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Overview

Endothelin axis and apoptosis

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Abstract

Endothelin axis (endothelin-1, -2 and -3, and endothelin receptors ETAR and ETBR) plays the key role in the various functions of the organism: acts as a modulator of vascular tone, tissue differentiation, growth and development, cell proliferation, and hormone synthesis. In addition to its physiological role, endothelin axis or individual components of the system, can have a significant effect on the tissue remodelling process and tumorigenesis. For example, endothelin-1 modulates mitosis, apoptosis and angiogenesis and can stimulate tumour invasion and metastasis. Increased endothelin-1 expression has been demonstrated in breast, ovarian, prostate and colorectal cancers. In tumour cells, the binding of endothelin-1 to ETA receptor induces signalling pathway for survival. Endothelin-1 triggers the anti-apoptotic signal through phosphatidylinositol 3-kinase (PI3-K)-dependent Akt phosphorylation. Endothelin receptors are coupled with G-proteins and they differ in sensitivity to antagonists. G-proteins are involved in cell signalling via adenylate cyclase, ion channels, PLC, PLA2, PKC, intracellular Ca^{2+}, calcineurin and MAP kinase. It is believed that this activity is mediated by ETAR, since the application of their specific antagonist BQ-123 causes a reverse effect. Endothelin receptor antagonists such as A-127722, BQ-123, BQ-788, etc. act specifically on either proliferation or apoptosis depending on the cell type. Today, certain endothelin receptor antagonists, such as atrasentan, are applied in the treatment of lung and prostate cancer or are at different phases of clinical trials.

INTRODUCTION

Apoptosis is a genetically regulated process, which is defined both in time and in space, and plays a significant role in homeostasis during growth and development of the organism. Disturbances in the regulation of programmed cell death may be the primary cause of various diseases including tumours, autoimmune diseases and various degenerative disorders.

Apoptosis was first described in 1972, as a type of cell death which leads to reduction of the cell volume, condensation and fragmentation of nuclei, swelling of the cell membrane and the loss of adhesive interaction with neighbouring cells *(1)*. Biochemical changes that occur in apoptosis include internucleosomal DNA fragmentation, expression of phosphatidylserine on the outer side of the plasma membrane and activated intracellular proteases *(2, 3)*.

Tumour cells showed increased expression of specific proteins that prevent apoptosis and allow proliferation. Increased expression of anti-apoptotic proteins can cause aggressive and rapid growth of tumour

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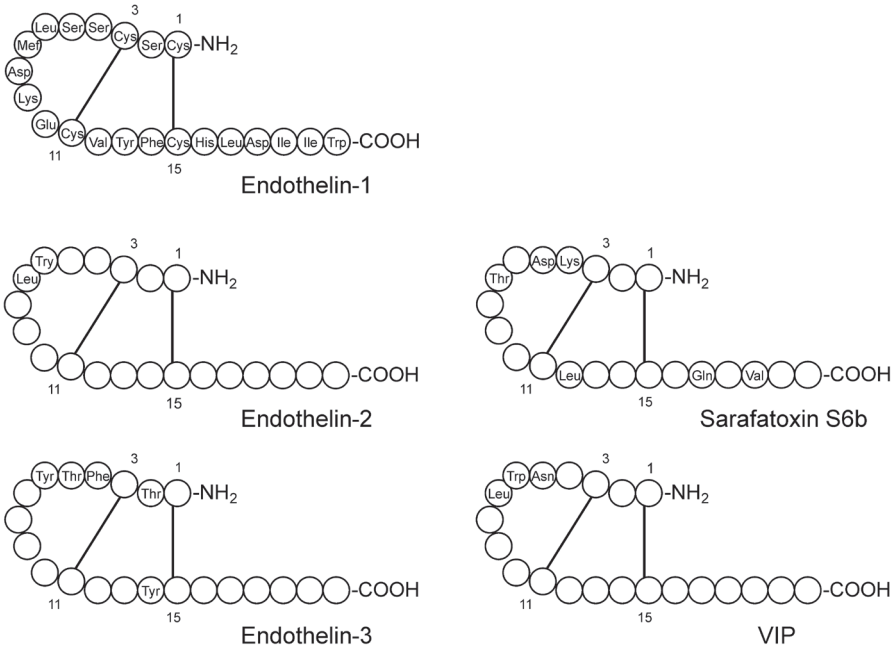

Figure 1. *Molecular structure of endothelin-1, -2, and -3, sarafatoxin and vasoactive intestinal polypeptide.*

tissue and resistance to anticancer drugs. Members of the Bcl-2 family proteins, members of the family of inhibitors of apoptosis (IAP), and heat shock proteins (HSP) are key factors that have been shown to contribute to the protection of tumour cells of spontaneous or induced apoptosis *(4, 5, 6, 7, 8)*.

Over the past 20 years, numerous studies aimed at finding the ways of prevention and treatment of malignant disease. Several common characteristics of different types of tumours were found, which are: constant proliferation, insensitivity to growth control signals, opposing to cell death, secretion of proinflammatory molecules, resisting to immune rejection, unlimited duplication of DNA, induction of angiogenesis, invasion into healthy tissue and metastasis, aerobic glycolysis, genome instability *(9)*.

Endothelin axis (endothelin-1, -2 and -3, and endothelin receptors ETAR and ETBR) plays the key role in the various functions of the organism: acts as a modulator of vascular tone, tissue differentiation, growth and development, cell proliferation and hormone synthesis *(10, 11)*. In addition to its physiological role, endothelin axis or individual components of the system can have a significant effect on tissue remodelling and in the process of tumorigenesis. Endothelin receptors act via G-proteins and activate specific signalling pathways. Binding of endothelin-1 (ET-1) to ETA receptor tiggers signalling pathway for survival *(12)* in different tumour cells. ET-1 induces the anti-apoptotic signal through phosphatidylinositol 3-kinase (PI3-K)-dependent Akt phosphorylation, depending on the time of exposure and the dose *(13-15)*. On the other hand, the binding of ET-1 to ETB receptor enhances the clearance of circulating ET-1 and delays apoptosis. Endothelin receptor antagonists such as atrasentan, BQ-123, BQ-788, etc. act on either proliferation or apoptosis depending on the cell type *(16-19)*. Certainly, endothelin receptor antagonists, such as atrasentan, zibotentan, YM-598, bosentan, and macitentan are applied in the treatment or are currently involved in different phases of clinical trials *(20-24)*.

ENDOTHELIN AXIS

Endothelins (ETs) are potent vasoconstricting peptides consisting of 21 amino acids. In 1988, Yanagisawa and his colleagues isolated and purified this peptide from cultivated porcine vascular endothelial cells, and named it endothelin *(25)*. Cloning and sequence analysis of the endothelin genes showed that ETs comprise the peptide family consisting of three isopeptides, namely ET-1, ET-2 and ET-3, and corresponding genes are located on chromosomes 6, 1 and 20 *(26, 27)*. Each of the three ET isopeptides shows a high degree of sequence homology having the same aminoacides in 10 positions *(2)*. ETs show similarities to sarafatoxin both in molecular structure and in biological activity. Sarafatoxin is a peptide that was isolated from snake venom *Atractaspis engaddensis (28)*. Vasoactive intestinal polypeptide (VIP) also shows structural similarity to ET. These vasoactive peptides are shown in *Figure 1*.

ET-1 is the strongest known vasoconstrictor. It contains two disulfide bonds between Cys1- Cys15 and Cys3-Cys11 in polypeptide molecules. Disulfide bonds contribute to the structural stability of the molecule. It is believed that these disulfide bonds are vital to high affinity binding

to ETA receptor class, but they are less important in identifying ETB receptors class *(11).* Expression of ETs may be tissue-specific. In rats, the tissue amounts of ET-1 are generally higher than ET-3, except in the pituitary gland where ET-3 is higher, and in renal inner core where the concentration of these isopeptides is equal. ET-2 is prevalent only in the gastrointestinal tract. ETs are widely present in mammals, and have been found in several species of fish and invertebrates, which suggests that ETs found in humans have a long evolutionary history *(11).* In humans, preproendothelin-1 consisting of 212 amino acids is synthesises first, and after proteolytical cleveage with dibasic pair-specific endopeptidase big ET-1 composed of 38 amino acids is formed *(10, 11, 26).* Big ET-1, is transformed into mature ET-1 (1-21) by the cleavage between Trp21-Val22, with the endothelin converting enzyme (ECE) or by chymase into ET-1 (1-31).

Vascular endothelial and other cells secrete ET-1 in response to various stimuli. According to in vitro studies, the release ET is stimulated by: haemodynamic stress, hypoxia, inflammatory cytokines (tumour necrosis factor, interleukin-1 and transforming growth factor-β), vasoactive hormones (angiotensin II, vasopressin, bradykinin and adrenaline) and thrombin. Natriuretic peptide and heparin reduce the release of ET-1. It is assumed that ETs function as local hormones, and they act in autocrine, paracrine and endocrine manner (11, 12, 26). Endothelial cells can rapidly adapt synthesis of ET-1 to the demand for regulation of vascular tone because of the short half-life of ET-1 (15-20 minutes for ET-1 mRNA, 4-7 minutes for ET-1 in plasma). An increased amount of ET-1 occurs after the binding of thrombin, angiotensin II and vasopressin to the receptor. Therefore, through the cascades of secondary messengers, intracellular Ca^{2+} is increased and protein kinase C (PKC) is activated. Through AP-1 regulation locus in the promotor region they stimulate transcription of mRNA for ET-1. Creation of ET-1 mRNA is inhibited by nitric oxide (NO), prostacyclin and atrial natriuretic peptide, possibly via cGMP which inhibits phosphatidyl inositol mechanism. On the other hand, the synthesis of ET-1 is stimulated by thrombin, hypercholesterolemia, TGF β, bradykinin, epidermal growth factor, vasopressin, hypoxia, mechanical stress, cyclosporine, oxidized LDL, angiotensin II, Ca^{2+}, etc. (12, 29-31).

To date, only two ET receptors, ETAR and ETBR, have been identified; however, a certain speculations on the existence of the ETC receptor also exists. The known ETB receptor subtypes are ETB1 and ETB2. ETA receptor contains 427 amino acids and exhibits the highest affinity for ET-1 and the lowest for the ET-3. On the other hand, ETB receptor shows equal affinity for all three ETs. Both receptors are coupled with G-protein. Binding of ET to the appropriate receptor activates a number of signalling molecules, resulting in short-term or long-term changes in the target cells. Signalling pathways that mediate short-term changes of cellular functions include: connection of receptor with G-proteins, phospholipase C (PLC), signal transduction via Ca^{2+}, PKC, phospholipase A2 (PLA2), phospholipase D (PLD), Na^{+}/H^{+} exchange and alkalization of the cytoplasm and the creation of cAMP or cGMP, leading to vasoconstriction *(11, 12, 31).*

Hemodynamic changes characterized by changes in pressure and resistance to blood flow and hemodynamic control mechanisms involve neural, hormonal, renal, and local control. The vascular endothelium is considered to be a significant factor in local control, due to its anatomic location between circulating blood and vascular smooth muscles. Under physiological conditions as well as after activation of the neurotransmitters, hormones, and under the influence of physical stimuli, different vasoactive substances are produced in endothelial cells. They act as: 1) vasoconstrictive factors: endothelins-1 (ET-1), cyclooxygenase pathway products, angiotensin II, 2) vasodilatation factors: NO, hyperpolarisation factor, prostacyclins and others. ET-1 acts through the ETAR located on the cell membranes of the smooth muscle of blood vessels, wherein the activated PLC, raises the concentration of free intracellular Ca^{2+} and causes vasoconstriction. The thromboxane A2 (TXA2), prostaglandin H2 (PGH2) and superoxide anion are the most significant vasoconstrictive factors which arise via cyclooxygenase pathway. TX A2 and PGH2 have a direct constrictive effect, while the superoxide anion affects the inactivation of NO *(31).*

However, in addition to its physiological role endotelin axis or individual components of the system can have a significant effect in tissue remodelling but also in the process of tumorogenesis. For example, endothelin-1 modulates mitosis, apoptosis and angiogenesis and can stimulate tumour invasion and metastasis. Besides its vasoconstriction action, ET-1 has mitogenic effect: it phosphorylates a number of cytoplasmic and membrane-associated proteins, participants in transmembrane signalling pathways that lead to cell proliferation. Signalling pathways that mediate long-term changes in cellular functions include the creation of several proto-oncogenes (c-fos, c-jun, c-myc, VF-30) and growth factors (PDGF, EGF, TGF-β, insulin), and co-mitogenic action. Different mitogenic stimuli activate an intracellular cascade of kinases, including raf-1, mitogen-activated protein kinase, MAPK kinase and S6 kinase II, which carry messages from the cell membrane to the cell nucleus.

ROLE OF ENDOTHELIN AXIS IN MALIGNANT DISEASE

Numerous malignant tumours, including prostate, ovarian, colon, lung and breast cancer, are associated with the increased concentration of one or more of endothelin types and its receptors. *Table 1* shows the role of endothelin receptors in various tumours *(12).* The binding of ET-1 to the receptors activates signalling pathways that transmit mitogenic signals to the nucleus and promote cell

TABLE 1

Role of ET receptors in different tumors (modified, according to Bagnato and Rosano 2008).

	Endothelin receptors	Receptor antagonists and their effects
Prostate cancer *(56, 57)*	Expression of the ETAR increases with tumour stage and grade, and is associated with decreased ETBR expression. ZD4054 inhibited ETA-receptor-mediated anti-apoptotic events while allowing ETB-receptor-mediated pro-apoptotic signalling.	ETAR antagonist has demonstrated benefit in PSA progression, markers of bone turnover and pain. ZD4054 has been associated with a promising improvement in overall survival compared with placebo and was generally well tolerated in patients with metastatic hormone-resistant prostate cancer.
Ovarian cancer *(58, 59, 60)*	ETAR mRNA is present in 85% of primary and metastatic cancers, and correlates with tumour grade. ETAR mediates all ET-1-induced tumour promoting effects. ETAR and EGFR cross-talk enhances the metastatic potential of epithelial ovarian cancer.	In preclinical studies ETAR antagonists display anti-tumor effects and additive effects in combination with taxanes or gefitinib. Blockade of ETAR and EGFR with zibosentan and gefitinib is a potential new therapeutic opportunity for epithelial ovarian cancer treatment.
Bladder cancer *(61)*	Both ETAR and ETBR are expressed in bladder tumours.	ETAR antagonist decreases lung metastases.
Melanoma *(62)*	ETBR is over expressed and correlates with tumour progression. ETBR mediates all ET-1-induced tumour promoting effects.	ETBR antagonists inhibit melanoma cell growth *in vitro* and *in vivo*. The dual ETA/BR antagonist Bosentan has benefit in disease stabilization in metastatic melanoma patients.
Bone malignancies *(63)*	ETAR is expressed in osteoblasts.	ETAR antagonist significantly reduced osteoblastic bone metastases and tumour burden in bone.
Breast cancer *(55)*	ETAR expression correlates with several clinic-pathological parameters of aggressive carcinoma.	In preclinical studies ETAR antagonist inhibits tumour growth.
Renal cancer *(64)*	ETAR is expressed in different cell lines.	
Head and neck cancer *(65)*	ET-1 is able to stimulate the proliferation of HNSCC cells via both, ETAR and ETBR.	
Nasopharyngeal carcinoma *(66)*	ETAR is over expressed in 74% of tumours.	ETAR antagonist inhibits tumour growth and metastasis and shows synergistic effects in combination with cytotoxic drugs.
Colon cancer *(67)*	Increased expression of ETAR and ETBR.	Dual ETA/BR antagonist Bosentan induces apoptosis or sensitises cells to Fas-induced apoptosis.
Cervical cancer *(68)*	HPV-positive cervical carcinoma cells predominantly express functional ETAR.	ETAR antagonist inhibits tumor growth in monotherapy as well as in association with taxane.
Kaposi's sarcoma *(69)*	Both ETAR and ETBR are expressed.	Dual ETA/BR antagonist inhibits tumor growth in nude mice.
Glioblastoma *(70, 71)*	ETBR is expressed in cancer cells. Endothelin B receptor antagonists block proliferation and induce apoptosis in glioma cells.	Dual ETA/BR antagonist induced apoptosis. BQ788 and A192621 trigger apoptotic processes mainly via activation of the intrinsic mitochondrial pathway involving caspase-9 activation.

proliferation. Those signalling pathways involve PLC (elevation of intracellular Ca, activation of PKC, activation of PI3-K and consequentially Akt pathway) and MAPKs; they are overlapping and partly synergistic. By applying ETAR and ETBR antagonists, the inhibition of growth is noticed only in the case of application of ETAR antagonist and it is proved that ET-1 affects proliferation only through ETAR. Also, the mitogenic activity of ET-1 is amplified by simultaneous application of growth factors such as EGF, bFGF, insulin, IGF, PDGF, TGF, IL-6 *(12).*

Endothelins are mitogenic for endothelial cells, smooth muscle vessels cells, fibroblasts and pericytes and, therefore, they act as activation of angiogenesis factors. Mitogenesis of endothelial cells is mediated via ETRB and mitogenesis of smooth muscle cells and pericytes vessels through ETAR. ET-1 is included in all stages of neovascularization, from proliferation to migration and tissue formation. Elevated levels of ET-1 are associated with increased microvessel density and the expression of vascular endothelial growth factor (VEGF) in tumour cells, depending on the dose and time, especially in a state of hypoxia *(12).* Increased expression of endothelin occurs during hypoxia by the action of transcription factor HIF-1-β (hypoxia inducible factor-1β). More precisely, endothelins stabilize HIF-1-β transcriptional complex that drives expression of angiogenic molecules such as VEGF and leads to cancer progression *(33).* During normal tissue oxygenation, but even more so in the state of hypoxia, ET-1 increases the expression of cyclooxygenases COX-1 and COX-2, and the formation of prostaglandin PGE2, which is, in addition to being a vasodilator, a possible regulator of tumour growth. Therefore, COX inhibitors reduce the creation of prostaglandin and VEGF, matrix metalloproteinases (MMPs) activation and invasiveness. Thus, HIF-1-β and COX are the downstream signalling molecules of the pathway that starts with ET-1 *(12).*

ET-1 is an anti-apoptotic factor. It can control signalling pathways in the regulation of of cell survival, such as PI3K/Akt pathway. Addition of ET-1 inhibits apoptosis in tumour cells, induced by paclitaxel, by causing phosphorylation of the Bcl-2 family, depending on the concentration. ETAR antagonists block this effect. This means that ET-1 contributes to resistance to paclitaxel, acting through ETAR *(34).* Endothelin receptor antagonists, apart from affecting the reduction of tumour growth by inhibiting proliferation and inducing apoptosis, may contribute to eliminating the frequent occurrence of resistance to some conventional drugs such as paclitaxel, by increasing the sensitivity to the drug and inducing apoptosis *(33, 35, 36).*

Acting via ETRA, ET-1 activates two families of proteinases related to the process of metastasis, MMPs and urokinase type plasminogen activator (uPA) in tumour cells. Furthermore, ET-1 stimulates the FAK (focal adhesion kinase) and phosphorylation of paxillin, which is an intracellular protein, the mediator in the interaction of the cytoskeleton and extracellular matrix *(12).*

Given that tumour vascularisation correlates with the expression of ET-1 in ovarian cancer and in colorectal tumours *(37, 38)* and brain tumours *(39),* additional tests were conducted and they showed that activation of ETAR by ET -1 stimulates production of VEGF. VEGF stimulates tumour growth and angiogenesis by increasing levels of hypoxia-induced factor-1α (HIF-1α) *(40).* In normal oxygenation ET-1 activates signalling pathway sensitive to hypoxia, with the increase of concentration and / or reinforcement of stability of HIF-1α. HIF-1 transcription complex (HIF-1α/β), binds to the binding site to hypoxia sensitive element (HRE), causes an increased expression of genes and stimulates angiogenesis *(15).*

ENDOTHELINS, ENDOTHELIN RECEPTOR ANTAGONISTS AND APOPTOSIS

Apoptosis is a genetically controlled process of programmed cell death *(1, 41).* This process allows the temporally and spatially predetermined removal of redundant or morphologically altered cells from the body, ensuring homeostasis. The mechanism of apoptosis is maintained by equilibrium between pro-apoptotic and anti-apoptotic factors *(42).* The process is necessary for the normal functioning of the body, and a significant number of pathological conditions associated with rapid or slow process of apoptosis.

Activators of apoptosis, through various intermediaries, lead to activation of proteolytic caspase cascade. Extracellular activators may be hormones, growth factors, nitric oxide II *(43),* cytokines or toxins that bind to membrane receptors or diffuse through the membrane. Intracellular activators are released as a result of irreversible damage to DNA and other important cellular structures created under the influence of physical factors, hypoxia, aging, free radicals, increasing the concentration of Ca^{2+} ions and many other factors.

Most literature data confirms that ETs inhibit apoptosis. It was found that ET-1 acts as an anti-apoptotic factor in tumour cells. However, ET-1 shows the same effect in endothelial cells and vascular smooth muscle cells, which contributes to maintaining the integrity of newly formed blood vessels *(44, 45).*

In tumour cells, the binding of ET-1 to ETA receptor causes induced signalling pathway for survival. ET-1 triggers the antiapoptotic signal through phosphatidylinositol 3-kinase (PI3-K)- dependent Akt phosphorylation, depending on the time of exposure and the dose *(14, 34).* On the other hand, the binding of ET-1 to ETB receptor enhances the clearance of circulating ET- 1 and delays apoptosis. Resistance to paclitaxel-mediated apoptosis caused by ET-1 (resistance) can be reversed by ETA recep-

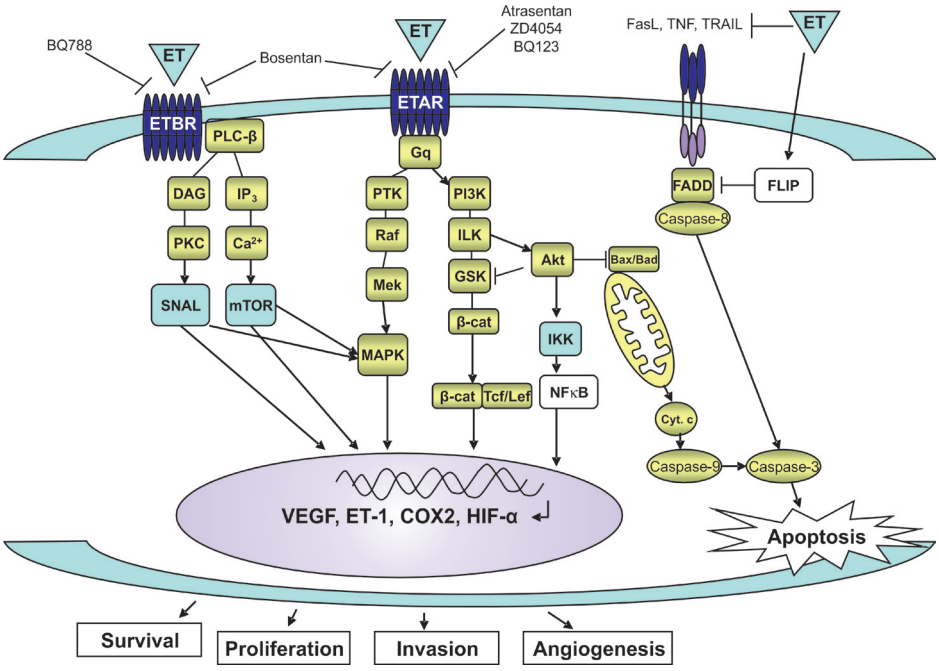

Figure 2. *Endothelins, their receptors, antagonists of endothelin receptors, and signalling pathways. Figure modified from previous schemes (Tamkus 2009, Wang 2011).*

tor antagonists. In preclinical studies ETA receptor antagonists cause 65% inhibition of tumour growth *(46)*. *Figure 2* shows the action of ET-1 through both endothelin and death receptors. ET-1 acts as an anti-apoptotic factor by stabilizing the short arm of Fas ligand (FasL) inhibitory protein (FLIP) *(47)*. Also, ET-1 can inhibit FasL-induced apoptosis by binding to high affinity sites *(48)*. In human glioblastoma cells, blockade of ET sensitizes cancer cells to FasL-mediated apoptosis *(15, 49)*.

The pharmacological effect of ET can be explained by exogenous appliance of ET, but to clarify its physiological role, ET receptor antagonists are needed. So far, a large number of receptor antagonists, specific for ETA and ETB receptors, or both, have been synthesized and it allows for a better insight into the biological activity of ET in various tissues and their role in disease development *(11, 31)*. Atrasentan is pharmacologically active enantiomer A-127722 *(50)*. After oral application the bioavailability of ETAR antagonist, with prolonged exposure period, shows great affinity (the ETA receptor, Ki = 34 pM) and selectivity (1000 times greater selectivity for ETA than ETB receptors). Atrasentan blocks the signalling pathways involved in cancer cell proliferation and other processes that promote tumour growth *(51)*. Significant results were reported in metastatic hormone-refractory prostate cancer treated with atrasentan, which in phase II clinical trials slowed tumour progression *(52)*. The results of phase III clinical trials were omitted *(53)* as a promising reduction in tumour progression was not reflected in the overall survival benefit. Despite these results, atrasentan in combination with docetaxel may provide an alternative treatment option of this disease *(54)*. ETA receptor antagonists show synergistic pro-apoptotic and anti-angiogenetic effect in combination with paclitaxel, leading to inhibition of tumour growth by 90% to 40% of all tested cases *(55)*.

CONCLUSION

Association between ET-1 and various types of cancer suggests a key role of ET-1 in the initiation or progression of tumours, defining endothelin axis as a potential therapeutic target. This fact has led to the development of several approaches targeting endothelin axis in cancer therapy. According to Rosano *et al. (20),* the potential advantage of dual ETRA and ETRB antagonists is that they can target not only cancer cell (which typically express ETRA) but also tumour-associated stromal elements, such as vascular, lymphatic and inflammatory cells and fibroblasts, which all express ETBR. ET-1 acts as tumour-stimulating anti-apoptotic factor by binding to high affinity sites of FasL or by stabilization of FLIP. ET-1 also suppresses apoptosis via PI3-K/Akt pathway. On other hand, ETs lead to the stimulation of the new blood vessels. In addition to inhibition of the synthesis of ET-1 or inhibition of ECE activity, the blocking of endothelin receptors represents the most promising approach in the field of control of multiple effects of ET-1, which are vital in creating a malignant phenotype.

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