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Journal: Clinical cancer research : an official journal of the American Association for Cancer Research

Year: 2009 Jul 15

Issue: 15

Volume: 14

Pages: 4521-8

DOI: 10.1158/1078-0432.CCR-08-0543

Published in final edited form as:
Clin Cancer Res. 2009 July 15; 15(14): 4521–4528. doi:10.1158/1078-0432.CCR-08-0543.

Endothelin B Receptor, a New Target in Cancer Immune Therapy

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Abstract

The endothelins and their G protein-coupled receptors A and B have been implicated innumerous diseases and have recently emerged as pivotal players in a variety of malignancies. Tumors over-express the endothelin 1 (ET-1) ligand and the endothelin-A-receptor (ET_AR). Their interaction induces tumor growth and metastasis by promoting tumor cell survival and proliferation, angiogenesis, and tissue remodeling. On the basis of results from xenograft models, drug development efforts have focused on antagonizing the autocrine-paracrine effects mediated by ET-1/ ET_AR. In this review, we discuss a novel role of the endothelin-B-receptor (ET_BR) in tumorigenesis and the effect of its blockade during cancer immune therapy. We highlight key characteristics of the B receptor such as its specific overexpression in the tumor compartment; and specifically, in the tumor endothelium, where its activation by ET-1 suppresses T-cell adhesion and homing to tumors. We also review our recent findings on the effects of ET_BR-specific blockade in increasing T-cell homing to tumors and enhancing the efficacy of otherwise ineffective immunotherapy.

Background

The endothelin system

The endothelin system comprises four endothelin (ET) peptide ligands, ET-1, 2, 3 (1), and the more recently discovered ET-4 (2); their two G protein-coupled receptors (GPCR), ET_AR (3) and ET_BR (4); and the endothelin-converting enzymes (ECEs), which catalyze the generation of the biologically active ETs. ETs derive from precursor proteins after cleavage by membrane-bound metalloproteinase ECEs (5) and are well known for their overall vasoconstricting activity. Among them, ET-1 is the most potent ligand and the most widely expressed in endothelial cells (6). The endothelin peptides exert their function through binding to their cognate receptors A and B, whereby they trigger divergent intracellular effects by activating numerous downstream signaling pathways. Members of the endothelin system have been identified in neuronal, renal, and vascular tissues, and their involvement has been well documented in an array of physiological processes such as embryonic development, reproduction, angiogenesis, and cardiovascular homeostasis (4,7–9).

Role of the endothelin system in disease

The role of the endothelin system has been well characterized in cardiovascular and renal disorders (10–13). ET-1 is produced by endothelial cells and exerts autocrine-paracrine functions by binding to ET_AR and ET_BR on vascular endothelial cells and pericytes. Balanced activation of the two receptors maintains vascular tone and regulates endothelial cell proliferation (14,15), whereas imbalance in this system contributes to the onset of

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

hemodynamic disorders. The same applies to the renal vasculature, in which endothelins play a major role in maintaining normal vascular tone through both the A (13,16) and B receptor (17). Endothelins and their receptors have also been implicated in pulmonary hypertension (18), asthma (19), and pulmonary fibrosis. ET-1 immunostaining was detected in normal lung epithelium and vasculature (20). ET_AR is found on vascular and airway smooth muscle, whereas ET_BR is mostly often found on the endothelium and smooth muscle cells. Activation of both A and B receptors on lung smooth muscle cells results in vasoconstriction, whereas ET_BR activation alone leads to bronchoconstriction (21).

ET_AR and ET_BR are also involved in inflammatory processes. Both ET_AR and ET_BR expression in bronchial smooth muscle cells is increased upon experimentally induced airway inflammation (22). ET_AR activation is also required for endotoxin-induced inflammation (23) or T-cell homing to the lungs after allergenic or inflammatory stimuli, whereas experimental airway inflammation is abrogated by ET_AR inhibition (24,25). The role of the endothelin axis in inflammation extends beyond the respiratory tract. ET_AR activation mediates renal inflammation and transforming growth factor-β (TGF-β) production in diabetes (26). Owing to its proinflammatory properties (27,28), ET-1 contributes to the progression of various diseases like glomerulosclerosis and atherosclerosis and the pathogenesis of autoimmune diseases such as scleroderma and lupus erythematosus (29,30). Importantly, ET-1 is synthesized by lymphocytes and other leukocytes, and has been shown to activate the proinflammatory transcriptional factor nuclear factor-κB (NF-κB) in human monocytes via ET_BR and to stimulate the production of inflammatory interleukins and tumor necrosis factor-α (TNF-β) (ref. 31). ET-1 is also a chemoattractant for monocytes *in vitro*, via stimulation of IL-8/CXCL8 and monocyte chemoattractant protein-1 (MCP-1)/CCL2 (32,33).

Role of the endothelin system in cancer

Endothelin 1—The role of the endothelin system in cancer has been reviewed extensively by Bagnato and colleagues (34) and others in the field. Kusuhara and colleagues were among the first to report ET-1 overexpression by breast, colon, stomach, prostate, and glioblastoma cell lines (35,36). Ovarian, neuroblastoma, and human papilloma virus (HPV)-positive human cervical carcinoma cell lines also overexpress ET-1 (37,38), whereas increased immunopositivity for ET-1 was detected *in vivo* in human colorectal cancer (39). Compiling clinical evidence shows elevated plasma ET-1 levels in patients diagnosed with various solid tumors, including hepatocellular, gastric, and prostate cancer (40–42). Interestingly, condensed breath of patients with non small cell lung carcinoma (NSCLC) showed increased ET-1 levels (43), proposing ET-1 as an early detection marker (44). Finally, in ovarian carcinoma, high ET-1 levels were detected in ascites (45). In summary, the endothelin 1 ligand is overexpressed by many tumors.

Strong evidence suggests a role for members of the endothelin system in the growth and progression of multiple tumors. Exogenous addition of ET-1 to a range of cell lines promotes various aspects of tumorigenesis. In prostate cancer cell lines, ET-1 increased survival and proliferation (42,46). Exposure of breast cancer cells to ET-1 led to invasive phenotype, which involved matrix metalloproteinase (MMP) activity (47). The same mechanism occurred in osteosarcoma, in which ET-1 was shown to promote MMP-2 and MMP-9 induction (48). Lastly, in colon cancer ET-1 overexpression was shown to rescue cancer cells from apoptosis and growth arrest by promoting the oncogene β-catenin (49).

ET_AR—The effects of ET-1 on cancer cells are mostly mediated by ET_AR. Importantly, ET_AR was shown to be overexpressed in renal and cervical cancer cell lines (50,51) as well as several cancer types *in vivo* including colorectal, bladder, prostate, and nasopharyngeal carcinomas (46,52–55). ET_AR is also overexpressed in approximately 85% of primary and metastatic ovarian carcinomas. In this study, all ovarian carcinoma-derived cell lines were positive for both ET-1 and ET_AR mRNA (56). Concomitant up-regulation of ET-1 and ET_AR on tumor cells contributes to cell autonomy and malignant progression and triggers complex pathways driving tumorigenesis, including cell proliferation, inhibition of apoptosis, matrix remodeling, invasion, and metastatic dissemination (57). ET-1/ET_AR also increases invasion and migration of tumor cells through downstream effects on MMPs, cadherins, connexins, and integrins. Increased cell proliferation is mediated by increased Ca^{2+} uptake and activation of the PKC, PLC, MAPK, and AKT pathways. ET-1/ET_AR interaction can also activate the AKT and the NF-?B pathways to promote tumor cell survival (34).

The mitogenic activity of ET-1 can also be augmented by growth factors (57). One example is the cross-signaling between ET_AR and the epidermal growth factor receptor (EGFR) (ref. 34). EGFR has been identified as a downstream mediator of ET_A-receptor activation by ET-1 in ovarian cancer (58). The mechanism is triggered by ET-1, which causes EGFR transactivation. This event leads to activation of the RAS/MAPK pathway and AKT activation through the formation of Shc/Grb-2 complexes (45,58), subsequently contributing to the mitogenic signaling induced by ET-1. This cross-signaling between the EGFR and ET_AR pathways provides the rationale for combining EGFR inhibitors with ET_AR antagonists to treat ovarian carcinoma. It has been shown that ZD4054, a specific ET_AR antagonist, reduces ET-1-induced EGFR transactivation, whereas the EGFR inhibitor gefitinib significantly inhibited EGF and ET-1 induced EGFR phosphorylation (59). This drug combination simultaneously disables multiple signaling pathways, offering improvements in ovarian carcinoma treatment (59).

ET-1 increases the expression of cyclooxygenase (COX)-1 and COX-2, prostaglandin (PG) E_2, and VEGF production by ovarian cancer cells via ET_AR activation (60). The effect of ET-1 on VEGF expression is mediated through HIF-1α (61). Elevated expression of ET-1 has been associated with increased VEGF expression, lymphatic vessel invasion, and unfavorable outcome in invasive ductal breast carcinoma (62). A correlation between ET-1 expression and VEGF expression has also been shown in lung cancer (63). Additionally, inhibition of human ovarian tumor growth in nude mice after treatment with the potent $ET_A R$ -selective antagonist ABT-627 was associated with reduced COX-2 and VEGF expression by the tumor (60). Thus, over-expression of the ligand ET-1 and its receptor, ET_AR, account for autocrine-paracrine activation of the endothelin axis in many solid tumors, which plays important and multifaceted roles in tumor cell progression. The ET_AR is therefore a very attractive target for cancer therapy.

ET_BR—Investigation of the role of ET-1/ET_BR in tumor cell biology has been more limited. In normal cells, ET_BR counter-regulates ET-1/ET_AR activity through multiple mechanisms including increasing production of nitric oxide, promoting ET-1 clearance, triggering apoptotic pathways, and blocking cell growth; but it is unclear whether such antagonism also operates in tumor cells (64). Interestingly, ET_BR is overexpressed and correlates with melanoma development and progression. Expression profiling of human melanoma biopsies indicated ET_BR overexpression to be associated with aggressive tumor phenotype (65), and ET_BR was proposed as tumor progression marker (66). Underscoring the role of ET_BR in melanoma growth, the specific antagonist BQ-788 was found to inhibit the growth of human melanoma cell lines and to reduce human melanoma tumor growth in a nude mouse model (67,68). The B receptor is also expressed in Kaposi's sarcoma and glioblastoma (69–71).

The role of ET_BR in cancer angiogenesis has been thoroughly investigated and reviewed by Bagnato and colleagues (34). ET-1 has been shown to directly promote tumor angiogenesis by inducing endothelial cell survival, proliferation, and invasion through ET_BR (61). ET-1 promotes angiogenesis also indirectly, by upregulating VEGF production in the vasculature, also through ET_BR activation (72), and increases vascular permeability through VEGF in response to tissue hypoxia (73). Furthermore, ET-1 upregulates expression of the extra domain-B containing fibronectin (EDB^+ FN) in human vascular endothelial cells (74,75). EDB^+ FN is a recently proposed marker of angiogenesis expressed in human cancers and in ocular neovascularization in patients with proliferative diabetic retinopathy. There is a strong correlation between ET_BR and VEGF expression in a number of different tumor specimens (76).

Clinical-Translational Advances

ET_AR and ET_BR antagonists in cancer therapy

ET_AR and ET_BR represent interesting targets for cancer chemoprevention and therapy. Many receptor antagonists have been developed and undergone preclinical and clinical testing. Some compounds have preferential A or B receptor inhibitory activity, whereas others exhibit mixed A and B antagonism. Given the prominent role of ET_AR in tumor cell biology, ET_AR -selective antagonists have been developed more extensively than ET_BR antagonists to treat malignancy (see Table 1). The first ET_AR -selective peptide antagonist, BQ-123 (77), was shown to inhibit cervical cancer growth in preclinical models (78). Furthermore, nonpeptide ET_AR antagonists such as Atrasentan, ZD4054, and YM598 have been shown to have a static effect on ovarian tumor growth in xenograft models (79); to delay progression of prostate cancer (80,81); and to attenuate growth and metastasis of human gastric carcinoma (82). ET_AR inhibitors are currently undergoing clinical testing for various cancer indications. A list of endothelin antagonists under preclinical or clinical development for cancer and various other indications is provided in Table 1. Notably, phase II results with Atrasentan in hormone refractory prostate cancer (HRPC) were encouraging (83). However, subsequent phase III trials in metastatic and nonmetastatic HRPC showed no significant therapeutic effects despite evidence of biologic effects on serum markers of disease burden (84,85). Large geographic differences in the median time to progression were also noted; U.S. patients showed less gain in time to progression relative to non-U.S. patients (85). Given the role of ET_BR in melanoma cells, ET_BR antagonists have been tested in melanoma, in which they proved efficacious (67,68). Interestingly, IRL1620, an ET_BR agonist, was shown to improve both delivery and therapeutic efficacy of paclitaxel in breast tumor-bearing mice (86).

ET_BR and the tumor endothelial barrier to T-cell homing

The focus of cancer therapy targeting ET-1 to date has been to antagonize the autocrine-paracrine effects of ET-1 on tumor cells, mediated mainly by ET_AR. Our laboratory recently showed a novel application of ET_BR blockade in tumor therapy. Specifically, ET_BR blockade at the tumor endothelium proved to be therapeutically efficient for tumor immune therapy (Fig. 1) (ref. 87). The success of immune therapy depends on the ability of effector T cells to infiltrate tumors. Although current tumor vaccines have proven effective in producing an antitumor immune response as measured by blood assays, they have fallen short of clinical expectations. Endothelium is a crucial controller of T-cell trafficking in homeostasis, autoimmunity, and transplantation in humans. We showed that the endothelial barrier also exists in tumors and is partly mediated by ET_BR.

This mechanism was uncovered in human ovarian cancer, in which endothelial cells were microdissected from tumors with brisk tumor-infiltrating lymphocytes (TILs) and tumors lacking TILs, to examine differences in their molecular profile. ET_BR emerged as one of the few genes overexpressed in tumor endothelial cells from tumors lacking TILs by Affymetrix array analysis (87). ET_BR was mostly localized to the endothelium and some stroma cells by immunostaining in human ovarian cancers. ET_BR mRNA or protein overexpression was associated with absence or paucity of TILs, especially of intraepithelial (also called intratumoral) T cells (87). These are T cells infiltrating the epithelial component of the tumor (tumor islets), which predict longer survival in ovarian cancer (88). ET-1 is overexpressed in ovarian cancer cells (34), and it was found that ET-1 mRNA was significantly higher in microdissected (cytokeratin-positive) tumor cells from tumors lacking TILs relative to tumors with brisk TILs. Thus, the entire ET-1/ET_BR (tumor-endothelial) paracrine axis seems upregulated in ovarian cancers lacking TILs. Importantly, we showed that recombinant human ET-1 blocks adhesion of activated T cells to human umbilical vein endothelial cells *in vitro*. These results establish a vascular mechanism of tumor immune evasion mediated by the endothelin system (87).

TNF-α is a major inflammatory cytokine implicated in carcinogenesis, tumor angiogenesis, and progression, and it is up-regulated in ovarian cancer (89). It has been previously reported that the overall TNF-α mRNA levels are similar in ovarian tumors with or without intraepithelial T cells (88). This was counterintuitive, as TNF-α is a major factor activating endothelium and promoting adhesion of T cells. It has been now found that ET-1 efficiently blocks adhesion of T cells to endothelial cells even when endothelial cells are activated with TNF-α (87). This observation explains the paradox of how tumors may exhibit inflammation yet be prohibitive to T-cell infiltration, thus establishing immune privilege even in the face of inflammation.

ET-1 was found to abrogate T-cell adhesion to endothelium via ET_BR and through suppression of endothelial intercellular adhesion molecule-1 (ICAM-1) expression at base line as well as following endothelial activation with TNF-α. Furthermore, it was found that ET_B*R* -induced suppression of ICAM-1 expression and surface clustering was mediated by nitric oxide (NO). ET_BR blockade with the selective antagonist BQ-788 upregulated endothelial ICAM-1 expression, promoted ICAM-1 clustering at the cell surface, and restored adhesion of T cells to ET-1-treated endothelial cells. ICAM-1 neutralizing antibody abrogated the effect of ET_BR blockade to promote T-cell adhesion to endothelium *in vitro* (87). These observations indicate that the endothelin system is crucial for controlling lymphocyte homing in tumors and that endothelial ET_BR overexpression, which can sway the vascular ET_AR/ET_BR balance toward ET_BR hyperactivity, results in suppression of T-cell homing. This evidence is substantiated by complementary data in lung inflammation; ET_AR activation is required for endotoxin-induced inflammation (23), whereas T-cell homing to lungs in response to an inflammatory stimulus is abrogated by ET_AR blockade (24,25). Thus, vascular ET_AR activation results in increased T-cell homing, whereas increased ET_BR signaling facilitates immune privileged status.

ET_BR blockade in cancer immune therapy

To test the activity of ET_BR in controlling T-cell homing to tumors and the effects of its blockade *in vivo* in the context of immunotherapy, vaccine approaches that have no efficacy in delaying tumor growth were used. It was found that vaccine failure was associated with poor accumulation of T cells at the tumor site, in spite of detectable systemic antitumor immune response. ET_BR blockade with specific antagonist BQ-788 greatly enhanced the efficacy of prevention and therapeutic vaccines. BQ-788 did not increase systemic immune response to the vaccine *in vivo*, but rather greatly enhanced T-cell infiltration in tumors following vaccine (87). This was attenuated by ICAM-1 neutralizing antibody, confirming the requirement for adhesive interactions mediated by ICAM-1 following ET_BR blockade *in vivo*. Furthermore, BQ-788 markedly increased homing of T-cells to tumors after adoptive transfer in mice. Thus, in many tumors there is hyperactivation of a paracrine ET-1/ET_BR axis established between tumor cells and endothelium, whereby tumor cells overexpress and release ET-1 whereas the tumor endothelium overexpresses ET_BR. This axis tonically suppresses T-cell homing (even in the presence of tumor inflammation), and can be disrupted by ET_BR blockade, which *in* *vivo* markedly enhances tumor immune therapy (87). This mechanism may not be unique to ovarian cancer. For example, ET_BR is also overexpressed in breast cancer vasculature (86). Interestingly, ET_BR upregulation predicts poor outcome both in breast and ovarian cancer (47,90). The mechanisms underlying ET_BR overexpression in tumor endothelium are not fully understood, but VEGF may be implicated (91,92).

Our results argue that ET_BR antagonists warrant testing in combination with passive or adoptive immunotherapy. There are unique features that render ET_BR blockade an attractive strategy in cancer immunotherapy. First, as outlined above, the axis ET-1/ET_BR seems to be selectively upregulated in the tumor compartment but not in normal tissues. Indeed, in mouse experiments, ET_BR blockade by BQ-788 did not result in systemic inflammation or illness, and frequency of $CD45^+$ lymphocytes or $CD3^+$ T cells in liver, spleen, lungs, or kidneys after vaccine or adoptive T-cell transfer was not affected by BQ-788 (87). This is in contrast to current immunomodulatory approaches, which achieve systemic activation of effector cells by attenuating peripheral tolerance or other homeostatic checkpoint mechanisms and can result in significant autoimmune toxicity (93). Second, ET_BR -selective antagonists, including BQ-788, have been tested in humans and are well tolerated even in patients with cardiovascular disease (94–96). Thus, ET_BR can be pharmacologically perturbed with existing drugs to enhance the efficacy of immune therapy. Third, ET_BR blockade is likely to have also direct antiangiogenic effects through suppression of endothelial nitric oxide. Unlike in patients with sepsis (97), NO inhibition is safe and has been well tolerated in cancer patients (98). Although the anticancer effect of ET_BR (or NO) blockade as monotherapy may be modest, the concomitant administration of immunotherapy may act synergistically against angiogenesis (86,99).

Implications for pure ET_AR antagonists

Currently, on the basis of results obtained mostly with xenograft tumor models in immunodeficient mice, cancer therapy targeting ET is focused on ET_AR blockade. However, previous evidence shows that ET_AR signaling is required for T-cell homing (24,25), whereas our work indicates that increased ET_BR activity in tumor endothelium results in reduced T-cell homing and ET_BR blockade is required to improve T-cell homing to tumors. Because of the tonic antagonism between ET_AR and ET_BR signaling in the vasculature, pharmacologic ET_AR blockade could tilt the balance toward increased ET_BR signaling in the tumor vasculature. This could possibly result in increased angiogenesis and, on the basis of our work, could suppress T-cell homing to tumors. It has been previously shown that patients with ovarian cancer whose tumors are infiltrated by intraepithelial T cells survive longer (88), a concept validated by several groups (100–103). Similar observations were made in other solid tumors. In colon cancer, tumor-infiltrating T cells predict survival better than conventional anatomical staging (104), whereas in prostate cancer TIL represent a strong independent prognosticator of longer survival (105). Although the function of tumor-infiltrating T cells is not fully understood, it is possible that they contribute to controlling tumor growth during or after conventional cancer therapy. For example, the long-term therapeutic effects of VEGF receptor 2 blockade, a major antiangiogenic pharmacologic intervention, were fully depended on $CD8^+$ T cell infiltration in tumors (106). Furthermore, conventional chemotherapy agents have immunomodulatory effects and their long term efficacy may depend in part on immune effector mechanisms (107). If this were the case, ET_AR blockade alone might increase ET_BR signaling and reduce T-cell infiltration in tumors. This could negate some of the potential efficacy of cancer therapies and explain in part the failure of pure ET_AR antagonists to produce significant clinical results in tumors in which TIL may affect survival. Our results argue that ET_AR/ET_BR mixed antagonists might offer the advantage of simultaneously targeting the tumor cell (through ET_AR) and enhancing antitumor immune mechanisms (through vascular ET_BR) and should be the focus of future therapy.

Acknowledgments

Grant support: National Institutes of Health (NIH) NIHR01-CA098951; NIHP50-CA083638 Ovarian Cancer SPORE; and NIHR01-CA112162. AF was supported by the National Ovarian Cancer Coalition. RJB was supported by NIH/NICHD K12-HD43459 Career Development in Women's Health Research, and the Ovarian Cancer Research Fund.

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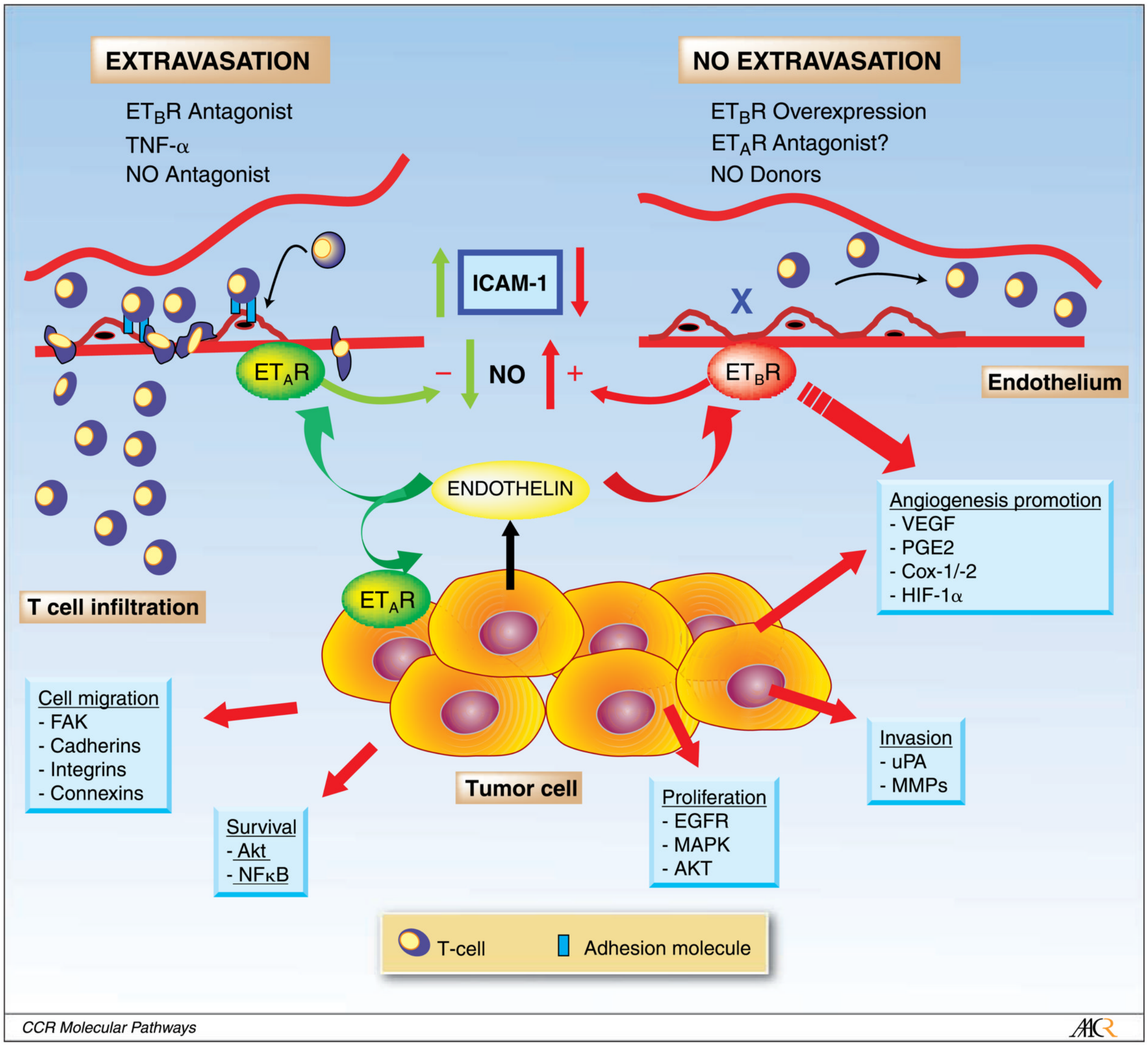

Fig. 1.

Control of T-cell homing to tumors by endothelin 1 expressed by tumor cells. Opposite paracrine effects are exerted by endothelin on tumor endothelium through ET_AR and ET_BR. The balance between ET_AR and ET_BR determines the fate of antitumor T-cell response. Overexpression of ET_BR in tumor endothelium results in increased angiogenesis but also suppression of ICAM-1 expression on endothelial cells, and inhibition of T-cell transendothelial migration and homing. This effect is largely mediated by NO. NO donors and possibly ET_AR inhibitors exert similar effects. ET_BR blockade results in increased expression of endothelial ICAM-1 and increased binding and transendothelial migration of T cells to the tumor. NO antagonists and TNF-α exert similar effects. Additional tumor-promoting autocrine effects of endothelin are established by binding to ET_AR on tumor cells, resulting in enhanced tumor cell survival, proliferation, migration, and invasion. Each of these effects entail multiple signaling pathways.

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Table 1

Endothelin targeting agents and their indications

Compound	Target	Dosing	Clinical testing-indication	Phase	Cancer preclinical testing	Company
Atrasentan	ET_AR	Oral	Prostate cancer	II–III	51, 79, 80	Abbott
			Renal carcinoma	II		
			Ovarian cancer	II		
			FT cancer	II		
			Brain tumors	I		
			NSCLC	III–IV		
			Malignant glioma	I		
			ED	III		
			HT	II		
			CVD	Approved		
Ambrisentan	ET_AR	Oral	PAH	Approved	—	Gilead Sciences
			IPF	II		
			USS	I		
Avosentan	ET_AR	Oral	CVD	II	—	Speedel
			DN	I–II		
BQ123	ET_AR	IV	HI	I	36, 108, 109	Merck Biosciences
			PAH	II		
			MI	II		
			CKD	I		
			HT	I		
Clazosentan	ET_AR	IV	SAH	Approved	—	Actelion
Darusentan	ET_AR	Oral	HT	III	—	Gilead Sciences
			CAD	II		
			ED	II		
Edonentan	ET_AR	Oral	CHF	II	—	Bristol-Myers Squibb
			END	II		
Sitaxsentan	ET_AR	IV-Oral	PAH	III	—	Encysive Pharmaceuticals/ICOS Texas Biotech
			CHF	II		

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Compound	Target	Dosing	Clinical testing-indication	Phase	Cancer preclinical testing	Company
S-0139	ET_AR	Oral	CHF	II	—	Shionogi-GSK
TBC-3711	ET_AR	Oral	HT	II	—	Encysive Pharmaceuticals
YM598	ET_AR	Oral	Prostate	II	82, 110	Astellas Pharma US
ZD4054	ET_AR	Oral	Prostate cancer	III	111, 112	AstraZeneca
			Bone metastasis	II		
			NSCLC	II		
BQ788	ET_BR	IV	CVD	I	67, 69, 113, 114	Merck Biosciences
IRL-1620	ET_BR agonist	IV	—	—	86, 99	Sigma
SPI-1620	ET_BR agonist	IV	Carcinoma	I	—	Spectrum Pharmaceuticals
			Short cervix	III		
Enrasentan	ET_AR/ET_BR	Oral	AHF	III	—	GlaxoSmithKline
Bosentan	ET_AR/ET_BR	Oral	Metastatic melanoma	II	115, 116	Actelion
			HT	II		
			PAH	Approved		
J104132	ET_AR/ET_BR	Oral	CHF-HT	II	—	Banyu/Merck

Abbreviations: AHF, acute heart failure; CAD, coronary artery disease; CHF, congestive heart failure; CKD, chronic kidney disease; CVD, cardiovascular diseases; DN, diabetic nephropathy; ED, erectile dysfunction; END, endothelial dysfunction; FT, fallopian tube; HD, heart disease; HI, hyperinsulinemia; HT, hypertension; IPF, idiopathic pulmonary fibrosis, MD, microvascular dysfunction, MI, myocardial infarction; PAH, pulmonary arterial hypertension; PC, prostate cancer, PH, pulmonary hypertension; SAH, subarachnoid hemorrhage; USS, ulcer scleroderma, systemic.