Role of Endothelin 1 on Proliferation and Migration of Human MCF-7 Cells

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ABSTRACT

Objective: The aim of this study was to explore the role of endothelin 1 (ET-1) in human breast cancer proliferation and migration and antagonism of endothelin receptor A (ETAR) and endothelin receptor B (ETBR) by using the non-selective dual ETA/ETB receptor antagonist bosentan and determine its anti-proliferative, anti-metastatic, and apoptotic effects demonstrated by nuclear factor kappa B (NF-kB), vascular endothelial growth factor (VEGF), Caspase 3 and Caspase 9 expression on endothelin-induced proliferation of MCF-7 cell line in vitro.

Materials and Methods: A total of 8,000 cells were seeded into e-plates 24 hours after the cells were incubated with or without 10-4 M BOS (1 hour before ET-1 treatment); 10-7, 10-8, and 10-9 M ET-1 for 1-4 days.

Results: Whether ET-1 is present or not in the tumor area, bosentan exerts anti-proliferative effect on breast cancer. However, ET-1 and bosentan group showed important inhibitory effect on tumor migration compared to bosentan alone, which can be attributed to increased activity of ET-1 axis in the presence of ET-1. The imbalance among the NF-kB, caspases, and VEGF, which are predictive factors of carcinogenesis significantly improved after bosentan administration.

Conclusion: Our study definitely demonstrated ET-1 and its critical role in cancer progression with apoptotic and anti-apoptotic pathways (NF-κB) and VEGF expression, and migration analyses were also performed. The second major finding was that bosentan inhibited ET-1-mediated effects on tumor proliferation and migration.

Keywords: Bosentan, cancer, ET-1, MCF-7, metastasis

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Introduction

Breast cancer is the most common type of cancer and is a significant cause of cancer deaths in women worldwide with approximately 1.5 million new cases diagnosed every year in Europe and the United States [1]. Major promoters of cell proliferation are estrogens in both normal and neoplastic breast epithelium, and their proliferative effect occurs via binding to the estrogen receptors [2]. In recent years, researchers around the world have been focused on finding new targets for drug development as better therapeutic strategies [3, 4]. New genes are being discovered in the development of breast cancer (BC), and studies are on to find new clinical biomarkers and a drug target for radical treatment [5].

Endothelin 1 (ET-1) is produced by the vascular endothelial cells and is also produced by breast epithelial cells [6], and it is found in three different forms in our body. ET-1 is a well-known isoform that is a potent vasoactive 21-amino acid peptide. Endothelin-1 (ET-1) binds to endothelin receptor A (ETAR) and endothelin receptor B (ETBR), and these are included in the category of G protein-coupled receptors (GPCR) [7]. ET-1 is also known to be a potent mitogen for different cell types such as endothelial cells keratinocytes, mesangial cells, osteoblasts, and human breast fibroblasts [8].

Previously, expression levels of ET-1 in human BC was demonstrated and discussed by different methods [9, 10]. Yamashita et al. and Alanen et al. have reported that ET-1 levels in

BC is higher than in normal breast tissue [9, 11]. Wulfing P et al. discussed that enhanced expression levels of ET-1, ETAR, and ETBR in tissue is associated with more aggressive breast tumors and decreased survival rates [12]. Another study about invasive breast carcinoma indicated that the expression level of ET-1 and its receptors is associated with raised VEGF expression and vascularity in biopsy material, which plays an important role in vascular cell growth and provokes tumor invasion [13]. Guise TA et al. reported that BC cell line inoculated animal model treated with a ETAR blocker showed a significant inhibition of osteoblastic bone metastases of breast cancer and decreased tumor mass compared with untreated animals [14]. In the current literature, different experimental studies put forth ET-1 axis and its role of breast cancers. While human breast cancer cell line MCF-7 suggested that protein secretion of ET-1 increased due to chemical hypoxia, and endogen ET-1 significantly elevated cell invasion, atrasentan is a selective ETAR antagonist that reduced cell invasion [15]. Anti-proliferative and anti-metastatic effects of bosentan were investigated on experimentally induced breast cancer metastasis to bone model both *in vivo* and *in vitro*. Bosentan administration decreased cancer proliferation and invasion. However, there is no evidence to show how bosentan exerts these effects and whether there is any apoptotic or other physiological role of ET-1 in breast cancer proliferation. Another study similar to this study investigated the relationship of cytokines and ET-1 *in vivo* and *in vitro*. Exogenously given ET-1 to 4T1 (mice breast cell line) did not stimulate cell proliferation and migration. ET-1 administration also did not enhance human MCF-7, MDA-MB468, and MDA-MB231 breast cancer cell lines; however, ET-1 significantly increased cell invasion as reported in the literature [16]. Interestingly, Paola et al. demonstrated that while exogenous ET-1 reduced invasiveness of 1833 cells in a dose-dependent manner, in MDA-MB231 cells, ET-1 increased cell invasiveness and tripled MMP 2 [17]. Although Et-1 has been known as an angiogenic agent in clinical trials, this information has been found to have contrasting results in studies with animal and human cell lines. and as a result, this has led to some speculation. Therefore, these different effects of ET-1 in breast cancer are interesting and suggest that cancer cell physiology could change depending on its origin, i.e., human or animal.

Main Points

- This study definitely introduced ET-1 and its critical role in cancer progression.
- Bosentan exerts anti-proliferative and anti-migratory effects on BC by inhibiting the ET-1 axis.
- The imbalance among the NF-kB, caspases, and VEGF, which are predictive factors of carcinogenesis significantly improved after bosentan administration.

This study aimed to explore the role of ET-1 in human breast cancer proliferation and migration, antagonism of the ETAR and ETBR by using the non-selective dual ETA/ETB receptor antagonist, bosentan, and determine its anti-proliferative, anti-metastatic, and apoptotic effects demonstrated by NF-kB, VEGF, Caspase 3, and Caspase 9 expression on endothelin-induced proliferation of MCF-7 cell line *in vitro*.

Materials and Methods

Chemicals and Reagents

Dimethyl sulfoxide and ET-1 were from Sigma-Aldrich (St. Louis, MO, USA) and bosentan from Hoffmann La Roche, Basel, Switzerland. Dulbecco's modified eagle medium (DMEM) and cell culture medium and reagents such as fetal bovine serum, penicillin/streptomycin, and trypsin-EDTA were obtained from GIBCO (Invitrogen Inc., NY, USA).

Cell Culture

The cell line used in our study was not a primary cell line but was purchased from American Type Culture Collection (ATCC, Manassas, VA. USA). Ethics committee approval is not required for these and similar studies. MCF-7 cells were purchased from ATCC and cultured in DMEM containing 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were grown in the presence of 5% CO_2 at 37°C.

Real-Time Monitoring of Cell Growth and Proliferation Assay

Cell proliferation was measured with the xCelligence system (Roche Diagnostics, Indianapolis, IN, USA), which is a label-free technique for dynamic monitoring of living cells [18]. First, 8,000 cells per well were seeded in hormone-free medium 24 hours after the cells were treated with 10^{-4}, 10^{-5}, and 10^{-6} M BOS (dissolved in 1% DMSO) in order to determine the optimal inhibitor concentration of bosentan for 24 hours. Secondly, 8,000 cells were seeded into the e-plates 24 hours after the cells were incubated with or without 10^{-4}M BOS (1 hour before ET-1 treatment), 10^{-7}, 10^{-8}, and 10^{-9}M ET-1 for 1–4 days. The xCelligence system employs 16-well plates with gold electrodes at the bottom of the wells. The interaction between cells and electrodes generates an impedance response that correlates linearly with a cell index to reflect cell number, adherence, and growth. Automatic measurements were performed every 15 minutes for the duration of the experiment. Data are presented as normalized cell index±standard error measurement (SEM).

Real-Time PCR

Total RNA Extraction and cDNA Synthesis

Six hours after incubation, cells were harvested and homogenized once during 2 minutes using the TissueLyser II (Qiagen). Total RNA was purified using RNeasy Mini Kit Qiagen according to the manufacturer's instructions in Qiaqube (Qiagen). The RNA samples were reverse-transcribed into complementary DNA by the high capacity cDNA Reverse Transcription Kit (Applied Biosystem). A total of 10 µl RNA was treated with 2 µl 10 X RT Buffer, 0.8 µl 25 X dNTPs mix, 2 µl 10X RT Random Primers, 1 µl MultiScribe Reverse Transcriptase, and 4.2 µl DEPC-H_2O. Reverse transcription was carried out at 25°C for 10 minutes, followed by 37°C for 120 minutes, and finally 85°C for 5 minutes using a Veriti 96-Well Thermal Cycler (Applied Biosystem). The cDNA concentration and quality was assessed and quantified by the Epoch Spectrophotometer System and Take3 Plate (Biotek).

Relative Quantification of Gene Expression

Relative ETRA, ETRB, CAS-3, CAS-9, NFKB, VEGF, mRNA expression analyses were performed with StepOne Plus Real-Time PCR System (Applied Biosystem) using cDNA synthesized from MCF-7 RNAs. Real-time PCR was performed by using primers generated for humans and shown in Table 1 (Primer Design Ltd., Southampton, UK). Results were expressed as relative-fold and compared to control groups. Expression data of β-actin in each cell group were used as endogenous controls. For each cell group, triplicate determinations were performed for both targets in a 96-well optical plate using 9 µl of cDNA (100 ng), 1 µl of Primer Perfect Probe mix, and 10 µl of QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany) in each 20 µl reaction. The plates were heated for 2 minutes at 50°C, 10 minutes at 95°C, and subsequent 40 cycles of 15 seconds at 94°C and 60 seconds at 60°C. All data were expressed as fold-change in expression compared to animal groups using the $2^{-\Delta\Delta Ct}$ method.

Wound Healing Motility Assay (HUVEC)

Human umbilical vein endothelial cells (HUVEC) were seeded onto 6-well dishes at 10^5/well. A single scratch wound was created using a p10 micropipette tip in to confluent cells. Cells were

washed 3 times with PBS to remove cell debris, supplemented with assay medium, treated with or without 10^{-4}M BOS and 10^{-7}, 10^{-8}, and 10^{-9}M ET-1. Cells were monitored and images were captured by inverted microscopy at 0, 12, and 24 hours post wounding.

Statistical Analysis

Columns are shown as mean±SD. Prism software (GraphPad Software Inc., San Diego, CA, USA) was used for data presentation and statistical analysis. Statistical analysis of more than 2 groups was performed by one-way analysis of variance (ANOVA) followed by Tukey testing.

Results

Cell Proliferation and Wound-Healing Migration Assay

The addition of bosentan at concentrations of 10^{-4}–10^{-6} M for 72 hours caused decrease in cell viability at all doses; however, the 10^{-4} M (100 µM) dose yielded the best results, and 10 µM and 1 µM bosentan did not show a significant difference after 48 and 72 hours (Figure 1) compared to untreated control cells. The addition of ET-1 at concentrations 10^{-7}–10^{-9}M for 1–4 days caused significant elevations in cell viability at all doses of ET-1. While 10^{-7} and 10^{-8}M ET-1 showed the best results for 1–4 days treatment, 10^{-9}M ET-1 did not show a significant difference after 4 days. ET-1 administration exogenously increased cell proliferation, which suggests that it has an important role in cancer proliferation. However, cell viability of 10^{-4} M bosentan group also decreased significantly on the fourth day. Interestingly, treating cells with bosentan and ET-1 administration caused a significant decrease in the proliferative effect of ET-1, at both doses of 10^{-7} M and 10^{-8} M, especially on the fourth day (Figure 2). Increased proliferative effect of ET-1 was inhibited by bosentan administration on all days. Therefore, bosentan may be good choice to inhibit the proliferative effect of ET-1 in patients with breast cancer.

Table 1. RT-PCR primer probe gene sequences

Gene	Sequences
ETRA	F:5′-TCGGGTTCTATTTCTGTATGCCC-3′
	R: 5′-TGTTTTTGCCACTTCTCGACG-3′
ETRB	F:5′-GCAAACCGCAGAGATAATGACG-3′
	R: 5′-GGACACAACCGTGTTGATGTATT-3′
CAS-3	F:5′-ACTGGGAATGAACCGCCTTTG-3′
	R: 5′-CTTCGCCACTGATAGGGATGC-3′
CAS-9	F:5′-TCGTTTCTGCGAACTAACAGG-3′
	R: 5′-GCACCACTGGGGTAAGGTTT-3′
NFKB	F: 5′ GTCAAAAACGCCACCTCTCAA-3′
	R: 5′- CTCGCATGGAATTTGGAACCG-3′
VEGF	F: 5′-GAGGAGCAGTTACGGTCTGTG-3′
	R: 5′-TCCTTTCCTTAGCTGACACTTGT-3′
β-actin	F: 5′-GCAAGCAGGAGTATGACGAGT -3′
	R: 5′-CAAGAAAGGGTGTAACGCAACTAA -3′

As shown in Figures 3, HUVECs migrated into the wound area following stimulation with 10^{-7} and 10^{-8} M ET-1 (Gap closure percent of 0.15±0.01 and 18.62±1.81, respectively) in a time-dependent manner when compared with the control groups (23.70±0.95). However, the effect of bosentan on migration of HUVEC cell showed deceleration under conditions of increasing concentration of ET-1 (52.93±6.82, 47.78±1.16, 47.65±1.55, respectively) for 24 hours compared to the control group. Furthermore, only bosentan administration showed an inhibitory effect on cell migration. These results suggest that ET-1 has a critical role in both cancer proliferation and migration. As bosentan shows a significant inhibitory effect against ET-1, it can be concluded that bosentan, an orphan medicine, may be used in breast cancer patients to prevent both proliferation and invasion of the cancer.

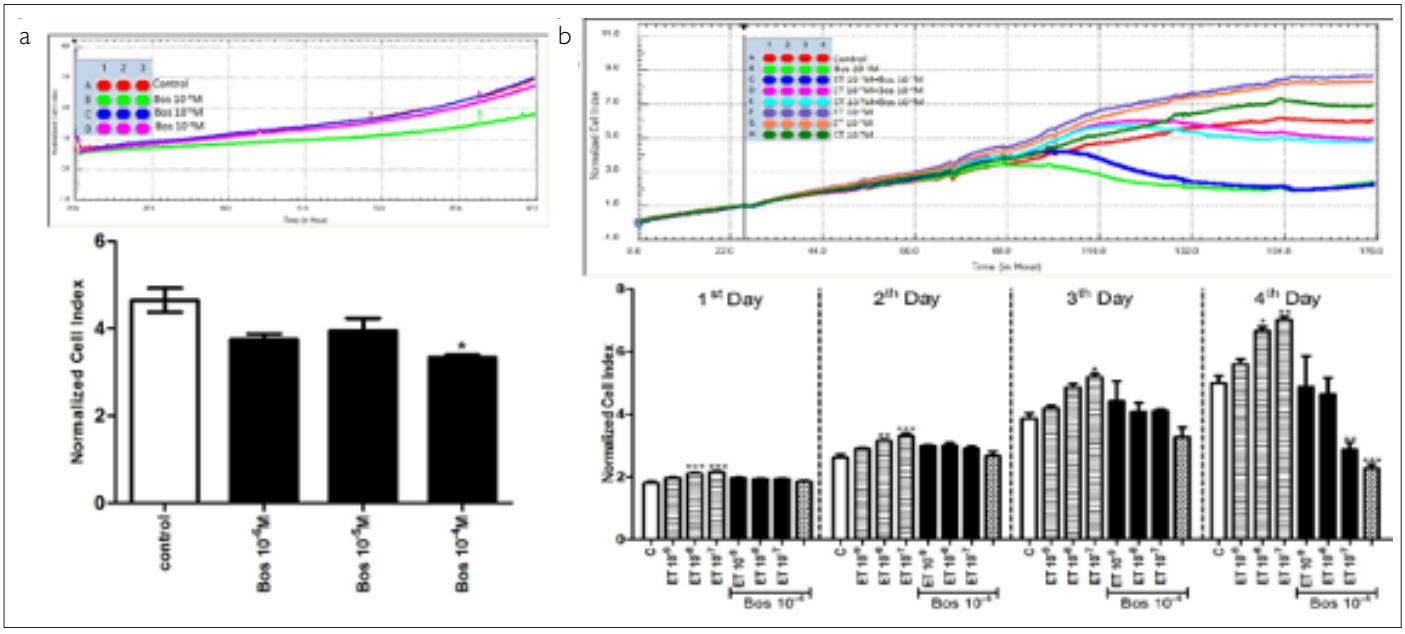

Figure 1. a, b. (a) Effects of bosentan on MCF-7 cells (dose dependent manner), (b) Effects of ET-1 and bosentan on tumor proliferation. Columns are shown as mean±SD, (*p<0.05, **p<0.01, ***p<0.001)

ET-1 Axis mRNA Expression

While ET-1 administration significantly up regulated ETRA mRNA expression compared to control ($p<0.05$), it was found ETRB mRNA expression (1.3-fold change) was statistically insignificant in ET-1 group ($p>0.05$). ETRA and ETRB expression was significantly down regulated by bosentan and ET-1+bosentan groups compared to ET-1 and control groups, respectively ($p<0.05$).

VEGF Expression

VEGF has an important role during carcinogenesis, both in growth and angiogenesis. In this study, increased VEGF expression in the control group was aggravated by ET-1 administration and this is in parallel with migration analyses ($p<0.05$). In contrast, bosentan administration significantly down regulated ET-1 induced VEGF expression compared to control and ET-1 groups ($p<0.05$). Therefore, bosentan provides anti-proliferative and anti-metastatic effects based on ET-1 induced VEGF expression.

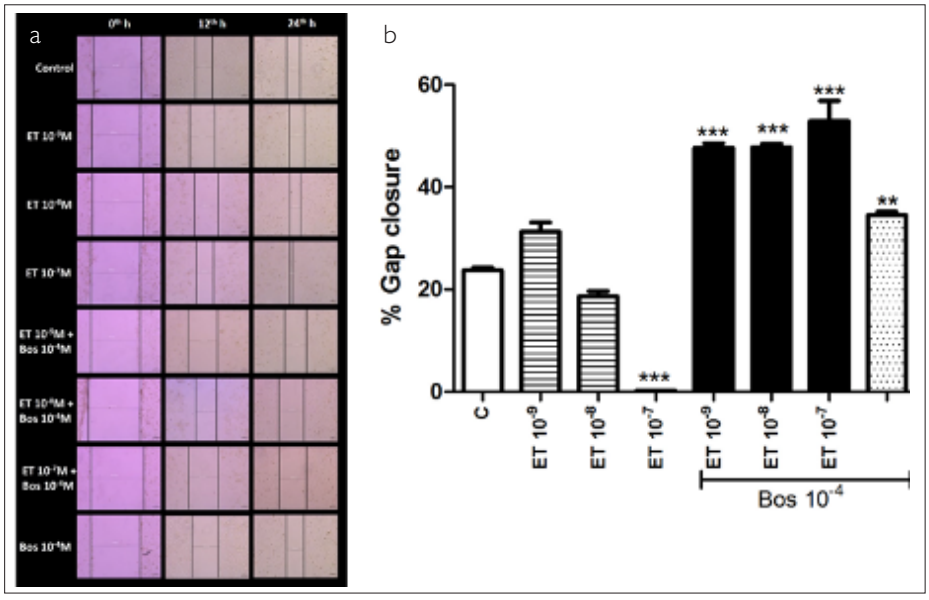

Figure 2. a, b. (a) Attenuation of migration ability of human umbilical vein endothelial cells under Bos and ET-1 conditions. Representative images at 0 hours, 12 hours, and 24 hours of the wound-healing assay are shown (×20). (b) Effects of ET-1 and bosentan on tumor migration by using wound healing model (% gap closure). Columns are shown as mean±SD, (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

Apoptotic Markers

Caspases 3 and 9 have an important role in inhibiting tumor proliferation. In breast cancer the persistent activation of the NFκB-signaling pathway leads to abnormal cell proliferation and differentiation, enhanced metastasis, and treatment resistance. In this study, NFκB expression was up regulated in the cancer cells, and ET-1 administration statistically increased NFκB expression compared to that of the control group ($p<0.05$). Bosentan administration after ET-1 induction exerts a significantly inhibitory effect on ET-1 induced NFκB expression ($p<0.05$). Bosentan administration also changed NFκB mRNA expression ($p>0.05$). While Caspase 9 down regulated ET-1 administration ($p<0.05$), Caspase 3 did not change after ET-1 administration (0.9-fold change, $p>0.05$). ET-1 exerts a high proliferative effect depending on activation VEGF and NFκB and inhibition of Caspase 9 expression compared to control. However, apoptotic protein expression significantly improved by bosentan administration ($p<0.05$),

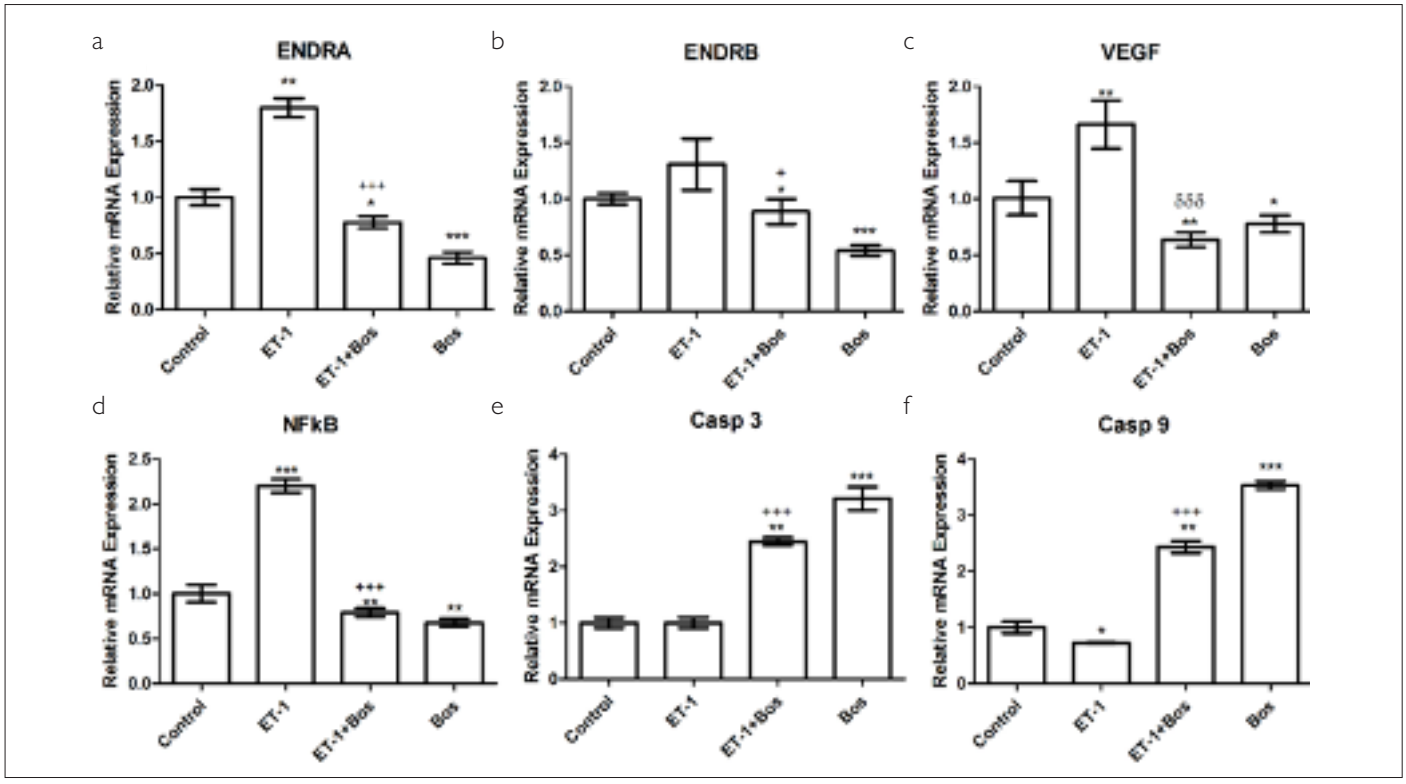

Figure 3. a-f. Effects of ET-1 and bosentan 6 hours after (a) ETRA, (b) ETRB, (c) VEGF, (d) NF-kB, (e) Cas3 and (f) Cas9 mRNA expressions of BC. Columns are shown as mean±SD, (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

and bosentan administration alone exerted a significant up regulatory effect on Caspases 3 and 9 compared to those of the control group ($p<0.05$).

Discussion

In this study, we demonstrated that ET-1 has a critical role in both tumor proliferation and migration on the MCF-7 cell line *in vitro*. Previous studies showed that ET-1 and its receptors are expressed in several tumor cells [19]. Particularly, ET-1 acts on pleiotropic effects of cell proliferation, apoptosis, migration, and neovascularization on BC [20].

ET-1 is produced by the vascular endothelial cells and also by hepatocytes, kidney mesangial cells, endometrial cells, neurons, sertoli cells, and breast epithelial cells [6]. It is present in 3 different forms in our body. ET-1 is a well-known isoform which is a potent vasoactive 21-amino acid peptide. ET-1 shows its effects via ETAR and ETBR, and these are included in the category of G protein-coupled receptors (GPCR) [7]. In addition to its vasoconstrictor effects, ET-1 is also known to be a potent mitogen for different cell types such as endothelial cells keratinocytes, mesangial cells, osteoblasts, and human breast fibroblasts [12, 21] via binding to the ETAR and ETBR in vascular and nonvascular cells and has a proliferative effect on numerous cell types [22, 23]. In addition, ET-1 mainly stimulates the vascular endothelial growth factor (VEGF) which is known as stimulatory protein for angiogenesis and mitogenesis [24]. ET-1 acts not only on the regulation of VEGF but also the other growth factors such as EGF, PDGF, bFGF, TGFs, and insulin, all of which play important roles in cellular transformation or replication [25].

Previously, it was believed that ETAR and its relationship to cancer was clear; however, ETRB and its role in cancer seems to be confusing. Vasodilatation after activation of ETRB in BC improves blood supply to the tumor area, which leads to tumor breeding and growth [26, 27]. In contrast, some studies have been shown that using ETRB agonists combined with chemotherapeutic agents enhanced anti-tumor efficiency [28]. Hence, activation or inhibition of ETBR in BC is still unclear. Selective ETAR blockers and ETRB agonists have been tried for the treatment of BC. However, is using a selective ETAR blocker for the treatment of BC beneficial? Since ETRB was still active when we used ETAR blockers, using dual ETR blockers may have a dual effect on BC.

Bosentan is one of the dual ETR blockers and approved for use in portal hypertension. Bosentan selectivity to ETRA is higher than ETRB; therefore, it has an advantage in anti-ET-1 activity. Anti-tumor and anti-metastatic effects of bosentan in BC was shown in previous studies [17, 29]. However, it has been concluded that the effects of ET-1 on BC proliferation and migration seems to be confusing.

NF-κB, a pro-inflammatory and pro-survival factor, is well known to be highly involved in the proliferation and migration of BC. Increased NF-κB plays an important anti-apoptotic role during BC proliferation [30]. However, the functional interaction between ET-1 and NF-κB in BC is poorly understood. In this study, ET-1 administration significantly up regulates NF-κB expression in BC compared to that of the control group, which implies that NF-κB mediates the effect of ET-1 on BC. Bosentan administration exerts a down regulatory effect on NF-κB gene expression. Therefore, increasing NF-κB is related to the aggressiveness of various cancers and cross-talk between different survival pathways [31, 32].

VEGF is another important factor on BC proliferation and migration, and VEGF gene expression is regulated by NF-κB. Previous studies suggest that NF-κB is involved in the up regulation of VEGF mRNA expression. Shibata et al. suggested that NF-kb inhibition decreases VEGF expression in BC. Preventing angiogenesis in BC by inhibition of NF-κB could be a new approach in the treatment of BC [33, 34]. In ET-1 induced groups, parallel with increased NF-κB, VEGF expression was higher compared to that of the control group. Presence of ET-1 in the culture of medium increased both proliferation and migration correlated with NF-κB and VEGF expression. These effects of ET-1 were significantly down regulated with bosentan administration.

Caspases are a family of protease enzymes that play an essential role in programmed cell death, particularly in cancer. However, cancer cells produce different endogenous agents to escape the immune system during carcinogenesis. Inhibiting apoptosis during carcinogenesis is an important role of the NF-κB pathway, which causes up regulation of key anti-apoptotic proteins and down regulation of apoptotic proteins [35]. Similar to previous studies, our study showed that Caspases 3 and 9 was significantly down regulated in ET-1 group compared to that of control group. In these groups, it has been demonstrated that ETAR and ETBR expression up regulated in line with NF-κB and VEGF expression. Increased Caspase 3 and 9 expressions significantly improved after bosentan administration.

Finally, bosentan exerts anti-proliferative and anti-migratory effects on BC by inhibiting ET-1 axis. However, these effects of bosentan varies based on the absence or presence of ET-1 in the tumor area. Whether ET-1 is present or not in the tumor area, bosentan exerts anti-proliferative effect on BC. ET-1 and bosentan together showed important inhibitory effects on tumor migration compared to bosentan alone, which can be attributed to increased activity of ET-1 axis in the presence of ET-1. The imbalance among the NF-kB, caspases, and VEGF, which are predictive factors of carcinogenesis, significantly improved after bosentan administration.

Limitations, drawbacks, or shortcomings: Analysis could be done using western blot (WB) method; however, our working budget was not sufficient for western blot analysis. Most of the similar studies also do not use the WB method, and our study had sufficient evidence for viability testing, wound healing model, and mRNA levels.

In conclusion, this study definitely proves the critical role of ET-1 in cancer progression with apoptotic and anti-apoptotic pathways (NF-κB) and VEGF expression. In addition, we also performed migration analyses. The second major finding was that bosentan inhibited ET-1 mediated effects on tumor proliferation and migration. The current findings add substantially to our understanding of ET-1 axis and its role in BC.

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