Efficacy of the specific endothelin A receptor antagonist zibotentan (ZD4054) in colorectal cancer: a preclinical study

Samer-ul Haque¹*, Michael R. Dashwood²*, Mohammed Heetun¹, Xu Shiwen³, Noreen Farooqi¹, Bala Ramesh¹, Hazel Welch¹, Felicity J. Savage¹, Olagunju Ogunbiyi¹, David J. Abraham³, Marilena Loizidou¹.

Cancer Nanotechnology Group, UCL Division of Surgery and Interventional Science¹; Department of Clinical Biochemistry, Royal Free Hospital²; Centre for Rheumatology and Connective Tissue Diseases, UCL Division of Medicine³, London, UK.
*S. Haque and M.R. Dashwood are joint first authors

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Corresponding author:
M. Loizidou
Division of Surgery and Interventional Science
UCL Medical School,
9th floor, Royal Free Hospital,
Pond Street,
London, NW3 2QG.
United Kingdom.
Email: m.loizidou@ucl.ac.uk
Telephone: 020 7794 0500 Ext.35575
Facsimile: 020 7472 6444

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ABSTRACT

Endothelin 1 (ET-1) is over-expressed in cancer, contributing to disease progression. We previously showed that ET-1 stimulated proliferative, migratory and contractile tumourogenic effects, via the ET\(_A\) receptor. Here, for the first time, we evaluate zibotentan, a specific ET\(_A\) receptor antagonist, in the setting of colorectal cancer (CRC), in cellular models. Pharmacological characteristics were further determined in patient tissues.

CRC lines (n=4) and fibroblast strains (n=6), isolated from uninvolved areas of CRC specimens, were exposed to ET-1 and/or ET\(_{A/B}\) receptor antagonists. Proliferation (methylene blue), migration (scratch wounds) and contraction (gel lattices) were assessed. Receptor distribution and binding characteristics (K\(_d\);B\(_{\text{max}}\)) were determined using autoradiography on tissue sections and homogenates and cytospun cells, supported by immunohistochemistry.

Proliferation was inhibited by ET\(_A\) (zibotentan>BQ123; p<0.05); migration by ET\(_B\)>ET\(_A\); and contraction by combined ET\(_A\) & ET\(_B\) antagonism. Intense ET-1 stromal binding correlated to fibroblasts and endothelial cells. CRC lines and fibroblasts revealed high density and affinity ET-1 binding (B\(_{\text{max}}=2.435\text{fmol}/1\times10^6\text{cells}, K_d=367.7\text{pmol/L}; B_{\text{max}}=3.03\text{fmol}/1\times10^6\text{cells}, K_d=213.6\text{pmol/L}). In cancer tissues, ET\(_A\) receptor antagonists (zibotentan; BQ123) reduced ET-1 binding more effectively (IC\(_{50}:0.1\mu\text{M}-10\mu\text{M}) than ET\(_B\) receptor antagonist BQ788 (∼IC\(_{50}:1\mu\text{M}).

ET-1 stimulated cancer-contributory processes. Its localisation to tumour stroma, with greatest binding/affinity to fibroblasts implicates these cells in tumour progression. ET\(_A\) receptor up-regulation in cancer tissues, and role in tumourogenic processes demonstrates the receptor's importance in therapeutic targeting. Zibotentan, the most specific ET\(_A\) receptor antagonist available, demonstrated the greatest inhibition of ET-1 binding. With its known safety profile, we provide evidence for Zibotentan’s potential role as adjuvant therapy in CRC.
INTRODUCTION

Colorectal cancer is one of the commonest malignancies in the West and a leading cause of worldwide morbidity and mortality. If detected at an early stage, the disease is potentially curable with surgical resection as the ‘gold standard’ treatment. However, up to 60% of patients will have regional or metastatic disease at initial presentation [1] limiting the potential for surgical intervention. For these patients, disease management focuses on the use of chemotherapeutic agents, either individually or in combination. Despite improvements in drug development, the success of these agents is limited.

Endothelin-1 (ET-1) is a small vasoactive peptide overexpressed in plasma and tissue samples from patients with various solid cancers [2] (Figure 1A). ET-1 effects are mediated by two distinct G protein-coupled receptors, ET_A and ET_B. Receptor binding and signalling often causes opposite effects on proliferation: in the majority of cells, binding to ET_A promotes growth, whereas binding to ET_B leads to apoptosis. Receptor expression is altered in a number of solid cancers, with ET_A reported as upregulated, and ET_B either up or down regulated, using various techniques. Using autoradiography, our previous results were similar to those reported in ovarian and prostate cancers [3,4], in that ET_A receptors were over-expressed while ET_B receptors were down-regulated in cancer tissue compared to normal colon. Specifically, binding to the ET_A receptor was greatest in cancer-associated fibroblasts and blood vessels and to a lesser extent in epithelial cancer cells. In contrast, ET_B receptors were the predominant receptors in normal colon and were markedly down-regulated in all cell types within cancer [5].

In vitro, ET-1 production has been detected in a number of human cancer cell lines, such as colorectal, stomach, breast and prostate [6,7]. Furthermore, exogenous ET-1 addition to ovarian, prostate and melanoma cancer cells stimulates proliferation [3,4,8]. ET-1 may also contribute to tumour progression by stimulating angiogenesis and desmoplasia and promoting invasion. Previously we investigated ET-1 effects on colorectal cancer cells and fibroblasts isolated from the colons of patients with cancer. ET-1 via ET_A provided a growth stimulus to colorectal cancer cells; the signal was also propagated via the transactivation of the epidermal growth factor receptor (EGFR), when this receptor was present [9]. Furthermore colonic fibroblasts, when exposed to ET-1 exhibited increased growth, migration and contraction [10]. Since the fibroblasts were isolated from macroscopically uninvolved areas of colorectal cancer specimens, our findings are consistent with ET-1 produced by cancer cells activating adjacent fibroblasts in a paracrine manner.
Clinically, specific ET$_A$ receptor blockade has been proposed as a potential target for anti-cancer therapy, however, despite encouraging pre-clinical data [11,12] and early clinical signals thus far, data obtained with this class of agent in prostate and non-small cell lung cancer (NSCLC) has been disappointing. To date, there are no data for the ET$_A$ receptor antagonist zibotentan (ZD4054) in colorectal cancer, either preclinically or clinically, to determine its usefulness either as monotherapy or in combination with cytotoxic drugs.

The purpose of the present study was to determine the effects of the specific ET$_A$ receptor antagonist zibotentan on cellular processes integral to cancer growth and progression, namely proliferation, migration, contraction and protein expression. These parameters were investigated in a panel of colorectal cancer cell lines and colonic fibroblasts, and compared to the standard laboratory ET receptor antagonists BQ123 (ET$_A$ selective) and BQ788 (ET$_B$ selective) (Figure 1). Furthermore, zibotentan pharmacological characteristics were determined in normal and cancer tissues and cells from patient specimens taken at surgery for colorectal cancer.

**MATERIALS AND METHODS**

**Biological materials**

**Cells:** The human colorectal cancer cell lines used were HT29, SW480 (moderately differentiated), SW620 (poorly differentiated) (European Collection of Cell Cultures, ECACC, Sigma-Aldrich Company Ltd, Dorset, UK); cell line authentication at source includes species and identity verification by DNA barcoding and profiling, and PCR of short tandem repeat sequences within chromosomal microsatellite DNA (STR-PCR); and the human colorectal cancer cell line LIM1215, isolated and immortalised at the Ludwig Institute for Cancer Research (HNPCC familial cancer; donated by Professor M O’Hare, Ludwig Institute for Cancer Research, London, UK). Further authentication was not undertaken in our laboratory. Submucosal fibroblasts were isolated from specimens of patients undergoing colorectal cancer resection and extracted from tissue adjacent to, but not macroscopically involved with, cancer. Briefly, after collagenase digestion, epithelial and endothelial cells were removed using antibody-coated magnetic beads and the remaining cells ($\beta$-actin positive) were grown and used (passages 4-12; untransformed cell strains; [10]). Cells were discarded thereafter due to loss of the fibroblastoid phenotype [13]. The strains were labelled CF (Colorectal Fibroblasts) and allocated numbers according to specimens received –here
we used CF35, CF42, CF56, CF65, CF75, CF78; isolated 2001-2004 with informed patient consent). Further authentication was not undertaken. All cell lines and cell strains were passaged for fewer than 6 months after resuscitation. Cells were cultured in a humidified atmosphere, 37°C, 5% CO₂/air, in Glutamine-enriched Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum and gentamicin (50μg/ml). For cytospins, cells (2x10⁶ - gross autoradiography; 0.5x10⁶ - microautoradiography) were loaded onto polylysine-coated slides, centrifuged, air dried and stored (-70°C; Shandon Cytospin3, WolfLabs Inc, Pocklington, UK).

**Tissues:** Specimens were obtained from patients (n=6) undergoing colorectal cancer resection; adjacent normal and cancer samples were snap frozen, stored (liquid N₂) and used for: (1) 5μm frozen sections on polylysine-coated slides (Leica CM3050 cryostat, Milton Keynes, UK); (2) Homogenates: Tissues were placed into a ball-bearing cage, dipped in liquid N₂ and homogenised (2min, Mikro-Dismembranator U, Braun Biotech, Melsungen, Germany). The resulting powder homogenates were diluted in molecular grade water (2ml) and stored (-70°C; Ethical approval, REC No 08/H0720/162, UCL Hospitals).

**Zibotentan ET₆ receptor antagonism – in vitro studies**

**Agents:** For colorectal cancer line studies, ET-1 was used at 10⁻⁸M; specific ET₆ receptor antagonists zibotentan (Astra Zeneca, Macclesfield, UK) and BQ123 (Bachem Ltd, St Helens, UK) and specific ET₆ receptor antagonist BQ788 (Bachem Ltd) were used at 10⁻⁷M. For fibroblast studies, ET-1 was used at 10⁻⁷M; zibotentan, BQ123 and BQ788 at 10⁻⁶M (Figure 1). Doses determined previously [9,14]. Other reagents were purchased from Sigma-Aldrich Co, unless otherwise specified.

**Growth:** Cells were seeded in 96-well plates (fibroblasts:10,000-15,000/well; HT29,SW620: 20,000/well), grown for 36h (60-70% confluence) and starved in serum free medium (24h). This was replaced by serum free medium containing ET-1 and/or ET₆/ET₆ receptor antagonists (zibotentan, BQ123, BQ788). Plates were incubated (48h), fixed (10% formal saline) and proliferation measured by the methylene blue assay [15], with absorbance 630nm (Anthos-2010 platereader, Biochrom Ltd, Cambridge, UK) proportional to cell number.

**Migration:** A modified “scratch wound” assay was used [16,17]. Cells were seeded (12-well plates; 100,000/well) in fully supplemented medium. At 90-100% confluence, a scratch was made through the centre of the well. Medium was replaced with serum-free medium (with mitomycin C to inhibit proliferation - 1μg/ml for fibroblasts; 0.5μg/ml for HT29, SW620) and ET-1 ± ET receptor antagonists (zibotentan, BQ123, BQ788). Photographs were taken
every 6 hours (0-24h). Means of 3 scratch-width measurements per well at fixed positions were used to calculate migration as the % reduction in width compared to that at time=0.

**Gel contraction:** Cells were grown in three-dimensional collagen gel lattices [18] set in 24-well plates (100,000 cells/lattice). Gels were impregnated with antagonists alone or in combination (zibotentan, BQ123; BQ788; zibotentan plus BQ788; 1h) and then ‘floated’ by the addition of 1ml serum-free ET-1. Control gels were floated by serum free medium. Gels were further incubated (72h) and fixed in 10% formaldehyde. To image, gels were placed on glass slides and scanned. Excess moisture was blotted off and gels weighed to determine weight loss proportional to contraction.

**Statistical analysis:** Data were analysed using one-way ANOVA (post-hoc Tukey’s HSD, p<0.05). For growth, six independent repeats were performed for each fibroblast strain (n=5) and each cancer line (n=2). For migration, six independent repeats were performed for each fibroblast strain (n=4) and each cancer line (n=2). Collagen gel contraction experiments were performed in three fibroblast strains and one cancer line (3 independent repeats). Graphical representations (growth, migration, contraction) combine data from all strains or cancer lines used (e.g., means of means, or normalizing data/converting control data sets to 100% to facilitate presentation/discussion).

**Zibotentan ET\(_A\) receptor binding characteristics**

**Saturation analysis:** Kd/Bmax determination: Receptor binding studies were performed as described previously [19]: cytospins and tissue homogenates were incubated in increasing \(^{125}\text{I}\)-ET-1 concentrations (GE, Amersham, UK, specific activity 2200 Ci/mmol: 3x10^{-12}-10^{-9}\text{M}) with non-specific binding established in the presence of unlabelled ET-1 (1µM). After incubation; slides were post-fixed in paraformaldehyde vapour; and homogenates filtered and washed (3x). \(^{125}\text{I}\) standards were prepared where 50µl aliquots of each of the serial dilutions of radioligand used were spotted onto filter paper and attached to microscope slides that were co-exposed to radio-sensitive film alongside the cytospins.

**Inhibition analysis:** Antagonist binding affinities were assessed by inhibition studies: cytospins/tissues were incubated in a fixed \(^{125}\text{I}\)-ET-1 concentration (150pM K\(_d\)) in the presence of increasing concentrations (3x10^{-9}-3x10^{-6}\text{M}) of the receptor antagonists - zibotentan, BQ123, BQ788. Two fixed concentrations (high=25µM; low=5µM) for each antagonist were used for autoradiography as described below.

**Autoradiography:** (1) Low resolution: Slide-mounted tissues and cytospins were apposed to Hyperfilm™MP (GE) in x-ray cassettes and exposed for 7-21 days (4°C) after which films were processed following manufacturer’s instructions and used for densitometry;
representative images were photographed. (2) **High resolution:** Selected slides were used for microscopic localisation of radioligand binding whereby tissues/cytospins were dipped in molten K2 emulsion (Ilford), stored in light-proof boxes (4°C, 7-21 days) and the emulsion processed following manufacturer’s instructions [14]. Tissues/cells were stained with haematoxylin & eosin (H&E), viewed (Olympus BX50 microscope) photographed (Zeiss Axiocam™ digital camera) and images stored (KS400 system, Imaging Associates, Bicester, UK). Additional to densitometry, radioligand binding was also assessed using a gamma counter (Perkin Elmer, Cambridge, UK) where - after film images of cytospins had been generated for densitometry - cells were digested (100µl 4M NaOH; ~10min) and radioactivity (counts per minute; CPM) measured.

**Calculation of binding characteristics:** Densitometric image analysis was performed on a Biospectrum® AC Imaging System (UltraViolet Products, UVP, Cambridge, UK) and analysed using VisionWorksLS Imaging software (version 6.4.3. UVP, 2007). Specific ^125^I-ET-1 binding was determined by subtracting non specific from total binding at each concentration. Maximum receptor binding (B_max) and affinity (K_d) were obtained using GraphPad Prism™ software (GraphPad, Santa Barbara, CA). Radioactivity counts (gamma counter; CPM) from cells removed from slides were similarly analysed.

**Immunohistochemistry:** Immunohistochemistry was performed using the Vectastain alkaline phosphatase kit (Vector Labs, Peterborough, UK) following manufacturer’s instructions as described previously [5,19]. Primary antibodies used were AS02/Thy-1 for fibroblasts, CD31 for vascular endothelial cells, anti-ET_A receptor and anti-ET_B receptor antibodies (Alomone Labs, Israel), Col11A1 for collagen Type XI (Santa Cruz Biotech, Inc, CA; 1:200 in PBS, 30min), followed by universal secondary antibody (1:100 in NHS/PBS, 10min). Sections were counterstained (haematoxylin) and photographed as above. H&E staining was performed on selected sections. ET_A receptors were further imaged by fluorescence of quantum dot (QD) - coupled BQ123 receptor antagonist (600nm emission). The water soluble QDs (manufactured in-house) were conjugated to BQ123 using the water soluble N-ethyl-N’-diaminopropyl-carbodiimide (EDC) method: Briefly, 200µl of QDs (1mg/ml in borate buffer (pH 7.4), were mixed with EDC (1mg/ml) for 30 min at room temperature. BQ123 (10^{-4}M) was then added to the above solution at ~ 100ug/ml and mixed for 1h at room temperature. After this reaction procedure, BQ123-QDs and unconjugated QDs were separated using centrifugal filter (Millipore, Cork, Ireland) with a cut off value of 10 kD membrane. After repeated centrifugations, purified and concentrated BQ123-QDs were obtained. BQ 123-QDs bioconjugates were applied to slide mounted tissues (unfixed, frozen sections) overnight (at 4°C), haematoxylin counterstained, and then observed under confocal microscopy.
RESULTS

Efficacy of zibotentan in in vitro models of colorectal cancer

The ability of the specific ETₐ receptor antagonist zibotentan to inhibit ET-1 stimulation of growth, migration and contraction was tested in colorectal cancer cells and colonic fibroblasts.

Proliferation: Growth effects were determined independently in five fibroblast strains in the presence of ET-1 alone, ET-1 and ETₐ or ET₈ receptor antagonists (zibotentan, BQ123; BQ788), and all three receptor antagonists alone (48h). Exogenous ET-1 stimulated growth in all strains, from a minimum of 28% to a maximum of 46%, compared to unstimulated controls (p<0.05; normalised results for all strains pooled in Figure 2A). This effect was reduced by the specific ETₐ receptor antagonists, zibotentan and BQ123 (p<0.05 v ET-1 group), but not the ET₈ receptor antagonist BQ788. Zibotentan had similar activity to that of the laboratory standard ETₐ receptor antagonist BQ123. Use of the antagonists alone did not produce a significant effect, although ET₈ receptor blockade (BQ788) appeared to produce a trend towards growth stimulation. A similar pattern of growth stimulation was seen for both the colorectal cancer cell lines HT29 and SW620, where addition of exogenous ET-1 produced an increase in proliferation of approximately 22%; results from independent repeats are pooled for graphical representation (p<0.05 v control, Figure 2B). ET-1-stimulated growth was significantly blocked by ETₐ (zibotentan, BQ123), but not by ET₈, receptor antagonists. Incubation with antagonists alone did not affect cell growth significantly.

Migration: Cell migration was tested in five fibroblast strains and both colorectal cancer cell lines. Fibroblast migration was recorded by light microscopy up to 24 hours after the initial scratch was created (Figure 3). For fibroblasts, the migration pattern was similar in four of the fibroblast strains, (one strain being unresponsive (CRF42)): By 18-24 hours fibroblasts completely obscured the scratch wound in the presence of ET-1 (100%) compared to untreated (13%). ET-1-stimulated migration was inhibited by both ETₐ and ET₈ antagonists, to varying degrees: BQ788 = 71%; zibotentan and BQ123 = 50-51%. However, combined blockade of ETₐ and ET₈ receptors with zibotentan and BQ788 produced an effect that was less than that observed with ET₈ blockade, but greater than that seen with ETₐ blockade (58.8% inhibition, resulting in 41.2% migration). Addition of antagonists alone did not
produce a noticeable effect (results not shown). Colorectal cancer cells did not migrate, regardless of treatment (24hrs).

**Contraction:** Contraction was investigated in rat collagen gels containing either fibroblasts or HT29 colorectal cancer cells. Contraction over 72 hours was recorded photographically and concomitant reduction in gel weight measured with control gels set at 100%. ET-1 caused gel contraction in the three fibroblast strains investigated, reducing the relative gel weight by approximately 40% i.e., 61.25% of the control tissues. (Figure 4). The addition of either ET\(_A\) or ET\(_B\), antagonists inhibited ET-1-induced contraction to varying degrees and in the following order: BQ123>BQ788>zibotentan. Combined ET\(_A\) and ET\(_B\) receptor antagonism (zibotentan plus BQ788) resulted in the most marked blockade of gel contraction, returning the ET-1-induced reduced gel weights to 89.71% of untreated controls. Addition of antagonists alone did not produce a noticeable effect (results not shown). Colorectal cancer cells did not cause gel contraction.

**Pharmacological characteristics of zibotentan in colorectal tissues, colorectal cancer cells and colonic fibroblasts**

Binding of \(^{125}\text{I}-\text{ET-1}\), in the presence or absence of receptor antagonists, was demonstrated by autoradiography in tissue sections and homogenates from patient specimens of colorectal cancer and normal bowel tissue; and also in cytospins of colorectal cancer cells or colonic fibroblasts.

**Localisation and distribution of ET-1 binding:** In high resolution autoradiographs of frozen tissue sections \(^{125}\text{I}-\text{ET-1}\) exhibited intense binding which was evident as white grains when viewed under dark field illumination; this correlated mostly with stromal regions as defined by H&E staining (Figure 5A). To identify associated structures, consecutive sections were stained immunohistochemically for: ET\(_A\) and ET\(_B\) receptors; stromal fibroblasts (Thy-1); endothelial cells (CD31) and collagen type XI (connective tissue) (Figure 5B). Normal colon tissue had preserved structural architecture with a well defined epithelial mucosal layer whereas that of tumours was disorganised. In normal colon specimens, microautoradiography revealed \(^{125}\text{I}-\text{ET-1}\)binding in the epithelial mucosa, submucosa and specific areas in the stroma; there was high ET\(_A\) and ET\(_B\) receptor immunostaining at regions of \(^{125}\text{I}-\text{ET-1}\)binding. \(^{125}\text{I}-\text{ET-1}\) bound strongly to regions that stained positively for endothelial cells and fibroblasts. In tumour tissues, both ET\(_A\) and ET\(_B\) receptors were present (ET\(_A\)>ET\(_B\)) and localised to areas of \(^{125}\text{I}-\text{ET-1}\) binding. Intense \(^{125}\text{I}-\text{ET-1}\)binding once
again correlated with both fibroblast and endothelial cell staining. Collagen type XI (Col XI), which was used to further define tumour stroma, was only present in tumour sections and not normal tissue, confirming previous reports of its association with colorectal pathology [20,21].

**Receptor subtype distribution:** To study ET\(_{A}\) and ET\(_{B}\) receptor distribution within normal and tumour sections, we inhibited \(^{125}\text{I}-\text{ET-1}\) binding using specific receptor antagonists, thereby demonstrating indirectly the presence of ET\(_{A}\) and ET\(_{B}\) receptors (Figure 6). The ET\(_{A}\) receptor antagonist, BQ123, in both normal and tumour sections, demonstrated a concentration dependent inhibitory effect with 25\(\mu\)M reducing \(^{125}\text{I}-\text{ET-1}\) binding to a greater degree than 5\(\mu\)M. Furthermore, the extent of inhibition was different between normal and tumour sections: most \(^{125}\text{I}-\text{ET-1}\) inhibition was observed in tumour sections, consistent with a higher ET\(_{A}\) receptor density in tumours compared to normal tissue. The ET\(_{B}\) receptor antagonist BQ788 also demonstrated a concentration dependent inhibition in both normal and tumour (25\(\mu\)M>5\(\mu\)M). Greater inhibition of ET\(_{B}\) receptor binding in normal colonic tissue suggests a higher ET\(_{B}\) receptor expression in healthy tissue than cancer. An interesting observation is the receptor distribution in normal mucosa where ET\(_{A}\) receptors appear localized closer to the luminal surface and ET\(_{B}\) receptors closer to the basal region. The extent of zibotentan concentration-dependent inhibition was not as great as either BQ123 or BQ788. On densitometry analysis there was more extensive inhibition observed in tumours than normal colonic tissue specimens, again consistent with a higher concentration of ET\(_{A}\) receptors in cancer. ET\(_{A}\) receptor overexpression in cancer tissues was corroborated by immunofluorescent detection of QD-conjugated BQ123 in patient specimens. Binding to ET\(_{A}\) receptors presented as a punctate pattern evident mostly in stromal areas surrounding epithelial glands (Figure 7A & 7B).

**Binding characteristics:** To examine the general binding characteristics of \(^{125}\text{I}-\text{ET-1}\) in normal and tumour sections we used tissue homogenates (not shown). The K\(d\) demonstrates that binding affinity of \(^{125}\text{I}-\text{ET-1}\) was similar in both the normal and tumour specimens (203pM and 204pM). Maximum binding (B\(\text{max}\)) was also similar in both normal and tumour samples (57.83fmol/mg and 58.31fmol/mg respectively -mg of tissue protein). Overall \(^{125}\text{I}-\text{ET-1}\) receptor binding to cytopsins of cancer cells and fibroblasts is shown in Figure 7C, with calculations of K\(d\) and B\(\text{max}\) in Figure 7D & 7E. The fibroblasts' combined K\(d\) value of 213.6pM (0.213nM) is regarded as high affinity (near 1nM or less = high affinity; 1\(\mu\)M or more = low affinity). Fibroblasts also demonstrated a relatively high maximal \(^{125}\text{I}-\text{ET-1}\)-receptor binding (3.03fmol/1x10^6 cells). The K\(d\) and B\(\text{max}\) values for all fibroblasts were
similar with only one strain (CF56) demonstrating lower values. The CRC cell lines (HT29, SW480) had a combined Bmax and Kd value of 2.43fmol/1x10^6 cells and 0.367nM respectively. Both cell lines had a similar Bmax to the fibroblasts with an ^125^I-ET-1 binding affinity which was marginally less (average HT29 & SW480: Bmax: 2.435fmol/1x10^6 cells; Kd: 0.367nM; average fibroblasts: Bmax: 3.03 fmol/1x10^5 cells; Kd: 0.213 nM. For receptor antagonist inhibition, the IC_{50} was determined using two methods, the first measuring radioactivity bound (CPM) in a gamma counter and the second through exposure to radiation-sensitive film and densitometric analysis, with readings categorised into high IC50 (<10μM), medium (10 – 100 μM) and low (>100 μM). Both methods gave similar IC_{50} results for fibroblasts (BQ123: 0.1-2.2μM; zibotentan: 10-15.1 μM & BQ788: 0.96-1mM). The IC_{50} of colorectal cancer lines also indicated that ETA receptor antagonists more effectively inhibited ^125^I-ET-1 binding (BQ123: 4.43- 10μM; zibotentan: 0.1-1.01μM & BQ788: 0.013-1mM).

DISCUSSION

The efficacy of the ETA receptor antagonist zibotentan against ET-1-driven proliferation, migration and contraction was investigated in a panel of colonic fibroblasts and colorectal cancer cell lines.

All ET-1-treated fibroblast strains demonstrated significant growth compared to controls. Previous work on colonic fibroblasts from our group reported only one of six strains showing ET-1-stimulated growth increase [10]. This discrepancy is explainable by (a) the inclusion of a 24-hour starvation period prior to ET-1 addition and that (b) here we only used two of the previously tested fibroblast strains. The only other study using colonic fibroblasts reported small non-significant increases in cell numbers in response to ET-1 [16]. On the other hand, Moraitis et al showed that ET-1 stimulated growth significantly in three fibroblast lines isolated from patients with ovarian cancers [22]. Differences may be explained both by variations in protocols and also by the nature of the specific fibroblasts: for example, ours were isolated from individual adult colons, while Kernochan et al employed a cell line (No Co18) isolated from the gastrointestinal tract of a 2.5 month old [16].

Fibroblast growth in the presence of exogenous ET-1 was reduced significantly by ETA receptor antagonists zibotentan and BQ123, with zibotentan as effective as BQ123, but not the ETB receptor antagonist BQ788, findings expanding on our previous work [10]. These findings are consistent with ETA as the major receptor responsible for propagating growth signals reported in a variety of cell types, including fibroblasts. Interestingly, in our series there was some response variation within the fibroblast strains to BQ788 antagonism: For
strain CF65, BQ788 antagonism resulted in a significant decrease in ET-driven cell growth. This is not obvious in the graphical representation (figure 2A) since the results were pooled from experiments conducted in five fibroblast strains. Conversely, in ovarian cancer-associated fibroblasts, ET-1-induced proliferation was inhibited by BQ123 and BQ788, suggesting that in the described model both ET receptors contribute to growth signalling [22]. Successful inhibition of proliferation by combined ET\textsubscript{A} and ET\textsubscript{B} receptor antagonism has been previously reported in lung myofibroblasts [23].

Treatment by receptor antagonists alone, especially ET\textsubscript{A} receptor antagonists zibotentan and BQ123, did not result in significant differences compared to controls. However, use of the ET\textsubscript{B} receptor antagonist BQ788 alone appeared to produce a trend towards increased proliferation in the fibroblast strains. The response of the five strains to BQ788 growth stimulation was mixed: in strain CF35 BQ788-stimulated increase in growth reached significance at p<0.05. In another strain, it just missed significance; while in the remaining three fibroblast strains there was minimal response to BQ788 stimulation. Explanations for the observed response centre around the availability of endogenous ET-1 after ET\textsubscript{B} receptor blockade. ET-1 produced by the fibroblasts themselves does not generally appear to constitute a major growth signal, since ET\textsubscript{A} receptor antagonism alone does not result in growth reduction. However, the ET\textsubscript{B} receptor is part of the ET-1 clearance pathway and therefore blockade by BQ788 would result in more endogenous ET-1 accumulating in the cell environment. The peptide would bind to ET\textsubscript{A} receptor and therefore contribute in a minor way to the proliferation signal. Differences in responses by individual fibroblast strains would be further determined by the original amounts of endogenous ET-1 produced by each individual strain. Although not measured in the fibroblast strains studied here, we have previously reported varied endogenous ET-1 production by different cell lines [19]. The present findings, considered in the wider context of fibroblast studies, demonstrate both strong overall patterns of colonic fibroblast response but also the importance of individual-specific differences. In the future such individual-specific data could inform personalised treatment.

ET-1-stimulated proliferation of colorectal cancer lines (HT29, SW620) was consistent with previous reports which also include similar results from LIM1215 [9,14]. Growth rates were reduced significantly by zibotentan and BQ123, but not BQ788; with zibotentan more effective than BQ123. Generally the majority of cancer cell types, e.g., prostate, ovarian, colorectal, show a reduction of ET-1-stimulated growth in response to ET\textsubscript{A} antagonism; the proliferative signal may be promoted further through EGF receptor transactivation [4,9,24].
The striking exception is melanoma, where ET-1 drives growth via ET$_B$ receptor [25]. Zibotentan inhibition of ET-1-induced growth was reported in ovarian cancer lines, with a reduction in ET$_A$-mediated angiogenesis and invasive mediators (VEGF, MMPs) [24,26]. Zibotentan also enhanced the apoptotic activity of the chemotherapeutic agent, Paclitaxel, in an in vitro and in vivo model of ovarian cancer [24], indicating a possible role for combination therapy.

ET-1-stimulated migration was clearly demonstrated in fibroblasts, but not cancer cells. It was inhibited by all three antagonists and most markedly by BQ788, consistent with previous findings [10,16]. Comparing ET$_A$ receptor antagonists, zibotentan produced a similar inhibitory effect on migration to BQ123. Combined ET$_A$ and ET$_B$ receptor blockade (zibotentan plus BQ788) produced a migratory effect in between that produced by ET$_B$ blockade and ET$_A$ blockade. Fibroblast migration and contraction due to various stimuli are well documented. For example, Shi-Wen et al demonstrated that on ET-1 exposure, normal lung fibroblasts up-regulated contractile proteins which promoted myofibroblast contraction and migration [27]. Fibroblasts from patients with scleroderma express high ET-1 levels suggesting a contributory role in connective tissue diseases.

Gel contraction was similarly a fibroblast-specific response and both ET receptors needed to be blocked for maximum inhibition, although the contractile response was not completely abolished. ET-1-stimulated contraction appears to be associated with an increase in cytosolic calcium and myosin phosphorylation and BQ123 and BQ788 were previously shown to inhibit this effect to various degrees [10,16]. In other tissues, ET-1 caused primary lung fibroblast contraction, although this response was mediated by ET$_A$ predominantly, rather than both receptors [27]. Although zibotentan was able to inhibit ET-1-mediated fibroblast contraction, it did so to a lesser degree than either BQ123 or BQ788.

To investigate zibotentan efficacy against $^{125}$I-ET-1 binding we carried out studies using specimens from patients with colorectal cancer. Autoradiography demonstrated $^{125}$I-ET-1 binding sites (ET$_A$ and ET$_B$ receptors) within normal colon and cancer tissues. Maximal binding was seen in stromal regions, which are densely populated by fibroblasts and blood vessels/endothelial cells, as demonstrated by immunohistochemistry (AS02, CD31). Positive collagen XI staining confirmed the pathological state of the stroma associated with cancer lesions [20]. This correlates with our previous work on human colon specimens which showed that radiolabelled ET-1, ET$_A$ receptor antagonist and ET$_B$ receptor agonist bound in a similar pattern within cancer and normal tissue [5,14]. Other groups have also reported strong stromal binding of $^{125}$I ET-1 in intestinal tissues [28,29]. Tissue homogenates were
used to determine binding characteristics. The $K_d$ and $B_{\text{max}}$ of $^{125}\text{I}-\text{ET-1}$ were similar in both normal and tumour homogenates. These figures are closely matched to previously published data regarding the characteristics of ET-1 binding in other tissues [30,31,32].

We previously demonstrated a change in the ratio of receptor subtypes, with up-regulation of $\text{ET}_A$ receptors and down-regulation of $\text{ET}_B$ receptors in colorectal cancer compared to normal colon [5,19]. In the present series we inhibited $^{125}\text{I}$- ET-1 binding with unlabelled receptor antagonists. BQ123 demonstrated a concentration dependent inhibition of $^{125}\text{I}$-ET-1 binding in both normal and tumour specimens (25$\mu$M>5$\mu$M). The most striking inhibition was observed in the tumour sections, consistent with a higher $\text{ET}_A$ receptor expression in cancer compared to normal colon, supporting previous findings: Specifically, Hoosein et al [5] used the selective radiolabelled $\text{ET}_A$ receptor antagonist $[^{125}\text{I}]\text{PD-151242}$ and demonstrated that binding on cancer tissues was increased by 55.5% when compared to normal colon. The $\text{ET}_B$ receptor antagonist BQ788 also inhibited binding in a concentration-dependent manner (25$\mu$M>5$\mu$M). The greater inhibition of binding to $\text{ET}_B$ receptors observed in normal colon than within tumour is consistent with higher $\text{ET}_B$ receptor expression in normal colon and a down-regulation in colorectal cancer [5,19]. In the latter study the specific $\text{ET}_B$ receptor agonist, $[^{125}\text{I}]\text{BQ3020}$, demonstrated a 45% decrease in $\text{ET}_B$ receptor expression in cancer compared to normal. However, this is contrary to findings by Egidy and colleagues [29] who described a quantitative increase in mRNA expression of both $\text{ET}_A$ and $\text{ET}_B$ receptors in colorectal cancer specimens. However, increases at the mRNA level are not necessarily followed by translation at the protein level. Zibotentan did not display a concentration-dependent inhibition to the extent observed with BQ123 and BQ788. There was overall greater inhibition observed in tumour specimens than normal tissues, in keeping with the over-expression of $\text{ET}_A$ receptors in cancer. The extent of inhibition of $^{125}\text{I}$- ET-1 binding may not have been as expected as zibotentan is a specific $\text{ET}_A$ receptor antagonist whilst BQ123 and BQ788 at higher concentrations are known to act on both receptor subtypes (lose selectivity) [33]. Therefore, use of these laboratory compounds may not be truly subtype-selective at high concentrations.

$^{125}\text{I}$-ET-1 binding to specific structures was further clarified by immunohistochemical localisation of $\text{ET}_A$ and $\text{ET}_B$ receptors, in addition to fibroblast, endothelial cell and collagen XI mapping. $\text{ET}_A$ and $\text{ET}_B$ immunostaining closely correlated to $^{125}\text{I}$-ET-1 binding in parallel tissue sections. Cancer tissues demonstrated increased $\text{ET}_A$ receptor staining localised to epithelial cells and tumour stroma, and reduced $\text{ET}_B$ receptor staining on the epithelial cell surface, compared to normal tissues. Similar alterations in receptor expression have been reported in autoradiographic studies on prostate and ovarian cancer [3,4]. $^{125}\text{I}$-ET-1 binding
to vascular regions, demonstrated here by co-localization with ET$_B$ expressing endothelial cells and its known association with ET$_A$ expressing vascular smooth muscle cells [30,31], highlights ET-1 angiogenic actions, such as VEGF production [34]. $^{125}$I-ET-1 also bound strongly to fibroblasts, which in cancer tissues express predominantly ET$_A$ receptors and often produce collagen type XI. Interestingly, ET-1 affects the APC/β-catenin and Wnt signalling which is the pathway involved in regulating COLXI and CTFG expression, molecules with tumourogenic associations [35].

Within normal mucosa, ET$_A$ receptors appear closer to the luminal surface with ET$_B$ receptors towards the muscularis mucosa and lamina propria (Figure 6), a distribution pattern not previously reported. This may reflect trophic signalling roles for ET receptors along the basal to apical direction of cell turnover and differentiation. Similar functions have been postulated for retinoic acid receptors and vitamin D receptors, which are denser at the apex; and ER$_\alpha$ found predominantly in basal regions [36]. This pattern is consistent with ET$_B$ associated with growth arrest signalling and ET$_A$ associated with differentiation signalling.

The combined fibroblasts Kd value (0.213nM) is regarded as high affinity (≤1nM) with the cells demonstrating a relatively high maximal $^{125}$I-ET-1 binding (3.03fmol/1x10$^6$ cells). The colorectal cancer lines exhibited a similar Bmax (2.43fmol/1x10$^6$ cells) and affinity that was marginally less than the fibroblasts (Kd: 0.367nM), suggesting that fibroblasts have more ET surface receptors than cancer cells; this is supported by our high resolution autoradiography (Figure 7E). Fibroblasts also had a higher binding affinity than cancer cells. These figures were similar to previous studies using human colonic mucosa and human skin fibroblasts with Kd values of 0.41nM and 0.4nM respectively [28,37,38]. This is the first time that individual cellular components of colonic tissue have been evaluated for ET-1 binding characteristics with our overall results suggesting a higher binding density/Bmax within fibroblasts.

Competition studies were performed using both radioactivity measurements and film densitometry. Both methods gave similar IC$_{50}$ results for fibroblasts (BQ123: high; zibotentan: medium; BQ788: low range). Only small sample numbers were used and, overall, the ET$_A$ receptor antagonists were much more effective at inhibiting $^{125}$I-ET-1 binding than ET$_B$ receptor antagonists. The ET$_A$ receptor antagonist BQ123 was ~10,000 fold more effective than the ET$_B$ receptor antagonist BQ788 while the orally active drug, zibotentan, was more effective at inhibiting $^{125}$I-ET-1 binding than BQ788 by ~1000 fold although ~1000 fold less effective than BQ123. This difference between ET$_A$ receptor affinity may once
again be due to a loss of BQ123 receptor selectivity when used at higher concentrations. Colorectal cancer cells exhibited IC$_{50}$ values similar to that of fibroblasts (zibotentan: high; BQ123: medium; BQ788: low range) with ET$_A$ receptor antagonists more effective at inhibiting $^{125}$I-ET-1 than the ET$_B$ receptor antagonist (BQ123 ~1,000 fold > BQ788). BQ788 IC$_{50}$ results were the same as that seen in fibroblasts, suggesting they may have similar universal functions such as an involvement in the ET-1 ‘clearance pathway’. When comparing the two ET$_A$ receptor antagonists, zibotentan was the most effective at inhibiting $^{125}$I-ET-1 binding in cancer cells.

In summary we demonstrated that ET-1 stimulates behaviour consistent with tumourigenesis in colorectal cancer cells and in colonic fibroblasts. Many of the actions are via the ET$_A$ receptor with various levels of contribution of the ET$_B$ receptor and this strengthens the evidence for the potential therapeutic use of zibotentan. Importantly ET-1 appears to drive colorectal tumourigenesis primarily via the stroma and relevant anti-ET treatment would provide a shift in treatment paradigm.
REFERENCES


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Figure legends:

**Figure 1.** Structures of (A) ET-1 peptide, (B) ET<sub>A</sub> receptors BQ123 and ZD4054, and (C) ET<sub>B</sub> receptor antagonist BQ788.

**Figure 2.** Growth response of (A) fibroblast strains and (B) colorectal cancer cell lines to ET-1 (10<sup>-7</sup>M for fibroblasts; 10<sup>-8</sup>M for colorectal cancer cells) and/or receptor antagonists (anti-ET<sub>A</sub> receptor: BQ123, zibotentan, anti-ET<sub>B</sub> receptor: BQ788; 10<sup>-6</sup>M for fibroblasts; 10<sup>-7</sup>M for colorectal cancer cells). Cell number (equivalent to absorbance at 650nm) was compared to controls (set at 100%). ET<sub>A</sub> receptor antagonists zibotentan and BQ123 significantly reduced growth in the presence (*p<0.05) of exogenous ET-1. ET<sub>B</sub> antagonist, BQ788, partially reduced ET-1 stimulated growth (NS). Combined results for 5 fibroblast strains (n=6 independent repeats for each strain) and 2 colorectal cancer cell lines (HT29, SW620, n=6 independent repeats for each line). Statistical analysis of original absorbance measurements using 1-way ANOVA with Tukey’s HSD post-hoc test. (** significant ET-1 growth v control).

**Figure 3.** Migration response of fibroblast strains to ET-1 (10<sup>-7</sup>M) or ET-1 plus receptor antagonists (zibotentan, BQ123, BQ788; 10<sup>-6</sup>M): (A) Migration images; (B) Migration as % of ET-1 induced migration (set at 100%); (C) Reduction of ET-1-induced migration. There is total migration of fibroblasts after 24 hours in wells exposed to ET-1 only (100% y-axis). This was significantly reduced by both ET<sub>A</sub> receptor antagonists zibotentan and BQ123, with an average migration of 50% and 51% respectively (*p<0.05 v ET-1). ET<sub>B</sub> receptor antagonist BQ788 produced the greatest inhibitory effect with a resultant average migration of 29% (*p<0.05 v ET-1). Combined ET<sub>A</sub> and ET<sub>B</sub> receptor blockade produced an in-between effect of that seen for ET<sub>A</sub> and ET<sub>B</sub> individually (p<0.05). Combined results for 4 fibroblast strains (n=6 independent repeats for each strain). Statistical analysis on original migration measurements, using 1-way ANOVA with Tukey’s HSD post-hoc test. (** significant ET-1 v control).
**Figure 4.** Contraction of fibroblast gels in response to ET-1 (10^{-7} M) or ET-1 plus receptor antagonists (zibotentan, BQ123, BQ788; 10^{-6} M): (A) Images of gel contraction. (B) and (C) Graph and table with weight change equivalent to contraction, as % of control (set at 100%). Relative weight changes compared to controls (100% weight) were calculated for each strain and the data combined (y-axis). ET-1 stimulated a 38% reduction in gel weight (p<0.05). This effect was blocked significantly by both ET_A receptor antagonists (BQ123 and zibotentan), and ET_B receptor antagonist BQ788 (p<0.05*). The effect is most marked with combined ET_A and ET_B blockade (p<0.05*). Combined results for 3 fibroblast strains (n=3 independent repeats for each strain). Statistical analysis on original contraction weight measurements, using 1-way ANOVA with Tukey’s HSD post-hoc test. (** significant ET-1 v control).

**Figure 5.** Microscopic localisation of ^{125}I-ET-1 in patient sections (A) showing dark field illumination of radiolabelled ^{125}I-ET-1 binding (left) and H&E staining of underlying sections (right). Binding is predominantly seen on connective tissues within both normal colon and tumour stroma. (B) IHC was used to identify cell type specific staining to regions of ^{125}I-ET-1 bound to normal (top panels) and tumour (bottom sections) tissue as visualised in low resolution autoradiography film. Thy-1 (fibroblast) had similar distribution to stromal ^{125}I-ET-1 bound regions in both normal and tumour sections. CD31 endothelial cell staining (tumour > normal) localised to ^{125}I-ET-1 dense regions. ET_A staining (tumour>normal) and ET_B staining (normal = tumour) also correlated with ^{125}I-ET-1 binding. Tumour specific collagen type XI was only seen in tumour stroma.

**Figure 6.** Receptor selective antagonists (BQ123, BQ788 or zibotentan; high conc. 25μM; low conc. 5μM) ability to inhibit ^{125}I-ET-1 (150pM) binding to normal (left) and tumour (right) slide-mounted tissue from patients. ^{125}I-ET-1 total binding in the absence of antagonist. Low resolution autoradiographs were produced by opposing the slide mounted tissues to
Hyperfilm™. Inhibition of $^{125}\text{I}}$-ET-1 binding was greatest with ET\textsubscript{A} receptor antagonists (BQ123> zibotentan) within tumour tissue. The ET\textsubscript{B} receptor antagonist (BQ788) had a greater effect reducing $^{125}\text{I}}$-ET-1 binding in normal tissue.

**Figure 7.** Localisation of Quantum Dot - BQ-123 conjugates in patient sections from normal (A) and tumour (B) specimens. Frozen sections were incubated with QD-BQ123 overnight (4°C) and visualised by confocal microscopy. Punctate yellow-orange QD-BQ123 binding was more abundant in tumour versus normal sections. Green tissue autofluorescence is present. Bar=50 μm. (C) Images generated by exposure of cytospins to film. Cytospun cells were incubated with increasing concentrations of $^{125}\text{I}}$-ET-1 (3x10\textsuperscript{-12} to 1x10\textsuperscript{-9} M) to determine total binding and in the presence of unlabelled ET-1 (1μM) to establish non-specific binding. (D) Cells were removed with NaOH and total and non-specific $^{125}\text{I}}$-ET-1 activity quantified by gamma counter. Graphs of specific binding were produced by subtracting non-specific from total $^{125}\text{I}}$-ET-1 binding. Prism™ was used to calculate fibroblast and cancer cell line $B_{\text{max}}$ (mean: 3.03fMol/1x10\textsuperscript{6}cells; 2.43fMol/1x10\textsuperscript{6} cells respectively) and $K_{d}$ (mean: 213.6pM [0.214nM]; 367.0pM [0.367nM] respectively). (E) Demonstrating specific radiolabelled $^{125}\text{I}}$-ET-1 (100pM) localisation to colorectal fibroblasts (top) and cancer cell lines (bottom). Total $^{125}\text{I}}$-ET-1 binding (left panels) and non-specific (right panels) demonstrates specific binding to cells.
Figure 1: ETA and ETB receptor antagonists. A) ET-1 peptide. B) ETA receptor antagonists: BQ123, ZD4054. C) ETB receptor antagonist: BQ788.
Figure 2

Panel A: Fibroblast Proliferation

- Control
- ET1
- ET1 + ZD4054
- ET1 + BQ123
- ET1 + BQ788
- BQ788

Reagents: ET-1 (10^{-7}) and Antagonists (10^{-6})

- Relative cell number
- **
- *

Panel B: Cancer Cell Line Proliferation

- Control
- ET1
- ET1 + ZD4054
- ET1 + BQ123
- BQ123
- BQ788
- BQ788

Reagents: ET-1 (10^{-8} M) and Antagonists (10^{-7} M)

- Relative cell number
- **
- *

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**Figure 3**

### Combined Fibroblast Migration

<table>
<thead>
<tr>
<th>Reagents: ET-1 (10^{-7}M) &amp; Antagonists (10^{-6}M)</th>
<th>Percentage Migration (%)</th>
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<tr>
<td>Control</td>
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<tr>
<td>ET-1</td>
<td>58.76 p&lt;0.05</td>
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<tr>
<td>ET-1 + BQ788</td>
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<tr>
<td>ET-1 + ZD4054/BQ788</td>
<td>58.76 p&lt;0.05</td>
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### Antagonist

<table>
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<th>% Reduction of ET-1 Induced Migration set at 100%</th>
<th>% Decrease</th>
<th>Significance</th>
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<tbody>
<tr>
<td>ZD4054</td>
<td>46.88 p&lt;0.05</td>
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<tr>
<td>BQ788</td>
<td>58.76 p&lt;0.05</td>
<td></td>
<td>p&lt;0.05</td>
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</table>

---

*ET-1 induced migration set at 100%*
# Figure 4

## Fibroblast Contraction

### C

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<th>Antagonist</th>
<th>% Contraction of Control Size Gel*</th>
<th>Significance</th>
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<tr>
<td>ET-1</td>
<td>61.25%</td>
<td>P&lt;0.05**</td>
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<tr>
<td>ET-1 + ZD4054</td>
<td>70.87%</td>
<td>P&lt;0.05*</td>
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<td>ET-1 + BQ123</td>
<td>77.36%</td>
<td>P&lt;0.05*</td>
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<tr>
<td>ET-1 + BQ788</td>
<td>76.51%</td>
<td>P&lt;0.05*</td>
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<tr>
<td>ET-1 + ZD4054+BQ788</td>
<td>89.71%</td>
<td>P&lt;0.05*</td>
</tr>
</tbody>
</table>

* = contraction compared to control  
** = inhibition compared to ET-1 induced contraction

---

**Reagents:** ET-1 (10^7) and Antagonists (10^-6)
Figure 7

(A) Fibroblasts

(B) Cancer Cell Lines

(C) Specific Binding (Combined)

(D) Specific Binding (Combined)

(E) Non-Specific Binding
Molecular Cancer Therapeutics

Efficacy of the specific endothelin A receptor antagonist zibotentan (ZD4054) in colorectal cancer: a preclinical study

Samer-ul Haque, Michael R Dashwood, Mohammed Heetun, et al.

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