REVIEW / SYNTHÈSE

The endothelin axis in cancer: the promise and the challenges of molecularly targeted therapy[1]

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Abstract: The endothelin (ET) axis, which includes ET-1, ET-2, ET-3, and 2 G protein-coupled receptor subtypes, ET_AR and ET_BR, promotes growth and progression of a variety of tumors, such as prostatic, ovarian, renal, pulmonary, colorectal, cervical, breast, lung, bladder, endometrial carcinoma, Kaposi's sarcoma, brain tumors, and melanoma. Acting on selective receptors, ET-1 regulates mitogenesis, cell survival, angiogenesis, bone remodeling, stimulation of nociceptors, tumor-infiltrating immune cells, epithelial-to-mesenchymal transition, invasion, and metastatic dissemination. At the molecular level, endothelin receptor antagonists, besides providing ideal tools for dissecting the ET axis, have demonstrated their potential in developing novel therapeutic strategies. Emerging experimental and clinical data demonstrate that interfering with endothelin receptors provides an opportunity for the development of rational combinatorial approaches using endothelin receptor antagonists in combination with chemotherapy or molecularly targeted therapy.

Key words: endothelin, endothelin receptor antagonist, G protein-coupled receptor, antitumor therapy.

Résumé : L'axe endothéline (ET), qui comprend les endothélines Et-1, Et-2 et ET-3, et deux récepteurs couplés aux protéines G, les récepteurs A (R_AET) et B (R_BET), favorise la croissance tumorale et la progression de diverses tumeurs, telles que les carcinomes prostatique, ovarien, rénal, pulmonaire, colorectal, cervical, mammaire, bronchique, vésical et endométrial, le sarcome de Kaposi, les tumeurs cérébrales et les mélanomes. Agissant sur un récepteur sélectif, l'ET-1 régule la mitogenèse, la survie des cellules, l'angiogenèse, le remodelage osseux, la stimulation des nocicepteurs, les cellules immunitaires infiltrant les tumeurs, la transition épithéliale-mésenchymateuse, l'invasion des métastases et leur dissémination. Sur le plan moléculaire, les antagonistes des récepteurs de l'endothéline, en plus d'être des outils idéaux pour l'analyse de l'axe ET, ont démontré leur potentiel dans l'élaboration d'une nouvelle stratégie thérapeutique. Des données cliniques et expérimentales émergentes démontrent que l'interférence avec un récepteur endothéline permet de développer des approches combinatoires rationnelles en utilisant des antagonistes des récepteurs de l'endothéline en association avec la chimiothérapie ou une thérapie ciblée.

Mots-clés : endothéline, antagoniste des récepteurs de l'endothéline, récepteur couplé aux protéines G, thérapie antitumorale.

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Introduction

The endothelins (ETs) comprise a family of 3 peptides, ET-1, ET-2, and ET-3, that are potent vasoconstricting peptides involved in the pathophysiology of many human malignancies (Levin 1995; Masaki 2000). Biological activity is mediated by the activation of 2 G protein-coupled receptor (GPCR) subtypes, ET_A and ET_B. Emerging experimental and clinical data increasingly underscore the role of GPCRs in cancer progression and metastasis. Malignant cells often hijack the normal physiologic function of GPCRs to proliferate autonomously, evade immune detection, increase their nutrients and oxygen supply, invade their surrounding tissues, and disseminate to others organs. The aberrant expression of GPCRs, such as endothelin receptors, and their autocrine and paracrine activation by agonists released by tumor or stromal cells represent the most frequent tactics used by tumor cells to stimulate GPCRs and their signaling networks (Dorsam and Gutkind 2007). The endothelin axis has a relevant role in a wide spectrum of malignancies (Nelson et al. 2003; Bagnato and Catt 1998) through the triggering of a highly interconnected signaling network that ultimately activates the "hallmarks of cancer" (Hanahan and Weinberg 2000), including aberrant cell proliferation, adhesion, migration, invasion, angiogenesis, and antiapoptotic activity (Fig. 1).

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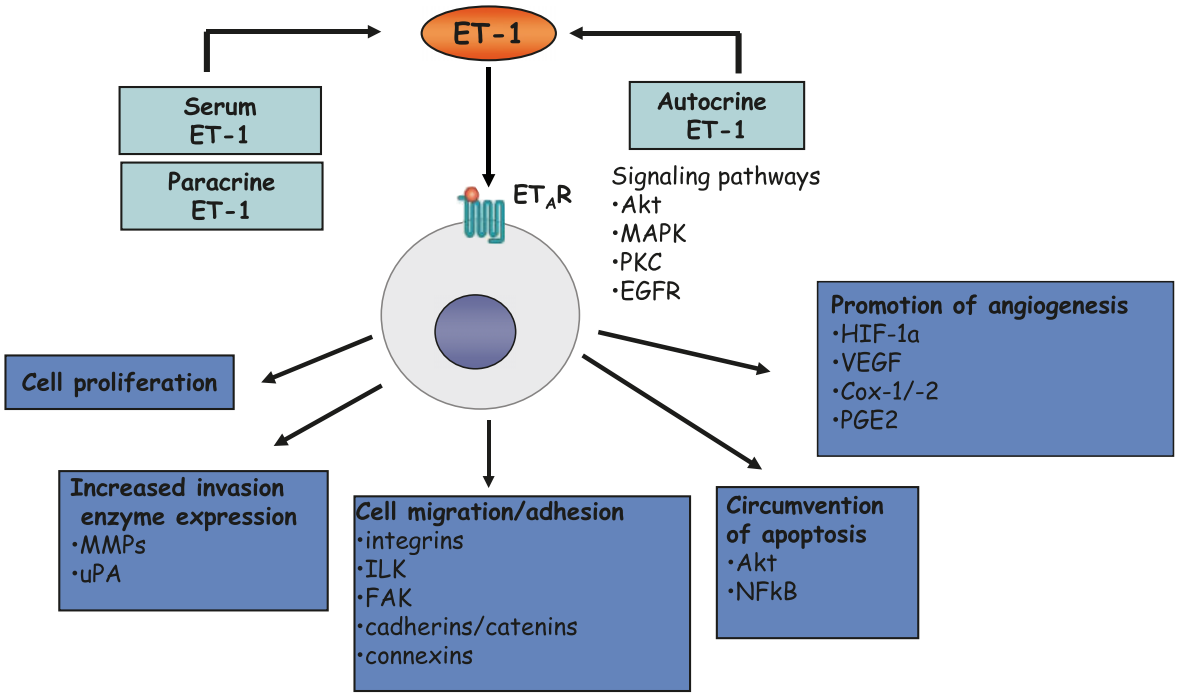
ET-1 synthesis

ET-1, ET-2, and ET-3, characterized by a single α-helix

Fig. 1. Role of ET-1 in tumor growth and progression. ET-1 is a growth factor, present in the plasma and produced by stromal and tumor cells, that activates different signaling pathways, such as Akt, MAPK, PKC, and EGFR, in an autocrine or paracrine fashion. These pathways regulate gene expression associated with cell proliferation, adhesion, migration, invasion, angiogenesis, and antiapoptotic activity, all of which are pivotal in the gain of malignant potential. Akt, serine–threonine kinase (protein kinase B); MAPK, mitogen-activated protein kinase; PKC, protein kinase C; EGFR, epidermal growth factor receptor; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; COX, cyclooxygenase; PGE_2, prostaglandin E_2; NF-κB, nuclear factor-κB; ILK, integrin-linked kinase; FAK, focal adhesion kinase; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator.



and 2 disulfide bridges, are encoded by distinct genes and are regulated at transcriptional and posttranscriptional levels. The primary translation product of the ET-1 gene is the 212-amino acid (aa) prepro-ET-1, which is cleaved by an endopeptidase to form the 38-aa big ET-1. ET-1 is synthesized from big ET-1, a biologically inactive precursor, by an unusual hydrolysis of the Trp21–Val22 bond by the endothelin-converting enzyme (ECE-1). In humans, there are 4 isoforms (ECE-1a–1d) derived from a single gene by the action of alternative promoters. Structurally, they differ only in the amino acid sequence of the extreme N-terminus. A second enzyme, ECE-2, also exists as 4 isoforms, differing from ECE-1 in requiring an acidic pH for optimal activity. As an alternative pathway, human chymase can also cleave big ET-1 to ET-1, which is cleaved, in turn, to the mature peptide (Xu et al. 1994). The half-life of ET-1 in circulation is 7 min (Rubin and Levin 1994). Two pathways have been described for clearance of endothelin: ET_B receptor-mediated uptake followed by lysosomal degradation (Burkhardt et al. 2000; Bremnes et al. 2000) or catabolism by the extracellular neutral endopeptidase (EC 3.4.24.11) neprilysin(NEP) (Vijayaraghavan et al. 1990; Battistini et al. 1993). ET-1 production is stimulated by a variety of stimuli, including interleukin (IL)-1β, transforming necrosis factor (TNF)-α, transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), vasopressin, hypoxia, and shear stress. Inhibitory factors include nitric oxide, prostacyclin, and atrial natriuretic peptide (Nelson et al. 2003).

Endothelin receptor signaling

ETs exert their activity by binding to 2 distinct GPCRs, ET_AR and ET_BR. ET_BR has equal affinity for all 3 ETs, whereas ET_AR exhibits an affinity for ET-3 that is 2 orders of magnitude lower than that for ET-1 and ET-2. Most importantly, ligand activation of ET_BR leads to induction of intracellular pathways that are counter-regulatory to ET_AR signaling. Owing to their differences in C-terminus sequences, which are pivotal for coupling of G proteins, the receptors induce divergent intracellular effects. GPCRs interact with heterotrimeric G proteins composed of α, β, and γ subunits. The α subunit of G proteins is divided into 4 subfamilies: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$. Each G protein activates several downstream effectors. Typically, $G\alpha_s$ coupling results in stimulation of adenylyl cyclase. $G\alpha_i$ coupling leads to inhibition of adenylyl cyclase and activation of Ca^{2+} channels. Coupling of $G\alpha_q$ entails activation of phospholipase C (PLC) (Masaki 2000), which cleaves phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol and inositol triphosphate (IP_3). $G\alpha_q$ and $G\alpha_{12}$ can also control the activity of key intracellular signal-transducing molecules, including mitogen-activated protein kinase (MAPK), and can induce various immediate early genes (Rozengurt 2007).

ET-1 and cell proliferation

The binding of endothelin to its cognate GPCR triggers

the activation of a network of multiple pathways rather than a linear sequence of intracellular signaling cascades. This network includes PLC activity, increased intracellular Ca^{2+} levels, and activation of protein kinase C and MAPK, which act in a synergistic and combinatorial fashion to relay the mitogenic signal to the nucleus and promote cell proliferation. Moreover, ET-1 stimulates phosphatidylinositol 3-kinase (PI3K)-mediated AKT activation, indicating that the endothelin axis drives a complex signaling network that is finely tuned during cell growth.

ET-1 stimulates DNA synthesis and cell proliferation in various epithelial tumor cells, including prostate, cervical, and ovarian cancer cells. In these cell lines, spontaneous growth is significantly inhibited in the presence of ET_AR antagonists, but not ET_BR antagonists, demonstrating that endogenous ET-1 acts as an autocrine modulator of cell proliferation only through ET_AR. The mitogenic activity of ET-1 can be amplified by synergistic interactions with other growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin, insulin-like growth factor (IGF), PDGF, TGF-β, and IL-6 (Nelson et al. 2003).

Cross-talk between cell surface receptors represents the main mechanism for expanding the cellular communication signaling network. A major pathway utilized by many GPCR agonists is the transactivation of epidermal growth factor receptors (EGFRs) that leads to RAS-dependent MAPK activation. GPCR-induced EGFR transactivation is mediated by release of precursor forms of EGFR ligands generated by activation of matrix metalloproteinases (MMPs). EGFR is also phosphorylated directly by c-Src (Buchanan et al. 2006). In colon cancer cells, for example, EGFR transactivation appears to proceed through an intracellular pathway involving GPCR, β-arrestin, and c-Src (Rozengurt 2007). In this context, ET-1 in ovarian cancer cells causes EGFR transactivation that is in part responsible for MAPK activation by c-Src. This event leads, through the formation of Shc/Grb-2 complexes, to activation of both the Ras/MAPK pathway and AKT (Vacca et al. 2000; Bagnato et al. 2005). The signaling cross-talk between the EGFR/ET_AR pathways provides a rationale for combining EGFR inhibitors with ET_AR antagonists. Recent work has shown that a specific ET_AR antagonist was able to reduce the ET-1-induced EGFR transactivation (Rosanò et al. 2007*a*). The EGFR inhibitor gefitinib significantly inhibited EGF- and ET-1-induced EGFR phosphorylation, but incompletely reduced the ET-1-induced activation of downstream targets, which indicates that dual alternative pathways, one EGFR-dependent and the other EGFR-independent, converge on MAPK and AKT activation. Concomitant blockade of ET_AR and EGFR resulted in a greater inhibition of EGFR, MAPK, and AKT phosphorylation, indicating the critical role of these interconnected signaling proteins and suggesting that a combinatorial approach, simultaneously disabling multiple signaling circuitries activated by EGFR and ET_AR, may be potentially advantageous in cancer therapy (Rosanò et al. 2007*a*).

ET-1 and tumor neovascularization

ETs, besides being mitogenic for epithelial cells, stimulate the growth of endothelial cells, vascular smooth muscle cells, fibroblasts, and pericytes and are also angiogenic factors. While mitogenesis of endothelial cells is mediated by ET_BR, mitogenesis of vascular smooth muscle cells and pericytes is mediated by ET_AR. ET-1 modulates various stages of neovascularization, including endothelial cell proliferation, migration, invasion, protease production, and tubule formation, and stimulates neovascularization in vivo. Elevated expression of ET-1 and its cognate receptor is significantly associated with microvessel density (MVD) and vascular endothelial growth factor (VEGF) expression in tumor cells. Thus, ET-1 increases VEGF mRNA expression and induces VEGF protein levels in a time- and dose-dependent fashion, and does so to a greater extent under hypoxia.

The transcriptional upregulation of VEGF is strictly controlled by hypoxia-inducible factor (HIF)-1α, the critical transcriptional factor that conveys signaling elicited by hypoxia and growth factors. Under normoxic conditions, ET-1 induces HIF-1α accumulation by inhibiting its degradation. In the nucleus, the ET-1-induced HIF-1 transcriptional complex is formed and binds to the hypoxia-responsive element (HRE), thereby transactivating VEGF. Thus, under hypoxic conditions, ET-1 potentiates hypoxia stimulus by amplifying HIF-1α stability and VEGF production (Bagnato et al. 2005). The ET-1-induced upregulation of VEGF expression and secretion as well as activation of HIF-1 transcription complex is blocked by a specific ET_AR antagonist. There is a reciprocal relationship between ET-1 expression and HIF-1α activity: not only does ET-1 stabilize HIF-1α during normoxia and to a greater extent in hypoxia leading to HIF-1α-mediated transcription of angiogenic genes, but also HIF-1α mediates transcription of ET-1 in different cell types. Therefore, expression of ETs is controlled by the tumor microenvironment, whilst the endothelins themselves modify the environment via HIF-1α (Grimshaw 2007).

In normoxic conditions, ET-1 significantly increases expression of cyclooxygenase (COX)-1 and COX-2 at the mRNA and protein level, as well as increasing COX-2 promoter activity and prostaglandin (PG)E_2 production, and it does so to a greater extent under hypoxia. COX-1 and COX-2 inhibitors block ET-1-induced PGE_2 and VEGF release, MMP activation, and cell invasion, demonstrating that both enzymes function as downstream mediators of ET-induced angiogenic and invasive properties. Endothelin receptor antagonists block these ET-mediated effects. Silencing of HIF-1α by small interfering RNA (siRNA) desensitizes COX-2 transcriptional activity, PGE_2 and VEGF production, and MMP activation in response to ET-1, implicating HIF-1α and COX as downstream targets of ET-1 signaling. Collectively, these results identify a new mechanism whereby the ET-1 axis can promote and interact with the HIF-1α-dependent machinery to amplify the signaling to tumor progression (Bagnato et al. 2005; Spinella et al. 2007).

Invasive tumor cells, including those of melanoma, prostate, breast, and ovarian carcinomas, have been shown to form de novo extracellular matrix-rich vascular channels expressing vascular-associated molecules (Folberg et al. 2000). Interestingly, tumor cell-lined vasculature is exhibited by

aggressive ovarian cancer cells. Approximately 30% of human ovarian cancers have some degree of tumor cell-lined vasculature that is associated with advanced stage, high tumor grade, development of distant metastasis, and poor overall survival (Sood et al. 2004). MMP-2 and MT1 (membrane type 1)-MMP appear to play a key role in the development of vasculogenic-like networks and matrix remodeling by aggressive ovarian cancer cells (Sood et al. 2004). It is noteworthy that invasive ovarian cancer cells that are capable of generating tubular networks in vitro express both MMPs and ET_AR and produce ET-1. Furthermore, ET_AR antagonist treatment prevents the formation of tumor-lined vascular channels (L. Rosanò, V. Di Castro, P.G. Natali, A. Bagnato, unpublished data). ET_AR blockade treatment could therefore also exert an antiangiogenic effect by acting on microvascular channels lined by tumor cells that overexpress ET_AR.

ET-1 and cell survival

ET-1 is an antiapoptotic factor in different cell types, indicating that this peptide may also modulate cell survival pathways, such as PI3K-dependent AKT activation. In different tumor cells, the addition of ET-1 markedly inhibited paclitaxel-induced apoptosis as a result of Bcl-2 phosphorylation. Interestingly, use of a specific ET_AR antagonist blocked this effect, indicating that ET-1 contributes to triggering resistance to paclitaxel through ET_AR binding activation of antiapoptotic signaling pathways such as Akt (Del Bufalo et al. 2002).

Specific ET_AR antagonists may therefore provide an improved approach in tumor treatment in which ET_AR blockade could result in tumor growth inhibition by reducing cell growth as well as by inducing apoptosis. Furthermore, when combined with conventional chemotherapy, the ET_AR antagonists would more effectively induce apoptosis by contributing to the reversal of paclitaxel resistance, as observed in ovarian, prostate, cervical, and nasopharyngeal carcinoma (Bagnato et al. 2002; Rosanò et al. 2007*b*; Akhavan et al. 2006).

ET-1 and tumor invasion

In tumor progression, changes in cadherin, connexin (Cx), MMP, and integrin expression have emerged as key factors. In this context, the ET-1 axis consistently induced the activity of 2 families of metastasis-related proteinases, the MMPs and the urokinase-type plasminogen activator (uPA) system, at several levels: mRNA transcription, zymogen secretion, and proenzyme activation, resulting in the highest invasive potential of tumor cells. Furthermore, in these cells, ET-1 stimulated focal adhesion kinase (FAK) and paxillin phosphorylation, suggesting that targeting the ET-1 axis can inhibit cell migration and possibly other FAK-associated processes that also contribute to invasion and metastasis. Moreover, EGFR transactivation by ET_AR is in part responsible for MMP activity and tumor cell invasion (Bagnato et al. 2005).

Defects in intercellular communication, including reduced or inappropriate expression of Cx, predominantly Cx43, represent an early step during invasion. The ET-1 axis induces a transient and dose-dependent reduction of gap junction intercellular communications (GJIC) and Cx43 tyrosine phosphorylation by c-Src (Bagnato et al. 2004, 2005). Once malignant transformation occurs, cell adherence to the extracellular matrix also becomes essential for the invasive process. In this regard, ET-1 enhances the expression of $\alpha_2\beta_1$- and $\alpha_3\beta_1$-integrins. The activity of integrin-linked kinase (ILK), a multidomain focal adhesion protein that conveys intracellular signaling elicited by β_1-integrin and growth factor receptors, increases as tumor cells adhere to type I collagen through a β_1-integrin signaling, and it increases even more upon ET-1 stimulation. ET-1 increases ILK expression and activity and an ILK inhibitor effectively blocks the phosphorylation of the downstream signals, AKT and glycogen synthase kinase (GSK)-3β. The blockade of ET-1/ET_AR-induced ILK activity results in inhibition of MMP activation, as well as cell motility and invasiveness, in a PI3K-dependent manner, indicating that ILK functions as a downstream mediator of the ET-1 axis in potentiating aggressive cellular behaviour (Rosanò et al. 2006).

In epithelial cancer, acquisition of invasiveness is often accompanied by loss of epithelial features and gain of a mesenchymal phenotype, a process known as epithelial-to-mesenchymal transition (EMT). A primary event that governs EMT is the disruption of E-cadherin-mediated stable interactions between the cells. In melanoma and in ovarian cancer cells, activation of the ET-1 axis induces downregulation in the expression of E-cadherin and associated β-catenin and induces concomitant upregulation of the mesenchymal N-cadherin. At the molecular level, the ET-1 axis drives inhibition of GSK-3β by a PI3K-dependent ILK-mediated signaling pathway to stabilize both the transcription factor Snail and β-catenin proteins in a coordinate fashion; this cooperatively engages transcriptional programs that control repression of E-cadherin leading to EMT (Rosanò et al. 2005; Bagnato et al. 2004; Jamal and Schneider 2002). Interestingly, in colon cancer cells, ET-1 is a downstream target of β-catenin and can rescue cells from apoptosis after β-catenin inhibition. Moreover, in both noncancerous and malignant prostate cells, β-catenin transcriptionally activates ET-1 expression (Kim et al. 2005). Meanwhile, ET-1 stimulates β-catenin signaling via a PI3K-dependent pathway. The positive interregulation between β-catenin and ET-1 signaling plays an important role in promoting proliferation and survival of cancer cells, thereby representing a novel mechanism that contributes to cancer progression (Sun et al. 2006).

ET-1 axis and osteogenesis

Osteoblastic metastases frequently develop in advanced cases of prostate cancer and in several other common malignancies, such as breast cancer, caused by stimulation of the osteoblasts by factors secreted by tumor cells. Several factors, including ET-1, have been implicated in this process. Osteoblasts display a high density of ET_AR, and respond to ET-1, driving the osteoblastic proliferation and new bone formation associated with metastatic breast and prostate tumors (Guise et al. 2006). Thus, ET-1 stimulates mitogenesis in osteoblasts and decreases osteoclastic bone resorption and osteoclast motility. Recent data contributing to our understanding of the molecular mechanism of ET-1-mediated

stimulation of osteoblasts have disclosed the involvement of a calcineurin/NFAT (nuclear factor of activated T cells) pathway (Van Sant et al. 2007). Experimental models of osteoblast proliferation and bone metastasis are inhibited by ET_AR antagonists in vivo, suggesting that the ET_AR is an attractive target for the management of tumors that metastasize to the bone (Yin et al. 2003; Nelson et al. 2003). The growing list of potential clinical applications for ET_AR blockade should include treatment and eventually prevention of osteoblastic bone metastases (Carducci and Jimeno 2006).

ET-1 axis and pain

Evidence is accumulating to suggest that ET-1 may contribute to pain states in certain metastatic cancers. Local cutaneous injection of ET-1 causes pain and excitation of nociceptors through ET_AR and concurrently produces analgesia through ET_BR by inducing the release of β-endorphin and the activation of the opioid pool (Khodorova et al. 2003). Thus, antagonists of ET_AR have been shown to ameliorate pain. This knowledge may lead to improved, targeted analgesia in patients with advanced cancer (Davar 2001; Hans et al. 2008).

ET-1 axis and immune modulation

ETs modulate trafficking, differentiation, and activation of tumor-infiltrating immune cells. ETs have a role in recruiting tumor-associated macrophages: macrophages express both endothelin receptors and chemotaxis towards ETs via ET_BR and a MAPK-mediated signaling pathway. Macrophages not only react to ETs but also produce ETs themselves; in contrast, no immunoreactive ET can be detected in cell extracts from human neutrophils and lymphocytes (Grimshaw 2007). Emerging evidence has been provided to demonstrate that the ET-1 axis represents a crucial modulator of lymphocyte homing and that overexpression of endothelial ET_BR in tumors prevents T cell homing. These results have important clinical implications by suggesting that ET_BR blockade could be therapeutically useful for tumor immune therapy by abrogating the endothelial barrier and increasing T cell homing to tumors (Buckanovich et al. 2008).

Targeting endothelin receptors as a novel approach in cancer treatment

The demonstration that ET-1 sustains many of the "hallmarks of cancer" indicates a role for ET-1 in tumor initiation and progression, thus identifying the ET-1 axis as a potential therapeutic target (Fig. 2). This has propelled the development of several approaches targeting the ET-1 axis in cancer therapy. One approach is represented by the inhibition of ET biosynthesis, for example using stimuli such as red wine bioactive polyphenols (Corder et al. 2001) and green tea (Spinella et al. 2006) or blocking ET production from big ETs with ECE inhibitors (Xu et al. 1994), or by the promotion of ET biodegradation via transfection of metallopeptidase neprilysin (NEP) (Kajiyama et al. 2005). Another approach is represented either by the potent and selective ET_AR, ET_BR, or mixed ET_AR/ET_BR antagonists, which abolish the ET-1-induced effects by blocking receptor activation, or by the selective ET_BR agonist, which enhances tumor perfusion and thus potentiates the therapeutic efficacy of anticancer agents (Rajeshkumar et al. 2005).

To date, endothelin receptor blockade represents the most rational targeted approach in controlling the pleiotropic activities of ET-1, which are all pivotal in the gain and maintenance of malignant phenotype (Table 1). In particular, the development of small molecules acting as specific ET_AR antagonists has contributed to our understanding of the pathophysiologic relevance of the ET-1 axis and its signaling circuitry in tumor progression and metastasis, paving the way for their evaluation in clinical trials. Among various ET_AR antagonists, ABT-627 (atrasentan) and ZD4054 are orally bioavailable ET_AR antagonists that potently and specifically bind to the ET_AR, blocking signal transduction pathways implicated in cancer cell proliferation and other host-dependent processes promoting cancer growth (Nelson et al. 2003; Rosanò et al. 2007*b*).

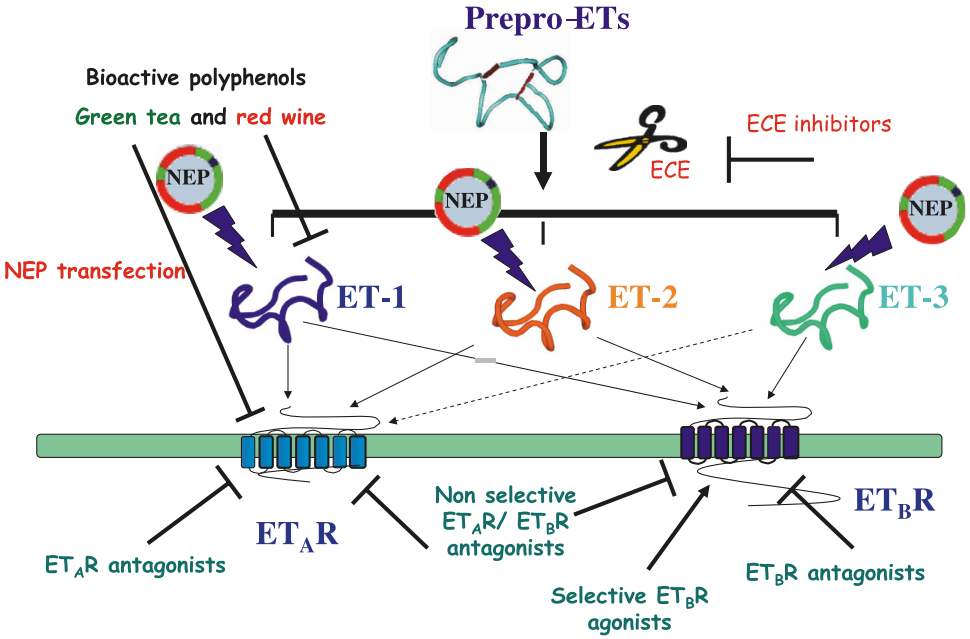
Ovarian carcinoma

ET-1 and ET_AR are overexpressed in 85% of primary and metastatic ovarian carcinomas, correlating with advanced stages of the disease. Levels of ET-1 are markedly elevated in the ascites of patients with epithelial ovarian cancer. Upon being activated, the ET_AR mediates pleiotropic cell signaling involved in the control of cell proliferation, survival, migration, and invasion (Bagnato et al. 2005). These findings complement and extend the recent analysis of gene expression profile of late-stage ovarian cancer whereby ET_AR has been identified as a metastasis-associated gene (Donninger et al. 2004). Moreover, the gene expression profile associated with response to chemotherapy identified ET_AR as one of the genes more highly expressed in post-chemotherapy samples than in samples of untreated primary ovarian tumors (Jazaeri et al. 2005).

In ovarian cancer, specific ET_AR antagonists, such as ABT-627 and ZD4054, inhibit in vitro cell proliferation, the ET-1-mediated protection against paclitaxel-induced apoptosis, and the release of VEGF. Cooperative proapoptotic and VEGF secretion inhibitory effects were observed when the ET_AR antagonist was used together with paclitaxel. Treatment with ZD4054 or ABT-627 produced tumor growth inhibition in ovarian cancer cell xenografts. Both treatments, which were generally well tolerated and had no detectable signs of acute or delayed toxicity, were long-lasting and achieved results comparable with those of paclitaxel. More marked and prolonged tumor growth inhibition was obtained by combined treatment of ET_AR antagonist with paclitaxel, with no toxicity and with tumor regressions in 40% of treated animals. A significant and complete inhibition of VEGF, MMP-2 expression, and tumor neovascularization and an increase in apoptosis were observed after the combined treatment (Rosanò et al. 2003*a*, 2005, 2007*b*).

The cross-signaling between the EGFR and ET_AR pathways strongly argued in favor of in vivo testing of a combination of EGFR inhibitor with ET_AR antagonist for the development of new effective therapeutic options for ovarian cancer. In ovarian carcinoma xenografts, coadministration of ZD4054 enhanced the efficacy of gefitinib, leading to partial or complete tumor regression. Antitumor effects

Fig. 2. The ET-1 axis as a therapeutic target in cancer. The discovery of the ET-1 axis components and their function in human cancer has propelled the development of a number of different approaches to target them. ET biosynthesis may be inhibited with stimuli, such as bioactive polyphenols in green tea and red wine, its production from big ET blocked by using endothelin-converting enzyme (ECE) inhibitors, or its biodegradation promoted by transfection of the metallopeptidase neprilysin (NEP). One of the most promising approaches is represented by the use of potent and selective ET_AR, ET_BR, or mixed ET_AR/ET_BR antagonists or selective ET_BR agonists, which enhance tumor perfusion, thus potentiating the therapeutic efficacy of anticancer agents.



were paralleled by biochemical and immunohistologic evidence of enhanced E-cadherin expression and decreased vascularization, K_i-67, MMP-2, VEGF, MAPK, and EGFR (Rosanò et al. 2007*a*).

A different approach is represented by transfection of NEP, a cell surface aminopeptidase capable of degrading a number of bioactive peptides, including ET-1. In ovarian carcinoma cells overexpressing NEP, there was a significant decrease in cell proliferation, survival, and invasiveness with a reduction of ET-1. Furthermore, tumorigenesis was reduced in vivo with the overexpression of NEP. This evidence suggests that NEP functionally suppresses the progression of ovarian carcinoma targeting ET-1 (Kajiyama et al. 2005).

Prostate carcinoma

The ET-1 axis has recently been identified as contributing to the pathophysiology of prostate cancer (Kopetz et al. 2002). ECE-1 is overexpressed in prostate cancer cells (Dawson et al. 2006). In the noncancerous prostate gland, ET-1 is produced by epithelial cells, the highest concentrations of ET-1 being found in the seminal fluid. In prostate cancer, key components of the ET-1 clearance pathway, such as ET_BR and NEP, are diminished, resulting in an increase in local ET-1 concentrations. Increased ET_AR expression is also seen in both primary and metastatic prostate cancer, correlating positively with advancing tumor stage and grade. In prostate cancer cell lines, ET-1 promotes cell proliferation and survival, new bone formation, and stimulation of nociceptors. Compared with monotherapy, combination treatment of ET_AR-overexpressing prostate cancer cells with ABT-627 and taxanes in vitro and in vivo was associated with lower cell viability and higher apoptotic rate. Selective ET_AR antagonists may block the proliferative effects of exogenous ET-1 in both prostate cancer cells and osteoblasts by direct effects on the tumor cells and by disrupting important bone–tumor interactions. Clinical testing of atrasentan has demonstrated benefit in prostate-specific antigen (PSA) progression, markers of bone turnover, and decreased pain in men with prostate cancer, but has not demonstrated significant improvement in survival or time to cancer progression (Carducci and Jimeno 2006). On the contrary, recent results from a phase II clinical trial indicate that ZD4054 could offer a significant improvement in overall survival in men with metastatic hormone-refractory prostate cancer (James et al. 2007), indicating that the role of this promising class of anticancer drugs remains to be defined by completion of future clinical trials.

Cervical carcinoma

Compared with noncancerous keratinocytes, human papillomavirus (HPV)-positive human cervical carcinoma cell lines overexpress ET-1 and ET_AR mRNA and secrete more ET-1 protein. Binding studies show that HPV-infected cells express increased numbers of functional ET_ARs and that specific ET_AR antagonists inhibit ET-1-induced proliferation, indicating the ET-1/ET_AR axis could be targeted for antitumor therapy in this tumor as well. Thus, atrasentan inhibited the growth of cervical carcinoma cell xenografts. Two cycles of treatment completely reversed tumor growth. Additive effects were displayed when atrasentan was administrated in combination with paclitaxel, supporting clinical

Table 1. Role of ET receptors in different types of tumors.

	Endothelin receptors	Receptor antagonists and their effects	References
Ovarian cancer	ET_AR mRNA is present in primary and metastatic cancers, correlating with tumor grade. ET_AR mediates all ET-1-induced tumor-promoting effects	In preclinical studies, ET_AR antagonists display antitumor activity and additive effects with taxanes or gefitinib	Bagnato et al. 2005; Rosanò et al. 2007*a*, 2007*b*
Renal cancer	ET_AR is expressed in renal cancer cell lines		Pflug et al. 2007
Endometrial cancer	Both ET_AR and ET_BR mRNA levels are lower in endometrial cancer tissue than in noncancerous endometrium		Pekonen et al. 1995
Prostate cancer	ET_AR expression increases with tumor stage and grade and is associated with decreased ET_BR expression	Clinical testing of atrasentan has demonstrated benefit in PSA progression, markers of bone turnover and pain, but not significant improvement in survival. ZD4054 improves overall survival in metastatic hormone refractory prostate cancer patients	Carducci and Jimeno 2006; James et al. 2007
Glioblastoma	ET_BR mediates the ET-1 effects in cell lines	Bosentan induces apoptosis in glioblastoma cell lines	Egidy et al. 2000*b*
Bladder cancer	Both ET_AR and ET_BR are expressed in bladder tumors	ET_AR antagonist decreases lung metastases	Herrmann et al. 2007
Melanoma	ET_BR is overexpressed and correlates with tumor progression. ET_BR mediates all ET-1-induced tumor-promoting effects	ET_BR antagonists inhibit melanoma cell growth in vitro and in vivo. Bosentan has benefit in disease stabilization in metastatic melanoma patients	Bagnato et al. 2004; Kefford et al. 2007
Bone malignancies	ET_AR is expressed in osteoblasts	ET_AR antagonist reduces osteoblastic bone metastases and tumor burden in bone	Guise et al. 2006
Breast cancer	ET_AR expression correlates with clinicopathologic parameters of aggressive carcinoma	In preclinical studies, ET_AR antagonist inhibits tumor growth	Smollich and Wulfing 2007
Nasopharyngeal carcinoma	ET_AR is overexpressed in 74% of tumors	ET_AR antagonist inhibits tumor growth and metastasis, showing synergistic effects with cytotoxic drugs	Mai et al. 2006*b*
Colon cancer	ET_AR and ET_BR are overexpressed	Bosentan induces apoptosis in colon cancer cells	Eberl et al. 2000
Cervical cancer	HPV-positive cervical carcinoma cells express functional ET_AR	In preclinical studies, ABT-627 inhibits tumor growth in monotherapy, as well as in association with taxanes	Bagnato et al. 2002
Kaposi's sarcoma	Both ET_AR and ET_BR are expressed	Dual ET_AR/ET_BR antagonist inhibits tumor growth in nude mice	Rosanò et al. 2003*b*
Lung cancer	ET_AR is overexpressed in tumor		Boldrini et al. 2005
Osteosarcoma	ET_AR is expressed in osteosarcoma cells	ET_AR mediates ET-1-induced cell invasion	Felx et al. 2006

use either in mono- or combination regimens (Venuti et al. 2000; Bagnato et al. 2002).

Breast carcinoma

ECE-1 is overexpressed in breast carcinoma and its expression is associated with an unfavourable outcome (Smollich et al. 2007). The expression of ET-1 and its receptors increase during the progression of breast carcinomas, correlating with the malignant potential (Smollich and Wulfing 2007). Moreover, elevated expression of members of the ET family, in particular the ET_AR, is associated with reduced disease-free survival and overall survival. Increased expression of the endothelin axis has been documented in invasive ductal carcinoma (IDC) of the breast compared with that in the healthy breast or in noninvasive ductal carcinoma in situ. Elevated expression of ET-1 is more common in IDCs of larger size and high histologic grade and in the presence of lymphovascular invasion, as well as in the serum of breast cancer patients with lymph node metastases (Smollich and Wulfing 2007).

Exposure of tumor cells to ETs leads to an invasive breast tumor cell phenotype in vitro via both ET_AR and ET_BR, involving increased activity of MMPs (Grimshaw et al. 2004). In vitro, the invasive capacity of breast tumor cell lines correlates with the level of expression of the ET-1 axis (Hagemann et al. 2005).

The spread and trafficking of tumor cells to potentially metastatic sites is controlled by chemokine expression in organs and by chemokine receptors in tumor cells. ET-1 through ET_AR induces mRNA and protein expression of the chemokine receptor CCR7 in breast tumor cells via HIF-1α, leading to increased invasion towards the CCR7 ligands CCL19 and CCL21 (Wilson et al. 2002; Grimshaw et al. 2004).

In biopsies of invasive breast cancer, the expression of ET-1, ET_AR, and ET_BR is associated with increased VEGF expression and vascularity (Smollich and Wulfing 2007).

Recently, it has been shown that bosentan, a dual endothelin receptor antagonist, inhibits tumor growth, vascularization, and bone metastasis in a model of breast carcinoma (Dreau et al. 2006). Increased expression of ET_AR in breast carcinomas is associated with resistance to chemotherapy, indicating that ET_AR status may serve as a predictive marker for identifying patients less likely to be responsive to conventional chemotherapy (Smollich and Wulfing 2007).

Kaposi's sarcoma

A different approach in targeting ET-1 receptors in cancer treatment is represented in the case of Kaposi's sarcoma (KS), in which ET-1 acts as an autocrine growth factor through both ET_AR and ET_BR. Binding of ET-1 and ET-3 to both receptors increases the proliferation, migration, and invasiveness of the KS-derived cells by stimulating secretion and activation of multiple tumor-associated proteases. Treatment of KS xenografts with A-182086, a mixed ET_AR/ET_BR antagonist, produced inhibition of tumor growth that was most likely related both to the antiproliferative effect on tumor cells and to the antiangiogenic effect on endothelial cells expressing ET_BR. Thus, ET-1 receptor antagonists may be effective for treatment of this malignancy because they are capable of interfering simultaneously with cell proliferation, invasiveness, and angiogenesis (Bagnato et al. 2001; Rosanò et al. 2003*b*).

Nasopharyngeal carcinoma

The ET_AR autocrine pathway is overexpressed in many malignancies, including nasopharyngeal carcinoma (NPC) (Mai et al. 2006*a*). In this tumor, ET_AR expression is an independent determinant of survival and a robust independent predictor of distant metastasis. Cell proliferation was inhibited by ABT-627 in 2 ET_AR-positive NPC cell lines, but not in ET_AR-negative NPC cell lines. ET_AR blockade also resulted in sensitization to the cytotoxic drug cisplatin and 5-fluorouracil-induced apoptosis. In nude mice, ABT-627 inhibited tumor growth, and combined treatment of ABT-627 with cisplatin or 5-fluorouracil produced additive antitumor effects. Of interest, the antitumor activity of ABT-627 was demonstrated also in an experimental lung metastasis model. These results support the rationale of combining ABT-627 with current standard chemotherapy to further improve the therapeutic ratio in the treatment of NPC (Mai et al. 2006*b*).

Melanoma

Transformed melanocytes express both ET_AR and ET_BR. Gene expression profiling (Bittner et al. 2000) and immunophenotyping of human cutaneous melanoma (Demunter et al. 2001) have recently identified ET_BR as critical in the progression of this malignancy. Through the same receptor, ET-1 acts as an antiapoptotic factor for melanoma cells and melanocytes. Thus, ET_BR blockade by the ET_BR peptide antagonist BQ788 results in growth inhibition and death of melanoma cells in vivo and in vitro (Lahav et al. 1999). ET-1 and ET-3 by ET_BR signaling induce inactivation of the gap junctions through phosphorylation of Cx43, which is responsible for contact-mediated regulatory control of keratinocytes. Additionally, activation of the ET_BR pathway by ET-1 and ET-3 contributes to disruption of normal host–tumor interactions by downregulating the expression of E-cadherin and associated β-catenin, with a concomitant significant increased expression of *Snail* and upregulation of N-cadherin. This latter change can mediate homotypic adhesive interactions as well as heterotypic melanoma cell–cell interactions. Concurrently, ETs increase $\alpha_v\beta_3$- and $\alpha_2\beta_1$-integrin expression and MMP-2 and MMP-9 activation. Downstream of ET_BR, activation of FAK and MAPK signaling pathways occurs, leading to enhanced cell proliferation, adhesion, migration, and MMP-dependent invasion (Jamal and Schneider 2002; Bagnato et al. 2004).

In melanoma, upregulation of HIF-1α is associated with neovascularization, VEGF expression, poor prognosis, and resistance to therapy. In hypoxic conditions, the PI3K–Akt pathway transforms melanocytes, indicating that hypoxia and HIF-1α are essential for melanocyte transformation (Bedogni et al. 2005). As reported for other tumors, ETs are also involved in angiogenesis in mouse models of melanoma (Lahav et al. 2004). In normoxic conditions, ET_BR activation by ET-1 and (or) ET-3 enhances HIF-1α expression and in turn upregulates VEGF, COX-1 and COX-2, and the PGE_2 pathway, and does so to a greater extent under hypoxia. Selective ET_BR antagonists block the ET-mediated effects. In human melanoma xenografts, the specific ET_BR antagonist A-192621 suppresses HIF-1α accumulation, tumor growth, neovascularization, and VEGF and MMP-2 expression (Spinella et al. 2007).

Blockade of both ET-1 receptors with bosentan decreased proliferation, induced apoptosis, and potentiated the effects of anticancer agents, suggesting that combination therapy of endothelin receptor antagonists with alkylating agents may improve their efficacy (Berger et al. 2006). A phase II study of bosentan as monotherapy in patients with stage IV metastatic melanoma showed disease stabilization in 6 of 32 patients (Kefford et al. 2007). New therapeutic strategies using specific ET_BR antagonist or nonselective endothelin receptor antagonist could therefore provide an improved approach to the treatment of melanoma.

Lung cancer

ET-1 has been proposed as a prognostic marker in non-small cell lung carcinoma (NSCLC) (Arun et al. 2004). There is higher expression of ET-1, ET_AR, and ECE-1 in lung tumors than in healthy tissue, whilst ET_BR is decreased. ET-1 expression is related to both VEGF expression and poor prognosis in NSCLC (Ahmed et al. 2000; Boldrini et al. 2005). Interestingly, ET-1 is increased in the breath condensate of NSCLC patients and this could potentially be used as a noninvasive test for early detection of NSCLC (Carpagnano et al. 2004).

Bladder cancer

The ET-1/ET_AR axis is overexpressed in bladder cancer. When metastatic bladder carcinoma cells were injected into mice treated with atrasentan, there was a dramatic reduction of metastases to the lungs (Titus et al. 2005).

Neuroblastoma

Human neuroblastoma cells express ECE-1 (Fisk et al. 2006), which has been suggested to play an important role in amyloid-β peptide metabolism as one of the amyloid-degrading enzymes. Hypoxia and oxidative stress decrease expression of ECE-1 at the protein level. Serum withdrawal from the incubation medium as well as addition of carbachol or PMA leads to a reduction in the levels of ECE-1 protein in neuroblastoma NB7 cells. In addition, human neuroblastoma cells express ET-1 that, via ET_AR, induces cell proliferation. Loss of ET_BR occurs in 42% of tumors and is prognostically associated with metastatic disease (Berry and Burchill 2002).

Osteosarcoma

New evidence suggests a role for ET-1 receptors as potential therapeutic targets in osteosarcoma. ET-1 promotes MMP-2 and MMP-9 induction involving the transcription factor NF-κB in human osteosarcoma (Felx et al. 2006). In osteosarcoma cells, ET_A is the receptor involved in their invasive capabilities.

Endometrial cancer

Expression of ET-1 has been found both in noncancerous human endometrium and in human endometrial adenocarcinoma (Economos et al. 1992). ET_AR and ET_BR expression is decreased in endometrial cancer tissue compared with that of normal endometrium (Pekonen et al. 1995). Moreover, expression levels of ECE-1 and NEP are also altered: in endometrial adenocarcinomas, expression of both ECE-1 and ET-1 is markedly increased while expression of NEP is downregulated (Pekonen et al. 1995; Arun et al. 2001).

Colon cancer

ECE-1, ET-1, and ET_AR are overexpressed in colon adenocarcinoma cells compared with noncancerous cells (Egidy et al. 2000*a*). In vitro, ET-1, acting via ET_AR, is a mitogen for colorectal cancer cells, and ET_AR expression is upregulated in all cell types compared with that of the healthy colon. Specifically, ET_AR binding was highest in cancer-associated blood vessels and fibroblasts and, to a lesser extent, in epithelial cancer cells. In contrast, ET_BR was the predominant receptor in the noncancerous colon and was markedly downregulated in cancer-associated blood vessels and fibroblasts and, to a lesser extent, in epithelial cells (Egidy et al. 2000*a*). The shift in ET receptor binding observed in epithelial cancer cells and in cancer-associated fibroblasts and endothelial cells may favour ET-1 signals, contributing to colorectal cancer growth and neovascularization via ET_AR. This may provide the basis for therapeutic use of specific ET_AR antagonists as adjuvant treatment of colorectal cancer (Hoosein et al. 2007).

Significantly elevated ET-1 expression occurs in 80% of primary human colon cancers. Furthermore, ET-1 is able to rescue colon cancer cells from growth arrest and apoptosis resulting from inhibition of β-catenin signaling, implicating a key role of ET-1 in promoting the oncogenic function of β-catenin. These results indicate that ET-1 overexpression is highly relevant in colon cancers (Kim et al. 2005).

Conclusions

The critical role of the ET axis and the therapeutic relevance of ET-1 receptor antagonists in a range of malignancies have lead to a new generation of molecularly targeted therapies for cancer (Table 1).

Resistance to apoptosis is a principal mechanism whereby tumors are able to overcome cytotoxicity induced by chemotherapy. ET_AR blockade sensitized tumor cells to the apoptotic potential of chemotherapeutic agents, resulting in tumor regression. The cooperative antitumor effect of combination therapy, in which ET_AR antagonists increase the commitment of tumor cells towards apoptosis and potentiate the therapeutic efficacy of conventional cytotoxic drugs, offers a rationale for its clinical evaluation in malignancies expressing ET_AR (Bagnato et al. 2005). Based on this rational approach, clinical trials using endothelin receptor antagonists are ongoing to evaluate the safety profiles and the potential for pharmacokinetic interactions with other cytotoxic drugs. For the time being, the completion of phase II clinical trials utilizing bosentan in combination with dacarbazine in melanoma patients, and atrasentan or ZD4054 in combination with docetaxel in prostate cancer patients, could open novel therapeutic possibilities for this promising class of drugs.

A novel approach to cancer therapy has emerged by the treatment with multiple selective inhibitors to various growth factor receptors or to key postreceptor signaling pathways. Engagement of the endothelin receptor by ET-1 induces tumor-promoting effects that are mediated by downstream effectors, such as EGFR, that may be the escaping pathways preferentially utilized by tumor cells. In this context, improved knowledge of the interconnected molecular mechanisms promoted by the ET-1 axis in cancer will certainly fuel the interest of basic and translational scientists in evaluating new treatment strategies that incorporate ET_AR blockade in combination with other molecularly targeted drugs to identify compensatory mechanisms of escape that can be eliminated therapeutically.

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