Efficacy of the Specific Endothelin A Receptor Antagonist Zibotentan (ZD4054) in Colorectal Cancer: A Preclinical Study

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Abstract
Endothelin 1 (ET-1) is overexpressed in cancer, contributing to disease progression. We previously showed that ET-1 stimulated proliferative, migratory, and contractile tumorigenic effects via the ETA receptor. Here, for the first time, we evaluate zibotentan, a specific ETA receptor antagonist, in the setting of colorectal cancer, in cellular models. Pharmacologic characteristics were further determined in patient tissues. Colorectal cancer lines (n = 4) and fibroblast strains (n = 6), isolated from uninvolved areas of colorectal cancer specimens, were exposed to ET-1 and/or ETA/B receptor antagonists. Proliferation (methylene blue), migration (scratch wounds), and contraction (gel lattices) were assessed. Receptor distribution and binding characteristics (Kd, Bmax) were determined using autoradiography on tissue sections and homogenates and cytosol cell samples, supported by immunohistochemistry. Proliferation was inhibited by ETA antagonist (zibotentan > BQ123; P < 0.05), migration by ETB > ETA, and contraction by combined ETA and ETB antagonism. Intense ET-1 stromal binding correlated with fibroblasts and endothelial cells. Colorectal cancer lines and fibroblasts revealed high density and affinity ET-1 binding (Bmax = 2.435 fmol/1 x 10^6 cells, Kd = 367.7 pmol/L; Bmax = 3.03 fmol/1 x 10^6 cells, Kd = 213.6 pmol/L). In cancer tissues, ETA receptor antagonists (zibotentan, BQ123) reduced ET-1 binding more effectively (IC50: 0.1–10 μmol/L) than ETB receptor antagonist BQ788 (~IC50, 1 mmol/L). ET-1 stimulated cancer-contributory processes. Its localization to tumor stroma, with greatest binding/affinity to fibroblasts, implicates these cells in tumor progression. ETA receptor upregulation in cancer tissues and its role in tumorigenic processes show the receptor’s importance in therapeutic targeting. Zibotentan, the most specific ETA receptor antagonist available, showed the greatest inhibition of ET-1 binding. With its known safety profile, we provide evidence for zibotentan’s potential role as adjuvant therapy in colorectal cancer.

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Introduction
Colorectal cancer is one of the most common 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 (Fig. 1A; ref. 2). ET-1 effects are mediated by two distinct G protein-coupled receptors, ETA and ETB. Receptor binding and signaling often causes opposite effects on proliferation: in the majority of cells, binding to ETA promotes growth, whereas binding to ETB leads to apoptosis. Receptor expression is altered in a number of solid cancers, with ETA reported as upregulated, and ETB, either up- or downregulated, using various techniques. Using autoradiography, our previous results were similar to those reported in ovarian and prostate cancers (3, 4), in that ETA receptors were overexpressed, whereas ETB receptors were downregulated in cancer tissue compared with normal colon. Specifically, binding to the ETA receptor was greatest in cancer-associated fibroblasts and blood vessels and to a lesser extent in epithelial cancer cells. In contrast, ETB receptors were the predominant receptors in normal colon and were markedly downregulated in all cell types within cancer (5).

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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 tumor 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 ETA provided a growth stimulus to colorectal cancer cells; the signal was also propagated via the trans-activation of the EGF receptor, when this receptor was present (9). Furthermore, colonic fibroblasts, when exposed to ET-1, exhibited increased growth, migration, and contraction (10). As 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_\text{A} receptor blockade has been proposed as a potential target for anticancer therapy; however, despite encouraging preclinical 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) have been disappointing. To date, there are no data for the ET_\text{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_\text{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 with the standard laboratory ET receptor antagonists BQ123 (ET_\text{A} selective) and BQ788 (ET_\text{B} selective; Fig. 1). Furthermore, pharmacologic characteristics of zibotentan were determined in normal and cancer tissues and cells from patient specimens taken during surgery for colorectal cancer.

Materials and Methods

**Biologic 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); 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 immortalized at the Ludwig Institute for Cancer Research (HNPCC familial cancer; donated by Professor M O’Hare, Ludwig Institute for Cancer Research, London, United Kingdom). 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 (β-actin positive) were grown and used (passages 4–12; untransformed cell strains; ref. 10). Cells were discarded thereafter due to loss of the fibroblastoid phenotype (13). The strains were labeled 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 less 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 supplemented with 10% fetal calf serum and gentamycin (50 μg/mL). For cytospins, cells (2 × 10^6 for gross autoradiography and 0.5 × 10^6 for microautoradiography) were loaded onto polylysine-coated slides, centrifuged, air dried, and stored (−70°C; Shandon Cytospin3, WolfLabs Inc).

**Tissues.** Specimens were obtained from patients (n = 9) undergoing colorectal cancer resection; adjacent normal and cancer samples were snap frozen, stored (liquid N2), and used for: (i) 5 μm frozen sections on polylysine-coated slides (Leica CM3050 cryostat, Milton Keynes); (ii) Homogenates: tissues were placed into a ball-bearing cage, dipped in liquid N2, and homogenized (2 minutes, Mikro-Dismembranator U, Braun Biotech). The resulting powder homogenates were diluted in molecular grade water (2 mL) and stored (−70°C; Shandon Cytospin3, WolfLabs Inc).

**Zibotentan ETₐ receptor antagonism—in vitro studies**

**Agents.** For colorectal cancer line studies, ET-1 was used at 10⁻⁶ mol/L; specific ETₐ receptor antagonists: zibotentan (AstraZeneca) and BQ123 (Bachem Ltd) and specific ETₐ receptor antagonist BQ788 (Bachem) were used at 10⁻⁷ mol/L. For fibroblast studies, ET-1 was used at 10⁻⁷ mol/L; zibotentan, BQ123, and BQ788 at 10⁻⁶ mol/L (Fig. 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 36 hours (60%–70% confluence) and starved in serum-free medium (24 hours). This was replaced by serum-free medium containing ET-1 and/or ETₐ/ETₐ receptor antagonists (zibotentan, BQ123, BQ788). Plates were incubated (48 hours), fixed (10% formal saline), and proliferation measured by the methylene blue assay (15), with absorbance 630 nm (Anthos-2010 platereader, Biochrom Ltd) 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 center of the well. Medium was replaced with serum-free medium (with mitomycin C to inhibit proliferation—1 μg/mL for fibroblasts; 0.5 μg/mL for HT29, SW620) and ET-1 ± ET receptor antagonists (zibotentan, BQ123, BQ788). Photographs were taken every 6 hours (0–24 hours). Means of 3 scratch-width measurements per well at fixed positions were used to calculate migration as the percentage of reduction in width compared with 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; 1 hour) and then “coated” by the addition of 1 mL serum-free ET-1. Control gels were floated by serum-free medium. Gels were further incubated (72 hours) and fixed in 10% formaldehyde. For imaging, 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 analyzed using one-way ANOVA [post hoc Tukey honestly significant difference (HSD), P < 0.05]. For growth, six independent repeats were conducted for each fibroblast strain (n = 5) and each cancer line (n = 2). For migration, six independent repeats were conducted for each fibroblast strain (n = 4) and each cancer line (n = 2). Collagen gel contraction experiments were carried out 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 datasets to 100% to facilitate presentation/discussion).

**Zibotentan ETₐ receptor binding characteristics**

**Saturation analysis: Kd/Bmax determination.** Receptor binding studies were conducted as described previously (19): cytospins and tissue homogenates were incubated in increasing ¹²⁵I-ET-1 concentrations (GE, specific activity 2,200 Ci/mmol; 3 × 10⁻¹²–10⁻⁹ mol/L) with nonspecific binding established in the presence of unlabeled ET-1 (10⁻⁹ mol/L). After incubation; slides were post-fixed in paraformaldehdy vapor; and homogenates filtered and washed (3 ×). ¹²⁵I standards were prepared where 50 μL aliquots of each of the serial dilutions of radioligand used were spotted onto filter paper and attached to microscope slides that were coexposed to radio-sensitive film alongside the cytospins.

**Inhibition analysis.** Antagonist-binding affinities were assessed by inhibition studies: cytospins/tissues were incubated in a fixed ¹²⁵I-ET-1 concentration (150 pmol/L Kd) in the presence of increasing concentrations (3 × 10⁻³–3 × 10⁻¹⁰ mol/L) of the receptor antagonists—zibotentan, BQ123, BQ788. Two fixed concentrations (high = 25 μmol/L; low = 5 μmol/L) for each antagonist were used for autoradiography as described below.

**Autoradiography.** Low resolution. Slide-mounted tissues and cytospins were apposed to Hyperfilm MP (GE) in X-ray cassettes and exposed for 7 to 21 days (4°C), after which films were processed following manufacturer’s instructions and used for densitometry; representative images were photographed.

**High resolution.** Selected slides were used for microscopic localization 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 hematoxylin and eosin (H&E), viewed (Olympus BX50 microscope), photographed (Zeiss Axiocam digital camera), and images stored (KS400 system, Imaging Associates). Additional to densitometry, radioligand binding was also assessed using a gamma counter (Perkin Elmer) where, after film images of cytospins had been generated for densitometry, cells were digested (100 μL 4 mol/L NaOH; ~10 minutes) and radioactivity (counts per minute; CPM) measured.

**Calculation of binding characteristics.** Densitometric image analysis was conducted on a Biospectrum AC Imaging System (UltraViolet Products) and analyzed using VisionWorksLS Imaging software (version 6.4.3. UVP, 2007). Specific $^{125}$I-ET-1 binding was determined by subtracting nonspecific from total binding at each concentration. Maximum receptor binding ($B_{max}$) and affinity ($K_d$) were obtained using GraphPad Prism software (GraphPad). Radioactivity counts (gamma counter; CPM) from cells removed from slides were similarly analyzed.

**Immunohistochemistry.** Immunohistochemistry was conducted using the Vectastain Alkaline Phosphatase Kit (Vector Labs) 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), Col11A1 for collagen Type XI (Santa Cruz Biotech, Inc; 1:200 in PBS; 30 minutes), followed by universal secondary antibody (1:100 in normal horse serum/PBS; 10 minutes). Sections were counterstained (hematoxylin) and photographed as above. H&E staining was conducted on selected sections. ET$_A$ receptors were further imaged by fluorescence of quantum dot (QD)-coupled BQ123 receptor antagonist (600 nm emission). The water soluble QDs (manufactured in-house) were conjugated to BQ123 using the water soluble N-ethyl-N’-diaminopropyl-carbodimide (EDC) method: Briefly, 200 μL of QDs (1 mg/mL in borate buffer (pH 7.4), were mixed with EDC (1 mg/mL) for 30 minutes at room temperature. BQ123 (10$^{-4}$ mol/L) was then added to the above solution at approximately 100 μg/mL and mixed for 1 hour at room temperature. After this reaction procedure, BQ123-QDs and unconjugated QDs were separated using centrifugal filter (Millipore) with a cut-off value of 10 kDa 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), hematoxylin counterstained, and then observed under confocal microscopy.

**Results**

**Efficacy of zibotentan in in vitro models of colorectal cancer**

The ability of the specific ET$_A$ 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 ET$_A$ or ET$_B$ receptor antagonists (zibotentan, BQ123, BQ788), and all three receptor antagonists alone (48 hours). Exogenous ET-1 stimulated growth in all strains, from a minimum of 28% to a maximum of 46%, compared with unstimulated controls ($P < 0.05$; normalized results for all strains pooled in Fig. 2A). This effect was reduced by the specific ET$_A$ receptor antagonists, zibotentan and BQ123 ($P < 0.05$ vs. ET-1 group), but not the ET$_B$ receptor antagonist BQ788. Zibotentan had similar activity to that of the laboratory standard ET$_A$ receptor antagonist BQ123. Use of the antagonists alone did not

![Figure 2](image-url)
produce a significant effect, although ET<sub>B</sub> receptor blockade (BQ788) seemed to produce a trend toward 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 ($P < 0.05$ vs. control, Fig. 2B). ET-1–stimulated growth was significantly blocked by ET<sub>A</sub> (zibotentan, BQ123), but not by ET<sub>B</sub> 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 (Fig. 3). For fibroblasts, the migration pattern was similar in all four of the fibroblast strains, (one strain being unresponsive (CRF42)): By 18 to 24 hours, fibroblasts completely obscured the scratch wound in the presence of ET-1 (100%) compared with untreated (13%). ET-1–stimulated migration was inhibited by both ET<sub>A</sub> and ET<sub>B</sub> antagonists, to varying degrees: BQ788 = 71%; zibotentan and BQ123 = 50–51%. However, combined blockade of ET<sub>A</sub> and ET<sub>B</sub> receptors with zibotentan and BQ788 produced an effect that was less than that observed with ET<sub>B</sub> blockade, but greater than that seen with ET<sub>A</sub> 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 (24 hours).

**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. (Fig. 4). The addition of either ET<sub>A</sub> or ET<sub>B</sub> antagonists inhibited ET-1–induced contraction to varying degrees and in the following order: BQ123>BQ788>zibotentan. Combined ET<sub>A</sub> and ET<sub>B</sub> 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 significant effect.
noticeable effect (results not shown). Colorectal cancer cells did not cause gel contraction.

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

Binding of $^{125}$I-ET-1, in the presence or absence of receptor antagonists, was shown 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.

Localization and distribution of ET-1 binding. In high-resolution autoradiographs of frozen tissue sections, $^{125}$I-ET-1 exhibited intense binding that was evident as white grains when viewed under dark field illumination; this correlated mostly with stromal regions as defined by H&E staining (Fig. 5A). To identify associated structures, consecutive sections were stained immunohistochemically for: ETA and ETB receptors, stromal fibroblasts (Thy-1), endothelial cells (CD31), and collagen type XI (Col XI), which was used to further define tumor stroma, was only present in tumor sections and not normal tissue, confirming previous reports of its association with colorectal pathology (20, 21).

Receptor subtype distribution. To study ETA and ETB receptor distribution within normal and tumor sections, we inhibited $^{125}$I-ET-1 binding using specific receptor antagonists, thereby showing indirectly the presence of ETA and ETB receptors (Fig. 6). The ETA receptor antagonist, BQ123, in both normal and tumor sections, showed a concentration-dependent inhibition in both fibroblast and endothelial cell staining. Collagen type XI (Col XI), which was used to further define tumor stroma, was only present in tumor sections and not normal tissue, confirming previous reports of its association with colorectal pathology (20, 21).

Receptors: ET-1 ($10^{-7}$ mol/L) and antagonists ($10^{-6}$ mol/L)

<table>
<thead>
<tr>
<th>Reagents: ET-1 ($10^{-7}$ mol/L) and antagonists ($10^{-6}$ mol/L)</th>
<th>Fibroblast contraction</th>
<th>Relative weight change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ET-1</td>
<td>61.25</td>
<td>0.05</td>
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<tr>
<td>ET-1 + ZD4054</td>
<td>70.87</td>
<td>0.05</td>
</tr>
<tr>
<td>ET-1 + BQ123</td>
<td>77.36</td>
<td>0.05</td>
</tr>
<tr>
<td>ET-1 + BQ788</td>
<td>76.51</td>
<td>0.05</td>
</tr>
</tbody>
</table>

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

For each strain). Statistical analysis on original contraction weight measurements, using one-way ANOVA with Tukey HSD post hoc test. (*; significant ET-1 vs. control).

In tumor tissues, both ETA and ETB receptors were present (ETA > ETB) and localized to areas of $^{125}$I-ET-1 binding. Intense $^{125}$I-ET-1 binding once again correlated with both fibroblast and endothelial cell staining. Collagen type XI (Col XI), which was used to further define tumor stroma, was only present in tumor sections and not normal tissue, confirming previous reports of its association with colorectal pathology (20, 21).

Receptor subtype distribution. To study ETA and ETB receptor distribution within normal and tumor sections, we inhibited $^{125}$I-ET-1 binding using specific receptor antagonists, thereby showing indirectly the presence of ETA and ETB receptors (Fig. 6). The ETA receptor antagonist, BQ123, in both normal and tumor sections, showed a concentration-dependent inhibitory effect with 25 μmol/L reducing $^{125}$I-ET-1 binding to a greater degree than 5 μmol/L. Furthermore, the extent of inhibition was different between normal and tumor sections: most $^{125}$I-ET-1 inhibition was observed in tumor sections, consistent with a higher ETA receptor density in tumors compared with normal tissue. The ETB receptor antagonist BQ788 also showed a concentration-dependent inhibition in both normal and tumor (25 μmol/L > 5 μmol/L). Greater inhibition of ETB receptor binding in normal colonic tissue suggests a higher ETB receptor expression in healthy tissue than cancer. An interesting observation is the receptor distribution in normal mucosa where ETA receptors appear localized closer to the luminal surface and ETB 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 tumors than normal colonic tissue specimens, again consistent with a higher concentration of ETA receptors in cancer. ETA receptor overexpression in cancer tissues was corroborated by immunofluorescent detection of QD-conjugated BQ123 in patient specimens. Binding to ETA receptors presented as a punctate pattern evident mostly in stromal areas surrounding epithelial glands (Fig. 7A and B).

**Binding characteristics.** To examine the general binding characteristics of 125I-ET-1 in normal and tumor sections, we used tissue homogenates (not shown). The 125I-ET-1 receptor binding affinity (Kd) was similar in both normal and tumor sections (203 pmol/L and 204 pmol/L). Maximum binding (Bmax) was also similar in both normal and tumor samples (57.83 fmol/mg and 58.31 fmol/mg, respectively; mg of tissue protein). Overall 125I-ET-1 receptor binding to cytospins of cancer cells and fibroblasts is shown in Fig. 7C, with calculations of Kd and Bmax in Fig. 7D and E. The fibroblasts' combined Kd value of 213.6 pmol/L (0.213 nmol/L) is regarded as high affinity (near 1 nmol/L or less = high affinity; 1 μmol/L or more = low affinity). Fibroblasts also showed a relatively high maximal 125I-ET-1 receptor binding (3.03 fmol/1 x 10⁶ cells). The Kd and Bmax values for all fibroblasts were similar with only one strain (CF56) showing lower values. The colorectal cancer cell lines (HT29, SW480) had a combined Bmax and Kd value of 2.43 fmol/1 x 10⁶ cells and 0.367 nmol/L respectively. Both cell lines had a similar Bmax to the fibroblasts with an 125I-ET-1–binding affinity that was marginally less (average HT29 and SW480: Bmax, 2.435 fmol/1 x 10⁶ cells; Kd, 0.367 nmol/L; average fibroblasts: Bmax, 3.03 fmol/1 x 10⁶ cells; Kd, 0.213 nmol/L). For receptor antagonist inhibition, the IC₅₀ 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 categorized into high IC50 (<10 μmol/L), medium (10–100 μmol/L), and low (>100 μmol/L). Both methods gave similar IC50 results for fibroblasts (BQ123, 0.1–2.2 μmol/L; zibotentan, 10–15.1 μmol/L; and BQ788, 0.96–1 mmol/L). The IC50 of colorectal cancer lines also indicated that ETA receptor antagonists more effectively inhibited 125I-ET-1 binding (BQ123, 4.43–10 μmol/L; zibotentan, 0.1–1.01 μmol/L and BQ788, 0.013–1 mmol/L).

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 showed significant growth compared with 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 (i) the inclusion of a 24-hour starvation period before ET-1 addition and that (ii) here we only used two of the previously tested fibroblast strains. The only other study using colonic cancer lines also indicated that ETA receptor antagonists more effectively inhibited 125I-ET-1 binding (BQ123, 4.43–10 μmol/L; zibotentan, 0.1–1.01 μmol/L and BQ788, 0.013–1 mmol/L).

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 as effective as the ETB receptor antagonist BQ788, findings that expand 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 (Fig. 2A), as 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 signaling (22). Successful inhibition of proliferation by combined ETA and ETB receptor antagonism has been previously reported in lung myofibroblasts (23).

Treatment by receptor antagonists alone, especially ETA receptor antagonists zibotentan and BQ123, did not result in significant differences compared with controls. However, use of the ETB receptor antagonist BQ788 alone seemed 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, whereas in the remaining three fibroblast strains there was minimal response to BQ788 stimulation. Explanations for the observed response center around the availability of endogenous ET-1 after ETB receptor blockade. ET-1 produced by the fibroblasts themselves does not generally seem to constitute a major growth signal, as ETA receptor antagonism alone does not result in growth reduction. However, the ETB 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 ETA 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, show 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 personalized treatment. ET-1–stimulated proliferation of colorectal cancer lines (HT29, SW620) was consistent with previous reports that 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, for example, prostate, ovarian, and colorectal cancers show a reduction of ET-1–stimulated growth in response to ETA antagonism; the proliferative signal may be promoted

Figure 7. Localization of QD-BQ-123 conjugates in patient sections from normal (A) and tumor (B) specimens. Frozen sections were incubated with QD-BQ123 overnight (4°C) and visualized by confocal microscopy. Punctate yellow-orange QD-BQ123 binding was more abundant in tumor versus normal sections. Green tissue autofluorescence is present. Bar, 50 μm. C, images generated by exposure of cytospons to film. Cytospun cells were incubated with increasing concentrations of $^{125}$I-ET-1 (3 × 10<sup>-12</sup> to 1 × 10<sup>-9</sup> mol/L) to determine total binding and in the presence of unlabeled ET-1 (1 μmol/L) to establish nonspecific binding. D, cells were removed with NaOH and total and nonspecific $^{125}$I-ET-1 activity quantified by gamma counter. Graphs of specific binding were produced by subtracting nonspecific from total $^{125}$I-ET-1 binding. Prism was used to calculate fibroblast and cancer cell line $B_{max}$ (mean, 3.03 fmol/10<sup>6</sup> cells; 2.43 fmol/10<sup>6</sup> cells, respectively) and $K_d$ [mean, 213.6 pmol/L (0.214 nmol/L); 367.0 pmol/L (0.367 nmol/L), respectively]. E, specific radiolabeled $^{125}$I-ET-1 (100 pmol/L) localization to colorectal fibroblasts (top) and cancer cell lines (bottom). Total $^{125}$I-ET-1 binding (left) and nonspecific (right) shows specific binding to cells.
further through EGF receptor transactivation (4, 9, 24). The striking exception is melanoma, where ET-1 drives growth via ETA receptor (25). Zibotentan inhibition of ET-1–induced growth was reported in ovarian cancer lines, with a reduction in ETα-mediated angiogenesis and invasive mediators (VEGF, matrix metalloproteinases; refs. 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 shown 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 ETα receptor antagonists, zibotentan produced a similar inhibitory effect on migration to BQ123. Combined ETA and ETβ receptor blockade (zibotentan plus BQ788) produced a migratory effect in between that produced by ETα blockade and ETβ blockade. Fibroblast migration and contraction due to various stimuli are well documented. For example, Shi-Wen and colleagues showed that on ET-1 exposure, normal lung fibroblasts upregulated 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 seems 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 ETβ 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 125I-ET-1 binding, we carried out studies using specimens from patients with colorectal cancer. Autoradiography showed 125I-ET-1–binding sites (ETA and ETβ 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 shown by immunohistochemistry (AS02, CD31). Positive collagen XI staining confirmed the pathologic state of the stroma associated with cancer lesions (20). This correlates with our previous work on human colon specimens, which showed that radiolabeled ET-1, ETA receptor antagonist, and ETβ receptor agonist bound in a similar pattern within cancer and normal tissue (5, 14). Other groups have also reported strong stromal binding of 125I-ET-1 in intestinal tissues (28, 29). Tissue homogenates were used to determine binding characteristics. The *KD* and *B* max of 125I-ET-1 were similar in both normal and tumor 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 showed a change in the ratio of receptor subtypes, with upregulation of ETA receptors and down-regulation of ETβ receptors in colorectal cancer compared with normal colon (5, 19). In the present series, we inhibited 125I-ET-1 binding with unlabeled receptor antagonists. BQ123 showed a concentration-dependent inhibition of 125I-ET-1 binding in both normal and tumor specimens (25 μmol/L > 5 μmol/L). The most striking inhibition was observed in the tumor sections, consistent with a higher ETA receptor expression in cancer compared with normal colon, supporting previous findings: Specifically, Hoosein and colleagues (5) used the selective radiolabeled ETA receptor agonist [125I]IPD-151242 and showed that binding on cancer tissues was increased by 55.5% when compared with normal colon. The ETβ receptor antagonist BQ788 also inhibited binding in a concentration-dependent manner (25 μmol/L > 5 μmol/L). The greater inhibition of binding to ETβ receptors observed in normal colon than within tumor is consistent with higher ETβ receptor expression in normal colon and a down-regulation in colorectal cancer (5, 19). In the latter study, the specific ETβ receptor agonist, [125I]IPQ3020, showed a 45% decrease in ETβ receptor expression in cancer compared with normal. However, this is contrary to the findings elucidated by Egidy and colleagues (29), who described a quantitative increase in mRNA expression of both ETA and ETβ 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 tumor specimens than normal tissues, in keeping with the over-expression of ETA receptors in cancer. The extent of inhibition of 125I-ET-1 binding may not have been as expected as zibotentan is a specific ETA receptor antagonist while BQ123 and BQ788 at higher concentrations are known to act on both receptor subtypes (lose selectivity; ref. 30). Therefore, use of these laboratory compounds may not be truly subtype-selective at high concentrations.

125I-ET-1 binding to specific structures was further clarified by immunohistochemical localization of ETA and ETβ receptors, in addition to fibroblast, endothelial cell, and collagen XI mapping. ETA and ETβ immunostaining closely correlated to 125I-ET-1 binding in parallel tissue sections. Cancer tissues showed increased ETA receptor staining localized to epithelial cells and tumor stroma, and reduced ETβ receptor staining on the epithelial cell surface, compared with normal tissues. Similar alterations in receptor expression have been reported in autoradiographic studies on prostate and ovarian cancer (3, 4). 125I-ET-1 binding to vascular regions, shown here by colocalization with ETβ-expressing endothelial cells and its known association with ETA-expressing vascular smooth muscle cells (30, 31), highlights ET-1 angiogenic actions, such as VEGF production (33). 125I-ET-1 also

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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 signaling, which is the pathway involved in regulating COLX1 and CTFG expression, molecules with tumorigenic associations (34).

Within normal mucosa, ET$_A$ receptors appear closer to the luminal surface with ET$_B$ receptors towards the muscularis mucosa and lamina propria (Fig. 6), a distribution pattern not previously reported. This may reflect trophic signaling 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 ERα found predominantly in basal regions (35). This pattern is consistent with ET$_B$ associated with growth arrest signaling and ET$_A$ associated with differentiation signaling.

The combined fibroblasts $K_d$ value (0.213 nmol/L) is regarded as high affinity ($<1$ nmol/L) with the cells showing a relatively high maximal $^{125}$I-ET-1 binding (3.03 fmol/1 × 10$^6$ cells). The colorectal cancer lines exhibited a similar $b_{max}$ (2.43 fmol/1 × 10$^6$ cells) and affinity that was marginally less than the fibroblasts ($K_d$: 0.367 nmol/L), suggesting that fibroblasts have more ET surface receptors than cancer cells; this is supported by our high-resolution autoradiography (Fig. 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 $K_d$ values of 0.41 and 0.4 nmol/L, respectively (28, 36, 37). 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 $b_{max}$ within fibroblasts.

Competition studies were conducted 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 approximately 10,000 fold more effective than the ET$_B$ receptor antagonist BQ788, whereas the orally active drug, zibotentan, was more effective at inhibiting $^{125}$I-ET-1 binding than BQ788 by approximately 1,000-fold although approximately 1,000-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 showed that ET-1 stimulates behavior consistent with tumorigenesis 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 tumorigenesis primarily via the stroma and relevant anti-ET treatment would provide a shift in treatment paradigm.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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