enrasentan the cardiac function was significantly preserved, compared to that of untreated animals [89]. Willette *et al.* have evaluated the effects of enrasentan, at low and high doses, in SHR-stroke prone rats maintained on a high-fat and high sodium diet, producing neurobehavioral deficits, cardiac hypertrophy, cardiac and renal damage and significant mortality within two months. At the end of the study overall mortality was reduced by the drug from 65% in the control group to 5% and 10% in the two enrasentan groups, although there was no significant difference in blood pressure in rats treated either with placebo or enrasentan. Moreover, the drug prevented the progressive deterioration of cardiac output, cardiac index and stroke volume observed in the control group and attenuated left ventricle hypertrophy induced by high blood pressure; plasma levels of aldosterone and atrial natriuretic peptide (ANP), recognized markers of cardiac dysfunction, were significantly reduced in rats treated with enrasentan [90]. Cosenzi *et al.* have demonstrated that in rats with hypertension due to a high fructose diet enrasentan not only reduces blood pressure but also the deposition of collagen III in the heart, which can contribute to the development of cardiac dysfunction [91].

In 1998 Süttsch *et al.* evaluated, in a double blind placebo-controlled fashion, the effect of acute and short term (2 weeks) administration of bosentan (1000 mg bid) in a small group of patients ( $n=36$ ) with heart failure (NYHA class III, left ventricle ejection fraction =  $22.4 \pm 4.5\%$ ). Patients continued concomitant treatment with ACE-Inhibitors, diuretics and digoxin throughout the study but  $\beta$ -blockers and nitrates were stopped. Acute administration of bosentan was followed by a decrease in systemic and pulmonary arterial pressure, in wedge pressure, in right atrial pressure and in systemic and pulmonary resistances and by an increase in cardiac output with unchanged heart rate; the drug was then administered for two weeks and the beneficial effects were further increased at the end of the study [92].

In the REACH-1 study 173 patients with heart failure (NYHA class III and IV, left ventricle ejection fraction <35% despite optimal treatment, hospitalization for heart failure within the previous 12 months or 6-min walking distance <375 m) were randomized one third to placebo, one third to slow up-titration to bosentan (125 mg daily for 1 week, followed by 250 mg for 1 week and subsequently to 500 mg for 24 weeks) and one-third to rapid up-titration of bosentan (250 mg for 1 week followed by 500 mg for 24 weeks). The study was interrupted following recommendations of the Data Safety Monitoring Committee due to concerns about elevated levels of hepatic

**Table 3. Randomized Clinical Trials Evaluating Endothelin Receptor Antagonists In Heart Failure**

Trial	Drug	Duration	Patients	Outcome
REACH-1	Bosentan 250 mg b.i.d		370 pts.; NYHA IIIb-IV	Early interrupted because of frequent liver toxicity. Slight trend to better outcome in patients with active treatment.
ENABLE I ENABLE II	Bosentan	9 months	1613 pts.; NYHA IIIb-IV	No difference
RITZ-1	Tezosentan 25 mg /hour i.v.	72 hours	669 pts.; requiring hospitalization for HF	No difference
RITZ-2	Tezosentan 50-100 mg/hour i.v.	6 hours	285 pts.; requiring hospitalization for HF; C.I. < 2.5 l/min	Improved hemodynamic parameters
RITZ-4	Tezosentan 25-50 mg/hour i.v.	24-48 hours	193pts.; HF + acute coronary syndrome	No difference. Frequent hypotension in active treatment group.
RITZ-5	Tezosentan 50-100 mg/hour i.v.	24 hours	84 pts.; acute pulmonary edema	No difference
ENCOR	Enrasentan 30-90 mg	9 months	419 pts.; NYHA II-III; EF <35%	Better outcome in placebo group
HEAT	Darusentan 30-300 mg	3 weeks	157 pts.; C.I. < 2.6 l/min/ m <sup>2</sup>	Significantly improved hemodynamic parameters in active treatment group
EARTH	Darusentan 10-300 mg	24 weeks	642 pts.; EF< 35% LVEDD>3.0cm/m <sup>2</sup>	No difference

LEGENDA: NYHA New York Heart Association functional class; pts= patients; i.v.= intravenously; EF= left ventricle ejection fraction; C.I.= cardiac index; HF=heart failure; LVEDD=left ventricle end diastolic diameter.

transaminases in patients receiving bosentan. At the end of the study only 50% of patients had completed the 6 months of double blind therapy; in this group a significantly greater clinical improvement was observed in the bosentan group in comparison with placebo. Increased levels of transaminases were observed in 10% of patients but these were asymptomatic and reversible [33].

In the ENABLE study, 1613 patients with heart failure (NYHA class III and IV) were treated with lower doses of bosentan for a mean period of 18 months. At the end of the study no difference could be demonstrated in the incidence of all-cause mortality, cardiac death and heart failure [Packer M, unpublished data].

The ENCOR study was a multicenter, randomized, double blind placebo controlled study evaluating the effect of enrasentan added to conventional treatment in patients with congestive heart failure. In this study 419 patients with stable congestive heart failure (NYHA class II-III) and ejection fraction < 35% (mean EF 25%) were assigned to one of the following treatment arms: 1) Conventional therapy + enrasentan 30 mg; 2) Conventional therapy + enrasentan 60 mg; 3) Conventional therapy + enrasentan 90 mg; 4) Conventional therapy + enalapril 10 mg; 5) Conventional therapy + placebo.

Conventional treatment included stable doses of digoxin, diuretics,  $\beta$ -blockers, and "standard doses" of angiotensin converting enzyme inhibitors (ACE-I) (enalapril 5-10 mg / day or equivalent doses of other ACE-I). The duration of the study was 9 months; the endpoints were clinical heart failure composite. The results of the study are disappointing. In enrasentan combined groups there was a trend favoring the placebo over the study drug. Enrasentan treated patients had a 3-fold greater likelihood of being hospitalized for heart

failure and a non-significant trend towards greater total mortality. In the combined endpoint of time of death, hospitalization and withdrawal due to worsening of heart failure there was a significantly better outcome in the placebo group than in the enrasentan group ( $p=0.007$ ). Moreover, the placebo was better tolerated than enrasentan [92].

The results of studies performed with mixed endothelin receptor antagonist failed to demonstrate an additional beneficial effect of these drugs in patients with heart failure in optimal treatment. It was argued that better results could be obtained by selectively blocking the ET<sub>A</sub> receptors in order not to interfere with the potentially favorable effects of ET<sub>B</sub> receptors (vasodilation, clearance of endothelins).

Spieker *et al.* have evaluated the effect of a single oral dose of the selective ET<sub>A</sub> receptor antagonist darusentan (1, 10, 30, 100 or 300 mg) in 95 patients with chronic heart failure and ejection fraction < 35%. All drugs with hemodynamic effects were discontinued in the morning of the study. Cardiac output was determined by thermodilution and other hemodynamic parameters were recorded with a Swan-Ganz catheter, measurements were performed baseline and repeated 6 times after administration of the drug. The results demonstrate that darusentan significantly, and dose dependently, increased the cardiac index and lowered mean arterial pressure, systemic vascular resistance, pulmonary wedge pressure, mean pulmonary artery pressure, pulmonary vascular resistance and right atrial resistance. Therefore the authors concluded that a single dose darusentan improved hemodynamics in a dose dependent manner without activation of other neurohumoral systems [93].

The randomized, double blind, placebo controlled HEAT trial has evaluated the hemodynamic and neurohumoral

effects of 30, 100 or 300 mg darusentan administered daily for 3 weeks in 157 patients with chronic heart failure, pulmonary capillary wedge pressure 12 mmHg and a cardiac index 2.6 L/min. m<sup>2</sup>. All patients were on ACE inhibition or angiotensin II antagonists, 70% on diuretics, 65% on digitalis glycosides, 65% on nitrates 46% on  $\beta$ -blockers and 24% on aldosterone antagonists. At the end of the study cardiac index and systemic vascular resistance had significantly improved whereas other hemodynamic parameters were unchanged. Neurohumoral activation was not observed and the drug was substantially well tolerated; side effects were those commonly observed in patients treated with this class of drugs. Four deaths occurred (2 unexplained sudden deaths, 1 cardiogenic shock and 1 ventricular fibrillation) but these events did not differ statistically with the placebo group. Moreover, early exacerbation of heart failure was observed in 36.7 % of patients receiving 300 mg darusentan, but in 12.1%, 13.9%, 15.4 % in the placebo, 30mg group and 100 mg group respectively [94].

In the multicenter, randomized, double blind, placebo controlled, parallel-dose ranging EARTH study 642 patients with chronic heart failure (left ventricle ejection fraction < 35%; left ventricle internal end-diastolic diameter > 3.0 cm/m<sup>2</sup> body surface area) were assigned to darusentan at 10, 25, 50, 100 or 300 mg daily or placebo for 24 weeks in addition to standard therapy. Primary endpoint was change in left ventricle end-systolic volume measured by MRI; symptoms and neurohumoral measurements were also evaluated. At the end of the study, data from 590 patients were available. There was a small decrease in LVESV over time in all groups with a weak relation to dose but there were no significant differences between baseline and endpoint or between treatment groups. Moreover, the treatment groups did not differ with respect in either cardiac or clinical variables. The NYHA class classification did not change significantly and there was no difference between the groups. The groups did not differ in global assessment score, nor in the composite score of death, hospital admission and change in quality of life. All neurohumoral parameters except ET-1 concentrations were unchanged. Blood pressure was lower in all active treatment groups. The drug was well tolerated although worsening of heart failure tended to be more frequent with the higher dosage groups, this trend was not statistically significant. Concentration of liver enzymes and bilirubin remained unchanged in all groups [95].

SLV306 is the first oral, dual Neutral Endopeptidase (NEP) and ECE inhibitor [96]. Blocking ECE prevents the passage from the inactive precursor Big-endothelin to the active endothelin. NEP, also known as Vasopeptidase, an enzyme involved in the metabolism of many peptides, including natriuretic peptides (ANP, BNP). Theoretically, blocking this enzyme makes it possible to obtain a higher concentration of ANP and BNP with significant diuretic and sodiuretic activity and without the secondary effects of traditional diuretics (loss of electrolytes, unfavorable metabolic effects, activation of the Renin Angiotensin Aldosterone System). Good results have been obtained in patients with heart failure or with hypertension treated with omapatrilat, a dual ACE- and NEP- inhibitor, although the superiority of this drug in comparison with pure ACE-I has

not been definitively demonstrated [97,98,99] Moreover, registration of omapatrilat has been blocked because of the frequent occurrence of angioedema in Afro-American patients. Discouraging results have been obtained from administering Candoxatril, a selective NEP-inhibitor, in 24 patients with chronic heart failure for 10 days. In contrast to the expected and desired effects, the drug induced an increase in systemic vascular resistance and a reduction in cardiac index. It must be considered that NEP has not a high substrate-specificity and is responsible also for degradation of ET-1. Hence one possible explanation of the results obtained with candoxatril might be that they are a consequence of the increased activity of ET-1 due to the inhibition of degradation of the vasoconstrictor peptide [100]. These results suggest a rationale for the potential utility of the concomitant blockade of NEP and ECE, which prevents the increase in ET-1 activity due to NEP inhibition. The acute effects of SLV306 have been evaluated in a randomized double-blind placebo controlled study in 75 patients with chronic heart failure who underwent right-sided heart catheterization. Pulmonary pressures and right atrial pressure significantly decreased. Plasma concentrations of ANP, BNP and Big-Endothelin (the inactive precursor of ET-1, as previously reported) significantly increased [101]. However, previous studies in patients with chronic heart failure have demonstrated that acute efficacy often is not confirmed in long-term studies. Chronic studies are therefore necessary to better evaluate the efficacy of SLV306 in patients with heart failure.

In conclusion, interesting observations of the involvement of endothelins in the pathogenesis of heart failure have been made in animal models; encouraging results have been obtained by administering endothelin antagonists in animals with heart failure. However, the results of clinical trials performed in patients with heart failure demonstrate that the addition of ET<sub>A</sub>-ET<sub>B</sub>, and also that of selective ET<sub>A</sub> receptor antagonists, to conventional treatment in patients with heart failure does not improve the clinical status. The reason for this discrepancy between experimental and clinical data has not been explained; it might also be hypothesized that optimal results are achieved in heart failure with conventional treatment comprising ACE-I, diuretics,  $\beta$ -blocker and digoxin and that there is no possibility of further improvement by adding new drugs.

## RENAL PROTECTION

### Chronic Renal Failure and Renal Ischemia

Goddard *et al.* have evaluated the effect of acute, selective ET<sub>A</sub> and ET<sub>B</sub> receptors antagonists in 8 patients with stable chronic renal failure (GFR 16 to 67 ml / min x 1.73 m<sup>2</sup> body surface) and in 8 healthy volunteers in a double blind, crossover, placebo-controlled study. BQ-123 (selective ET<sub>A</sub> receptor antagonist) or BQ-788 (selective ET<sub>B</sub> receptor antagonist) or their association or placebo were injected intravenously. The results demonstrated that BQ-123 reduced mean arterial pressure by 12.9  $\pm$  1.7 mmHg and total peripheral resistance in patients with chronic heart failure but not in healthy volunteers, whereas BQ-788 increased blood pressure in both groups. BQ-123 significantly increased renal blood flow and reduced renal

vascular resistance and filtration fraction without affecting glomerular filtration rate in patients and had a neutral effect in healthy volunteers. BQ-788 reduced renal blood flow and increased renal vascular resistance in both groups. Urinary sodium excretion was unchanged after both drugs and proteinuria, corrected for GFR, was significantly reduced by BQ-123 and not affected by BQ-788. The authors conclude that ET<sub>A</sub> receptor antagonism produced a substantial reduction in blood pressure associated with renal vasodilation in patients with chronic renal failure, thus showing that ET-1 plays a major role in regulating blood pressure and renal vascular resistance in chronic renal failure through ET<sub>A</sub> receptors. Additionally, reduction in filtration fraction and proteinuria suggests a potentially renoprotective action of ET<sub>A</sub> receptor antagonists [102].

Willette *et al.* have investigated the effect of ramipril, an ACE inhibitor, and that of enrasentan in rats after surgical inter-renal banding of abdominal aorta. In rats receiving vehicle cardiac hypertrophy, right (upstream) renal hypertrophy and left (downstream) renal atrophy were observed 4 weeks after the surgical procedure; ramipril prevented cardiac hypertrophy and had no effect on either kidneys; enrasentan prevented neither cardiac nor renal hypertrophy but completely eliminated left kidney atrophy distal to aortic stenosis. Radioligand binding studies demonstrated that the density of both ET<sub>A</sub> and ET<sub>B</sub> receptors was markedly higher in the atrophic kidney. *In situ* hybridization techniques revealed that ET<sub>B</sub> expression was highly upregulated in the atrophic kidney, particularly in the glomeruli. These data suggest that endothelins could contribute to the development of atrophy in the ischemic kidney. Potent vasoconstriction induced by these peptides could be responsible for ischemic atrophy [103].

### Proteinuria

Proteinuria is commonly observed in different nephropathies leading to renal failure and its reduction has a favourable effect on the progression of chronic renal disease. Gomez-Garre *et al.* [104] have demonstrated that bosentan significantly reduced proteinuria in a normotensive model of proliferative nephritis. Benigni *et al.* have observed that simultaneously blocking angiotensin II and ET-1 had a greater protective effect on proteinuria than single blocking of these receptors in an animal model of membranous nephropathy [105]. The same authors have reported that unselective blocking of endothelin receptors reduced urinary protein excretion in diabetic rats [106]. In contrast, Hoher *et al.* [107] have observed that the selective blocking of ET<sub>A</sub> receptors resulted in a non-significant decrease in proteinuria in diabetic rats. In rats with streptozotocin-induced diabetes, Cosenzi *et al.* have demonstrated that bosentan fully prevents an increase in urinary protein excretion observed one month after the onset of diabetes, this result was achieved without reducing blood pressure [108].

### Renal Fibrosis

ET-1 transgenic mice develop interstitial renal fibrosis and glomerulosclerosis with progressive heart failure, although blood pressure is not affected by transgene expression [109]. Other studies have reported that

endothelins induce the synthesis of collagen and/or fibronectin in the smooth muscle cells of porcine arteries [110] in cultured rabbit synovial cells [111] and in human fibroblasts [112]. In the kidney ET-1 is able to induce the gene expression of fibronectin and type IV collagen in cultured mesangial cells [104]. These observations suggest that endothelin receptor antagonism might be an effective tool to modify the deposition of extracellular matrix proteins in the kidney. This goal has been achieved by Ebihara *et al.* [113] in animals with puromycin aminonucleoside nephrosis and by Boffa *et al.* [114] in rats with vascular fibrosis induced by L-NAME. Cosenzi *et al.* have demonstrated that bosentan was effective in reducing renal deposits of collagen and fibronectin, in an animal model of hypertension and hyperinsulinism [108]; the same result was achieved by blocking AT-1 receptors [115] but not by reducing blood pressure by hydralazine. The renal production of endothelins increases in diabetes [116] and ET-1 gene expression is higher in the kidney of rats with streptozotocin-induced diabetes and also in primary cultured rat mesangial cells exposed to high glucose [14]; in this study glucose increases the activity of the ET-1 promoter in mesangial cells. As an alternative mechanism, Zoja *et al.* have observed that overloading proximal tubular cells with albumin induced a dose-dependent increase in the synthesis of ET-1, suggesting a link between proteinuria and the synthesis of this peptide [117]. Moreover, Khan *et al.* have demonstrated that ET<sub>A</sub> receptor binding sites were increased in the kidney of a small group of rats 6 months after the induction of diabetes [118]. It is also noteworthy that angiotensin II has been identified as a potent stimulator of endothelin synthesis [119] and the concept has developed that some of the effects of angiotensin II are dependent upon ET receptor stimulation [120]. In fact the synthesis of matrix protein induced by ET-1 could be prevented by an ACE-inhibitor in cultured mesangial cells and that the effect of angiotensin II in the same model could be prevented by an endothelin antagonist; accordingly, thus suggesting that the interaction between ET-1 and angiotensin II is important for the regulation of extracellular matrix turnover [104]. Cosenzi *et al.* have compared the effects of enrasentan with those of hydralazine in rats with hypertension, hyperinsulinemia and renal damage induced by high fructose diet (HFD). Both drugs completely controlled the HFD-induced increase in blood pressure; however, only enrasentan reduced the glomerular hypertrophy and the renal deposition of collagen 1, collagen 4 and fibronectin induced by the diet; a similar effect was observed in the heart. In this study organ protection could not be attributed solely to the antihypertensive effect, since it was absent in animals treated with hydralazine [121].

### Radiocontrast Agent Toxicity

Injection of contrast media can determine nephrotoxicity particularly in subjects with pre-existent renal insufficiency. Many pathogenic mechanisms have been proposed but there is no definitively accepted theory to explain this phenomenon. Administration of radiocontrast media in animals has been shown to increase the concentration of ET in plasma and urine [122, 123].

Fujisaki *et al.* have measured plasma concentration and urinary excretion of endothelins in normal subjects and in

patients with chronic renal failure after intravenous administration of radiocontrast medium. The authors report an increase in urinary endothelin excretion in the group with chronic renal failure in comparison with healthy subjects and suggest a possible role for endothelins in the pathogenesis of radiocontrast medium nephropathy [124].

Liss *et al.* have demonstrated that the injection of contrast media does not modify the renal superficial cortical flow but significantly reduces the outer medullary blood flow and the outer medullary oxygen tension in anesthetized Sprague-Dawley rats. Pre-treatment with the selective ET<sub>A</sub> receptor antagonist BQ123 did not prevent the decrease in outer medullary blood flow but the reduction in oxygen tension was significantly smaller and shorter in duration. The authors conclude that ET<sub>A</sub> receptors are partly involved in the depression of outer medullary oxygen tension induced by radiocontrast media and that the beneficial effect of BQ123 does not seem to be primarily mediated on the hemodynamic level but may involve tubular transport mechanism [125].

Wang *et al.* have evaluated the effect of SB209670, a mixed endothelin receptor antagonist, in 158 patients with chronic renal insufficiency (mean serum creatinine  $2.7 \pm 1.0$  mg/dl) undergoing angiography. The primary end point was a mean change in serum creatinine concentration from baseline to 48 hours. Unlike previous data obtained in animals, the results of this study show that SB209670 exacerbates radiocontrast nephrotoxicity in comparison with placebo [126]. Studies evaluating the effects of selective ET<sub>A</sub> receptor antagonists in patients receiving radiocontrast media are still not available but would be interesting.

## Conclusions

The results of the studies performed in animals and those of the single, acute study performed in patients with chronic renal failure can be considered promising, however, further chronic clinical studies are necessary to confirm a possible role for the antagonists of endothelin receptors in the prevention and treatment of kidney disease, considering the disappointing results obtained in chronic double blind studies in patients with heart failure.

## ARTERIAL HYPERTENSION

ET-1 is one of the most potent vasoconstrictor but it is also a trophic factor and a mitogen, two additional properties that are of interest considering the trend to improve end-organ remodelling in addition to normalizing blood pressure in hypertensive patients. For these reasons it has been suggested that endothelin receptor antagonists could play an important role as antihypertensive agents, although there is no convincing evidence that endothelins are involved in the pathogenesis of essential hypertension. Many studies, performed in animal models of hypertension, have demonstrated that these drugs are effective as hypotensive agents in many, but not all, models of arterial hypertension. Good results have been obtained in SHR-stroke prone rats, in Dahl-salt sensitive rats, in salt depleted squirrel monkeys, in DOCA salt rats, in rats on a high fructose diet, and in dogs with renal hypertension, whereas these compounds were ineffective in SHR, in renovascular hypertension (2K/1C) and in hypertension induced by 1-NAME [127, 121].

After the promising results obtained in animals, a clinical trial involving 293 patients with essential hypertension has been performed by Krum *et al.* [34]. In this study the hypotensive effect of bosentan was compared with that of enalapril, and showed a similar hypotensive effect. However, an asymptomatic increase in liver enzymes (ALT and AST) was observed in some patients receiving bosentan and this effect appeared to be dose related. However, it must be considered that the maximum dosage of the drug was very high (2000 mg/day); this dosage is not necessary since a flat dose/effect curve has been demonstrated over 500 mg/day. The effect was completely reversible after the end of the treatment. The mechanism of liver injury has been previously discussed.

Darusentan is a nonpeptidic endothelin receptor antagonist that binds to human ET<sub>A</sub> receptors with a K<sub>i</sub> of 1.4 nmol/L and to ET<sub>B</sub> receptors with a K<sub>i</sub> of 184 nmol/L, therefore it is considered a selective receptor antagonist. Nakov *et al.* have evaluated the antihypertensive effect of this drug in 392 patients. The study was a randomized, double blind, placebo controlled parallel group comparison. After a 2-week placebo run-in period the patients were randomized to treatment with 10, 30 or 100 mg darusentan or placebo administered once daily over 6 weeks. The primary efficacy parameter was a change in resting diastolic blood pressure. A dose dependent blood pressure reduction was observed at the end of the study. Diastolic blood pressure was reduced by 3.7 mmHg, 4.9 mmHg and 8.3 mmHg in the three groups treated with respectively 10, 30 or 100 mg darusentan in comparison with placebo. The change was statistically significant in all three groups. Moreover, diastolic blood pressure was normalized respectively in 37.2%, 38.8% and 55.8% of patients receiving darusentan. Hemoglobin concentration was slightly reduced (0.5 g/dl) at the end of the study in patients treated with darusentan in comparison with placebo. No relevant change was observed with regard to any of the other laboratory parameters [128].

As previously reported, BQ-123, a peptide selective ET<sub>A</sub> receptor antagonist, reduced blood pressure, improved renal blood flow and reduced proteinuria after acute injection in a small group of patients with chronic renal failure and did not affect blood pressure in healthy volunteers [102].

Calhoun *et al.* have evaluated the short-term efficacy of sitaxsentan, a receptor antagonist, in a small group (n=31) of hypertensive patients in an open label study. Patients were treated at one of three dose levels (160mg, 320 mg, 480 mg divided into two daily administrations) over a 2 week-period. At the end of the study, office blood pressure was significantly reduced in all three groups whereas 24-hour ambulatory blood pressure was reduced only in the 320 mg and 480 mg groups. The drug was well tolerated (data presented at the AHA 2000 – abs 104499- but not published).

In conclusion, the few studies performed in hypertensive patients, following the promising results obtained in animal models of hypertension, appear to confirm the hypotensive efficacy of both mixed and selective ET receptor antagonists. Hepatic tolerability appears to be a dose-related problem. However, it was not possible to show any significant advantage of these drugs over currently used

antihypertensive drugs and there are no ongoing, new trials involving larger samples of hypertensive patients and for longer periods; therefore, there would appear to be no near future for endothelin receptors as antihypertensive drugs.

## STROKE

Many authors have demonstrated an increased plasma level of endothelins in patients with ischemic stroke, compared to that of healthy controls [129,130]; Alioglu *et al.* have observed that the difference in plasma endothelin levels between patients and healthy controls is highly significant early after the onset of the stroke but cannot be found after 7 days [13]. Juvela *et al.* have reported that endothelin concentrations correlate with delayed cerebral ischemia and vasospasm after aneurysmal subarachnoid hemorrhage in a group of 70 patients. The author suggests that endothelin may be an important causal or contributing factor to vasospasm after subarachnoid hemorrhage [131]. Yamashita *et al.* have found an increased production of tissue endothelins after the event in SHR-stroke prone [132] rats. However, contradictory data are also reported in literature; Haapaniemi *et al.* have measured plasma endothelin levels in 101 patients with ischemic stroke at different periods after admission and found normal endothelin levels at admission, after 1 week, after 1 month and after 3 months [133]. Bhardway *et al.* evaluated the effect of Ro 61-1790, a selective ET<sub>A</sub> receptor antagonist, in cats 24 hours after reversible middle cerebral artery occlusion. The results suggested that selectively blocking these receptors is not beneficial to tissue or functional outcomes from experimental strokes in cat [134].

The efficacy of enrasentan, a mixed endothelin receptor antagonist, has been evaluated in a rat focal ischemia model. Forty-two SHR received enrasentan dissolved in drinking water (3 mg/kg or 15 mg/kg) or placebo for 1 week; thereafter the animals underwent surgical procedure for permanent middle cerebral artery occlusion. After 24 hours the animals were sacrificed and the extension of the ischemic area was quantified. Enrasentan, at the 15 mg/kg treatment regimen, significantly reduced both hemispheric infarction and infarct volume. A 30% reduction in ischemic brain injury was demonstrated. Accordingly, the authors suggested a potential role for this drug as therapy in focal stroke [135]. The discrepancies between the results of this study and those of the study of Bhardway may be, at least partially, explained as follows. Ro 61-1790 was administered after the stroke whereas the rats of this study were treated for 1 week before the stroke, so that receptor blocking had been achieved when the ischemic injury occurred. The second point is that Ro 61-1790 is a selective blocker of ET<sub>A</sub> receptor and enrasentan is a dual blocker. Willette *et al.* have demonstrated a late increase in ET<sub>B</sub> receptors after transient cerebral ischemia; these receptors are located on proliferating microglia and might be responsible for the delayed neuron death [136].

Since delayed cerebral ischemia is an important cause of death and disability in patients who have suffered subarachnoid hemorrhage Shaw *et al.* have evaluated the efficacy of TAK-044, a mixed endothelin receptor antagonist, in 420 patients with subarachnoid hemorrhage

due to aneurysm rupture. The study design was a multicenter, randomized, placebo controlled, parallel group and it was a phase II trial. The primary end point was whether a delayed ischemic event occurred 3 months after the first dose of the study drug. Secondary end points included determining whether a delayed ischemic event occurred within 10 days following the first dose of the drug, whether a new cerebral infarction was demonstrated by a CT scan or by necropsy within 3 months and the patients Glasgow Outcome Scale at the end of the study. The primary end point was achieved with a lower incidence of delayed ischemic events by the end of the study (29.5 vs. 36.6%), whereas no significant differences were observed in the secondary end points [137].

Although ET<sub>A</sub> receptors are responsible for vasospasm, the observations of Willette and those of Shaw suggest that blocking both ET receptors might be preferable to protect the neuronal cells from ischemic injury.

## CHRONIC LIVER DISEASE

The pathophysiological importance of the endothelin system in chronic liver disease has been the subject of many studies. Authors report that plasma endothelin levels are generally increased in patients with liver cirrhosis, in line with the severity of disease [15,138].

The stellate cells have been demonstrated to play a pivotal role in the regulation of intrahepatic resistance and blood flow. Following liver injury, stellate cells undergo a process that has been termed "activation" and exhibit an exaggerated contractile phenotype, since endothelins are the most active stimulators of these cells [139]. Moreover, an enhanced expression of both endothelins and endothelin receptors has been observed in hepatic *stellate* cells after activation [140]. These observations have suggested that endothelin antagonism would be a valuable tool to reduce portal hypertension.

The administration of the mixed endothelin receptor antagonist SB209670 decreased portal pressure in rats with biliary cirrhosis, by reducing portal venous system resistance [141]. Similar results were obtained using bosentan in rats with secondary biliary cirrhosis and in rats with CCl<sub>4</sub>-induced cirrhosis [142]. In contrast, Poo *et al.* observed no beneficial effects by administering RO 48-5695, another mixed receptor antagonist, in cirrhotic rats [143]. De Gottardi *et al.* have compared the effect of a selective antagonist of ET<sub>A</sub> receptors, atrasentan (ABT-627) with those of mixed antagonists and selective antagonist of ET<sub>B</sub> receptors administered for 11 days in rats with a partially ligated portal vein; they conclude that only the selective antagonist of ET<sub>A</sub> receptors ameliorates portal hypertension [144].

Endothelins appear to be involved in wound healing and it has been shown that endothelin receptor antagonist can reduce fibrosis in the heart and in the kidney of diabetic rats [91,108]. Accordingly, studies have investigated the possibility of preventing liver fibrosis by antagonizing ET receptors. Cho *et al.* have evaluated the effect on the parameters of fibrogenesis of a selective ET<sub>A</sub> receptor antagonist, LU135252, administered at 80mg/ kg daily for

six weeks in rats with bile duct occlusion. These authors conclude that selectively blocking ET<sub>A</sub> receptor can dramatically reduce collagen accumulation in rats with secondary biliary fibrosis [145]. More recently, Thirunavukkarasu *et al.* investigated the effect of the mixed antagonist TAK-044 in rats undergoing hepatic injury induced by carbon tetrachloride administered intraperitoneally for 4 or 8 weeks. The histopathological examination performed at the end of the study showed a significant arrest of progression to cirrhosis in the 4-week group and reversal of cirrhosis in the 8-week group. Moreover, portal hypertension was reduced in treated animals and an improvement of the indexes of hepatic damage and of those of hepatic synthesis was observed [146].

In conclusion, experimental studies performed in animal models of chronic liver disease and portal hypertension demonstrate the protective activity of these drugs, which reduce portal hypertension and liver fibrosis. One minor problem is that it is not clear whether a selective ET<sub>A</sub> or a mixed antagonist is preferable. However, by far, the major problem is that frequent reversible increases in hepatic aminotransferase and a single death due to fulminant hepatitis have been recorded in studies performed in patients with hypertension or heart failure treated with endothelin receptor antagonists. For this reason it is difficult to imagine clinical trials performed in patients with chronic liver disease to investigate the potential protective activity of this class of drugs, although the pharmacokinetics of bosentan are not modified in patients with mild liver impairment [38].

#### RESTENOSIS AFTER ANGIOPLASTY

Percutaneous transluminal coronary angioplasty (PTCA) was introduced by Gruentzig *et al.* in 1979 [147] and this procedure is now employed worldwide, substituting cardiac surgery in a large number of critical coronary stenoses undergoing treatment. However, the long-term efficacy of PTCA is reduced by the incidence of vascular restenosis. Endothelins are potent smooth-muscle mitogens and it has been suggested that these peptides could be involved in the pathogenesis of restenosis. In 1993 Douglas and Ohlstein observed that endothelin infusion after angioplasty in carotid arteries of rats caused a 65% greater neointima proliferation after 7 days compared to saline [148]. Wang *et al.* have demonstrated that the expression of ET-1, endothelin receptors and endothelin converting enzyme increases greatly after angioplasty in carotid arteries of rats [149]. As a consequence of these observations, the potential protective effect of enrasentan on restenosis after balloon angioplasty has been evaluated in rats. Angioplasty of the left carotid artery was performed in Sprague-Dawley rats; the animals were divided into two groups; 11 rats received enrasentan for 14 days, 12 rats received vehicle; thereafter they were sacrificed. Carotid arteries were evaluated *in vivo* with MRI and *post mortem* a histologic analysis was performed to calculate the intima-to-media ratio. Right carotid arteries were examined as control group. The results of the study have shown that enrasentan induced a 42% reduction in the neointimal growth in the injured carotid arteries and a 20% increase in the intraluminal volume in the treated animals [150].

#### OTHER APPLICATIONS

Pathophysiological studies have suggested a possible involvement of endothelins in many other pathological conditions such as cardiopulmonary by-pass, glaucoma, migraine; accordingly further possible applications can be hypothesized.

#### CONCLUSIONS

Compounds belonging to the class of endothelin receptor antagonists have undergone intensive experimental and clinical evaluation to verify effectiveness and safety in the treatment of several pathological conditions. After an initial period characterized by the evidence of an impressive amount of interesting data obtained in animal models of several diseases, opening great perspectives for the treatment of important pathological conditions, most of the subsequent clinical trials have shown disappointing results. The evaluation of the possible application of endothelin receptor antagonists in the treatment of heart failure appears to have concluded with a definitively negative result. The results obtained in patients with essential hypertension failed to show any significant advantage over currently used antihypertensive drugs. Liver toxicity has been observed as a not uncommon adverse effect, albeit usually asymptomatic and reversible. This effect was not predicted by experiments performed in rats. An important goal has been achieved by demonstrating the efficacy of bosentan for the treatment of arterial pulmonary hypertension. This result has been promptly followed by the worldwide registration of the drug for this condition. However, we cannot forget that this is a rare clinical condition and that previously only intravenous prostaglandins had proved to be an effective therapeutic approach. Moreover, the main advantage demonstrated by endothelin receptor antagonists in these patients is represented by a 30 to 40 m increase in the 6 min walking distance and not by a dramatic improvement in the quality of their life. Another unresolved question is represented by the theoretical advantage of selective ET<sub>A</sub> over mixed antagonists in the treatment of cardiovascular disease, for which clinical trials could provide no clear confirmation.

In the light of these considerations, are there further perspectives for endothelin receptor antagonists? The answer is probably affirmative. Trials performed in patients with advanced hormone-refractory prostate cancer demonstrate that selective ET<sub>A</sub> antagonist retard the progression of the illness, reduce pain and improve the quality of life. Clearly these drugs do not constitute curative treatment but a palliative approach, of no less importance at this stage of the disease, if we consider they are well-tolerated in comparison with traditional chemotherapy. As reported, other potential applications are at the stage of ongoing experimental evaluation, however some time will be required in order to demonstrate their clinical effectiveness.

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