The Contribution of Endothelin-1
In Colorectal Cancer
&
The Efficacy of the Novel Endothelin Receptor Antagonist ZD4054

By

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ABSTRACT

Introduction:

Endothelin-1 (ET-1) contributes to growth and progression of solid cancers, mainly through ET_A receptor (ET_AR). Hence, ET receptor antagonism is emerging as a potential cancer treatment. We evaluated the efficacy of the specific ET_AR antagonist zibotentan (ZD4054) in blocking ET-driven cellular and molecular effects in colorectal cancer (CRC).

Aims:

To determine the cellular response to ET-1 and effects of receptor antagonism on proliferation, migration and contraction of colonic fibroblasts and cancer cell lines. At the molecular level to identify novel genes that are regulated by ET-1 and whether antagonists including ZD4054 have potentially beneficial effects by blocking expression of these genes. Finally to determine ET-1 binding distribution by autoradiography in patient tumour sections and delineate binding characteristics of ET-1 and its receptor antagonists (B_{max}, K_d and IC_{50}).

Material and Methods:

To investigate ET-1 and its antagonistic effects at the cellular level, colorectal cancer cell lines (HT29;SW620) and colonic fibroblasts (isolated from patient colorectal cancer specimens: CF36; CF56; CF65; CF75) were studied. They were incubated with ET-1 with/without BQ123, zibotentan (ET_AR antagonists) and/or BQ788 (ET_BR antagonist). Growth was measured by methylene blue uptake; migration by scratch wound assay and contraction in collagen gels.

To identify novel key genes regulated by ET-1, Illumina micro-arrays determined differential gene expression post-ET-1 stimulation of 3 colorectal cancer cell lines (HT29, SW480; SW620) and the 4 human colonic fibroblast strains. To confirm expression of genes of interest, we examined time point induction mRNA levels (conventional RT-PCR; quantitative real-time RT-PCR). ET_A (Zibotentan, BQ123) and ET_B (BQ788) antagonistic effects were measured at the mRNA and protein levels (Immunoblotting). Silencing (SiRNA) was also used to confirm receptor involvement in regulation of these key genes.

ET-1 receptor distribution and binding characteristics (K_d; B_{max}) were determined using *in vitro* autoradiography on patient sections, tissue homogenates, CRC cell

lines and colonic fibroblasts. Effects of the ET_AR specific antagonist zibotentan (ZD4054) on ET-1 receptor binding (IC_{50}) were evaluated against laboratory-standard compounds. Immunohistochemistry (IHC) was used to identify stromal structures and receptor distribution (vascular CD31; Thy-1 fibroblasts; collagen type XI; ET_A and ET_B). Study was awarded ethical approval, REC No. 08/H0720/162, University College London Hospitals

Results:

ET-1 driven proliferation (26.2%-51.9%>control) was significantly inhibited ($p<0.05$) by ET_AR (not ET_BR) antagonism (BQ123=zibotentan; CRC & fibroblasts). ET-1 driven fibroblast migration and contraction were blocked by both ET_AR & ET_BR antagonism (zibotentan=BQ123). CRC cells did not demonstrate any migrate or contract.

Four-hour ET-1 induction had a significant effect on gene up/down-regulation ($p<0.01$ $\pm$ $\geq$1.5-fold) in all cancer cell lines (9 genes) and fibroblast strains (111 genes). We determined expression and effect of receptor antagonism of the following (table 1.1): (a) In cancer cells: (i) MT1X, maximum at 4hr, reversed by ET_A antagonism; (ii) MMP7, late maximum induction (24hr) (undetectable by 4hr microarray), reversed by ET_A antagonism; (iii)PPP2R5D, no significant up/down regulation, but levels were decreased by ET_A antagonism. (b) In fibroblasts: (i) CTGF and (ii) ADM maximum at 2-4hr, both reversed by ET_A & ET_B antagonism; (iii) STC1, transient up-regulation (1hr) followed by down-regulation (4hrs in-line with 4hr microarray data), reversed by ET_A & ET_B antagonism.

Gene	Name/Role	Peak	Antagonist
MT1X	**Metallothionein:** proliferation/migration/angiogenesis	4h	ET_A
MMP7	**Metalloproteinase 7:** migration/invasion	24h	ET_A
PPP2R5D	**Phosphatase 2Reg5Delta:** proliferation/migration	NS	ET_A
CTGF	**Connective Tissue Growth Factor:** proliferation/adhesion/migration/ angiogenesis	2-4h	ET_A/ET_B
ADM	**Adrenomedullin:** proliferation/survival/angiogenesis	2-4h	ET_A/ET_B
STC1	**Stanniocalcin 1:** proliferation/survival	1h	ET_A/ET_B

Table 1.1 *ET-1 regulated genes and their associated roles in tumourgenesis along with peak expression time and receptor antagonist that significantly affected expression levels.*

ET-1 binding to cancer and normal colon tissue had similar characteristics. There was greater ET_A than ET_B binding in CRC sections. Both cancer and normal tissues had strongest binding to stromal cells, particularly fibroblasts (IHC). Furthermore, characterising CRC cell lines and primary fibroblasts revealed high density and affinity ET-1 binding (B_{max} 1.11 fmol/1x10^6 cells; K_d 450.5 pmol/L and B_{max} 3.03 fmol/1x10^6 cells; K_d 213.6 pmol/L respectively). Inhibition studies showed ET_A antagonists (BQ123; Zibotentan) more effectively reduced ET-1 binding (approximate IC_{50} values in CRC: 10μM, 0.1μM respectively; fibroblasts: 0.1μM, 10μM respectively) than ET_B antagonism BQ788 (approximate IC_{50}; 1mM in both).

Conclusions:

The specific ET_AR antagonist zibotentan is at least as efficacious as BQ123 in blocking ET-1 driven growth, migration & contraction in CRC cell lines & colonic fibroblasts, which form the supporting tumour stroma.

ET-1 stimulates CRC cell line and cancer-associated fibroblasts to produce signals that promote cancer growth and formation of tumour stroma. ET_A and ET_B receptor antagonists block a number of these signals.

ET-1 bound strongly to CRC stromal structures with high affinity and density (fibroblasts; endothelial cells), and is consistent with ET-1 signalling contributing to tumourigenesis. We further demonstrated that the orally active ET_A antagonist Zibotentan reduces ET-1 binding to CRC tissues.

This study provides new and further evidence for the potential therapeutic use of the specific ET_A antagonist Zibotentan as an adjuvant treatment for CRC.

CONTENTS

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ABBREVIATIONS

ADM/AM	Adrenomedullin
AML/RUNX1	Acute Myeloid Leukaemia
bFGF	Basic fibroblast growth factor
B_{max}	Maximal binding Concentration
BMP	Bone Matrix Protein
BSA	Bovine Serum Albumin
CEA	Carcinoembryonic Antigen
COL11A1	Collagen Type XI
CTGF	Connective tissue growth factor
CRC	Colorectal Cancer
CYR61;CCN1	Cysteine-Rich 61
DAG	Diacylgycerol
DMEM	Dulbecco's modified eagle medium
ECE	Endothelin converting enzyme
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Estrogen Receptor
ET-1	Endothelin-1
ET_A	Endothelin Receptor A
ET_B	Endothelin Receptor B
EVI-1	Ecotropic Viral Integration Site 1
5-FU	5-Flurouracil
FAP	Familial polyposis coli
FCS	Foetal Calf Serum
FPCL	Fibroblast populated collagen lattices
H&E	Haematoxylin and Eosin
HIF	Hypoxia inducible factor
IC50	Inhibitory Concentration by 50 percent
IGF	Insulin growth factor
IL	Interleukin
IP_3	Inositol triphosphate
K_d	Binding affinity
LOH	Loss of Heterozygosity
LPA	Lysophosphatidic Acid

MAPK	Mitogen Activating Protein Kinase
MB	Methylene Blue
MMP	Matrix metalloproteinase
MTs	Metallothioneins
MT1X	Metallothionein 1X
PBS	Phosphate buffer solution
PDGF	Platelet derived growth factor
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
PLC/PLD/ PLA_2	Phospholipase C/D/A_2
PP2A	Phosphatase A2
RNAi	RNA interference
S1P	sphingosine-1-phosphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SiRNA	Short interfering RNA
STC-1	Stanniocalcin-1
TGF	Transforming growth fatctor
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tissue necrosis factor
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
VSMC	Vascular Smooth Muscle Cell

Chapter 1

Introduction to
Colorectal Cancer and Endothelin-1

1.1 INTRODUCTION

The work described in this thesis focuses on opposing the tumourigenic actions of the peptide Endothelin-1 in colorectal cancer, using Endothelin receptor antagonism. Endothelin-1 is a small vasoactive peptide which was first identified in 1988. It is one of a family of three endothelins which exert their action through two G-protein coupled receptors, ET_A and ET_B. This chapter reviews our knowledge of colorectal cancer and the evidence implicating ET-1 in tumorigenesis; In particular the role of ET-1 in mitogenesis, apoptosis, angiogenesis, tumour invasion and metastasis. Evidence relating to downstream effectors is presented, and trials relating to the potential for endothelin-system modulation as an adjuvant therapeutic strategy are reviewed. Aims and specific objectives of this work are described at the end of the chapter.

1.2 Colorectal Cancer

Colorectal cancer (CRC) is the third commonest cancer in the UK with about 36,000 new cases every year. Being one of the commonest malignancies in the developed world, it is the leading cause of morbidity and mortality worldwide due to cancer. According to Cancer Research UK in 2009, it is the second commonest cause of death in the UK, with a peak incidence in the sixth decade of life with over 80% arising in the over 60's. Environmental factors play a major role in the aetiology of most cancers. However there are inherited genetic factors that play a significant role in about 10-30% of cases. A highly penetrant dominant or recessive inherited syndrome is associated with up to 5% of all CRC cases (Haque *et al.,* 2008*).*

Research has shown that a number of colorectal cancers develop following mutations in oncogenes and tumour-suppressor genes. These mutations generally occur resulting in a well defined pathological sequence seen histologically. Indeed mutations not only occur in familial cancers at a younger age such as in Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colon Cancer HNPCC, but also account for 80-85% of all sporadic colorectal cancers (Vasen *et al*., 2008).

Fearon and Vogelstein in 1990 had proposed sequential mutations occur in the development of colorectal cancers. These mutations mainly target three main tumour suppressor mutations (APC, DCC and p53) and one oncogene (k-Ras) as described by Fodde *et al.*, 2001.

The staging of colorectal cancer is to determine prognosis and aid in the appropriate management of patients. The most widely used and accepted staging system used in the UK is the Duke's classification, providing a 5 year survival rate depending on extent of spread. With early detection, colorectal cancer is potentially curable with surgical intervention. However, up to 60% of patients will have regional or metastatic disease at initial presentation, limiting the potential for surgical resection. In advanced disease, and those with inoperable disease, management focuses on disease control with chemotherapeutic agents such as 5-Flurouracil, either individually or in combination with other agents.

Despite our increased knowledge of molecular mechanisms in colorectal cancer, success of new chemotherapeutic drugs and monoclonal antibodies is still limited. We therefore need to develop novel therapeutic strategies for patients with advanced or unresectable disease that can be used in the adjuvant setting following surgery. Recent strategies which have shown promise including the targeting of growth factor and angiogenic receptors, thought to be important in the promotion of colorectal cancer development and progression. Another increasingly recognised potential target is Endothelin-1 (ET-1) which has important autocrine and paracrine actions in a number of human cancers (Rosano *et al.,* 2005; Grant *et al.,* 2007).

1.3 Endothelin-1

The potent vasoconstrictor peptide endothelin 1 (ET-1) is one of a family of three multifunctional peptides (ET-1, 2, and 3). They comprise a 21 amino acid structure characterised by a single α-helix and 2 disulphide bridges, encoded on chromosomes 6, 1 and 20 respectively. Initially described in 1988 as a vasoconstrictive substance isolated from bovine aortic endothelial cells, ET-1 is the most extensively studied of the three peptides and the one most implicated in tumorigenesis (Sakurai *et al.,* 1992).

The ET physiological effect is exerted via ET_A and ET_B receptors, which are G-protein coupled trans-membrane receptors (GPCR) found in both vascular and

non-vascular tissues. The ET_A receptor has varying affinities for each isoform (higher for ET-1 then ET-2, with a two-fold lower affinity for ET-3) whereas the ET_B receptor shows no selective affinity for any of the ET subtypes (Sakamoto *et al*., 2001).

The endothelins have been implicated in numerous pathological conditions including hypertension and cardiac failure. Interest in the role of ET-1 in cancer has grown over the last decades, and currently there is evidence that ET-1 can modulate mitogenesis, apoptosis, angiogenesis, tumor invasion, and development of metastases.

1.3.1 ET-1 Associated Signal Transduction Pathways

Translation of the ET-1 gene produces a number of precursors which eventually result in the production of Big-ET-1. This has negligible biological activity and is converted by the metalloproteinase Endothelin Converting Enzyme 1 (ECE-1) into the active ET-1 peptide. Production of ET-1 is stimulated by Interleukin (IL-1ß), tumour necrosis factor (TNFα), transforming growth factor (TGFß), platelet derived growth factor (PDGF), vasopressin, hypoxia and shear stress. ET-1 inhibitory factors include nitric oxide, prostacyclins and atrial natriuretic peptide (ANP) (Nelson *et al*., 2003).

The consequences of ET-1 induced receptor stimulation are complex with many intracellular pathways being activated (figure 1.1). ET-1 binding to its receptor results in dissociation of the α and $\beta\gamma$ subunits of one of several possible associated G-proteins. The activated G-protein then phosphorylates one of several upstream pathway initiators, some of which include phospholipase C and D, phospholipase A_2, adenylate cyclase, and guanylate cyclase (Shome *et al*., 2000). The former two initiators activate IP3 and Protein Kinase C (PKC) which ultimately result in mitogen activating protein kinase (MAPK) pathway activation. This is achieved either directly by Ras activation through IP3 or indirectly via increased intracellular Ca2+ through PKC. Raf-1 (downstream in the MAPK pathway) is activated directly via PKC (Blaukat *et al*., 2000). Ultimately MAPK results in early response gene transcription of fos and jun. Resultant cellular effects include mitogenesis and motility.

Transactivation of other cell surface receptors as a result of ET receptor stimulation has been demonstrated: in Rat-1 fibroblasts (Daud *et al*., 1996), ovarian cancer and colorectal cancer (CRC) cells. The Tyrosine Kinase Epidermal Growth Factor Receptor (EGFR) is trans-activated and induces MAPK activity (Grant *et al*., 2007). ET-1 has also been shown to potentiate the effects of platelet-derived growth

factor (PDGF) on human smooth muscle cells, suggesting interactions with other tyrosine kinase receptors (Yang *et al*., 1999).

As ET_B receptor binding affinity is equal for all isoforms, this suggests a role in the clearance pathway via lysosomal degradation following receptor up-take (Bremnes *et al*., 2000). A second clearance pathway has been suggested involving catabolism by the extracellular neutral peptidase nepilysin (NEP).

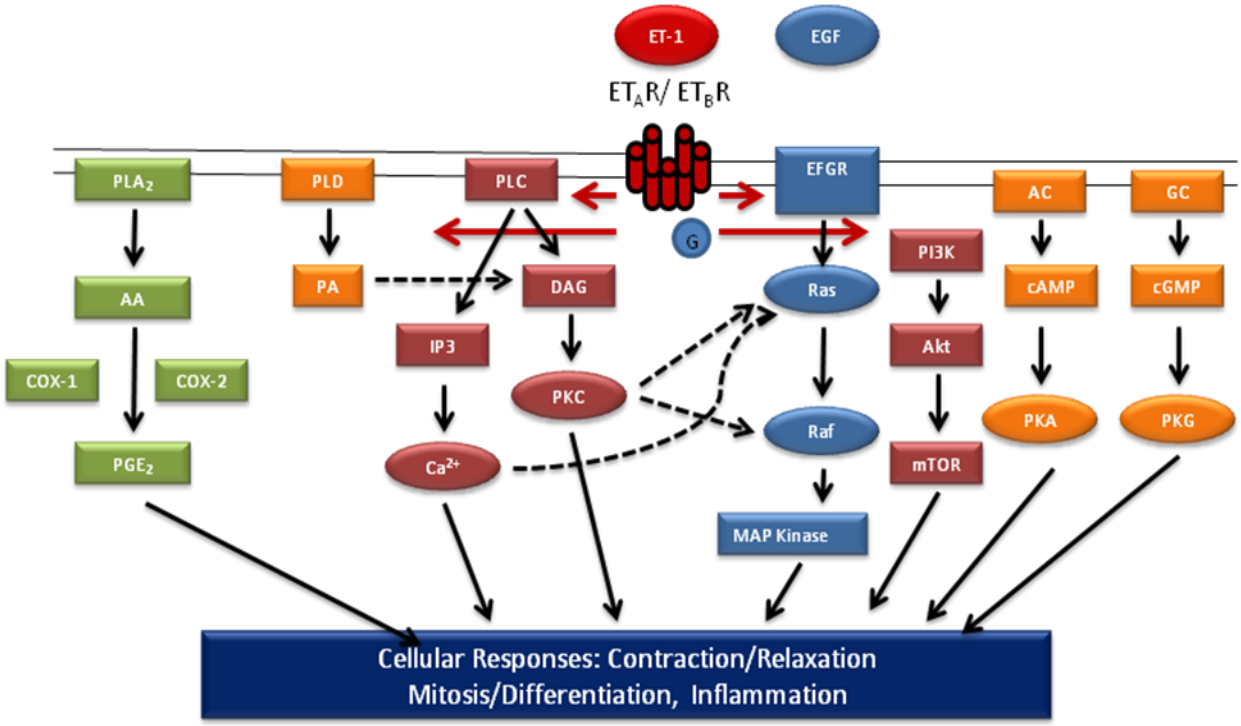


Figure 1.1 *Schematic representation of the signal transduction pathways associated with stimulation of the Endothelin receptors. Upstream transduction molecules activated (phosphorylated) by the G-Protein are shown at the top of each pathway and include the Epidermal Growth Factor Receptor (EGFR), Phospholipase A_2, C, D (PLA_2, PLC, PLD), Adenylate Cyclase (AC),Guanylate Cyclase (GC) and Phosphoinositide 3-Kinase (PI3K). Downstream effectors include phosphatidyl inositol 3 kinase (IP_3), diacylglycerol (DAG), phospholipase A (PA), cyclic GMP (cGMP), protein kinase A, C, G (PKA, PKC, PKG), Akt, mTOR, cyclooxygenase 1, 2 (COX-1, COX-2) and prostaglandin E_2 (PGE_2).*

1.3.2 ET-1 Expression in Cancer

Many human cancer cell lines have been shown to synthesize ET-1, including colonic, breast, stomach, prostate, and glioblastoma cells (Ali *et al*., 2000; Kusuhara *et al*., 1990). Similarly, *in vivo*, increased tissue immunoreactivity for ET-1 has been

demonstrated in several cancer types, including ovarian, breast and colorectal tumours (Bagnato *et al*., 1999).

In normal colorectal tissue, low expression of ET-1 and ECE-1 was reported. However, these molecules were significantly up-regulated in 80% of primary CRCs and in the majority of metastatic disease. Furthermore, ET-1 expression levels within tumour-adjacent stromal and endothelial cells were also raised in keeping with its known paracrine function (Asham *et al*., 2001; Shankar *et al*., 1998). Our group showed that plasma levels of ET-1 were increased in patients with both primary colorectal cancers with or without liver metastasis (Asham *et al*., 2001; Shankar *et al*., 1998). Other studies have found that both pre-operative and intra-operative portal plasma levels were significantly higher in metastatic disease. Elevated plasma levels of ET-1 have also been detected in patients with other various solid tumors, including gastric and prostate cancer, where levels are greatest in patients with metastastic, hormone refractory disease (Nelson *et al*., 1996).

ET-1 dysregulation as an early event in colorectal tumourigenesis was suggested by the observation that pre-malignant colorectal adenomas showed increased expression of Big ET-1 and ECE-1 mRNA when compared to normal colon. ET-1 immunoreactivity in breast ductal carcinoma in situ (DCIS) specimens is also significantly higher ($P<0.005$) than that of normal breast tissue, suggesting that modulation of the endothelin system may be an early phenomenon in tumorigenesis.

1.3.3 Endothelin Receptor Expression in Cancer

Varied expression levels of ET receptor subtypes in different cancers have been investigated by using immunohistochemistry, autoradiography and mRNA studies. Expression of the ET_A receptor is up-regulated predominately in colorectal, ovarian, renal and prostate cancers (Bagnato *et al*., 1999; Nelson *et al*., 1996; Ali *et al*., 2000), whilst ET_B receptors are down-regulated. On the other hand not only are ET_A receptors up-regulated, but ET_B receptors are also reported as up-regulated or have varied expression levels in lung and breast carcinomas. The question remains on whether the variety of techniques used to look at levels may give conflicting or unrealistic results for ET_B expression.

Specifically within CRCs, our group previously demonstrated that pharmacologically functional ET_A receptors were over-expressed in all colorectal cancer tissues, compared to normal. Most of this localisation was around blood vessels and fibroblasts, with less localisation to epithelial cancer cells. In contrast ET_B receptors were confirmed to be prominent in normal tissue with a marked down-

regulation in cancer associated blood vessels, fibroblasts and cancer epithelial cells (Hoosein *et al*., 2007). In prostate tumours, levels of receptor expression have been found to correlate with the presence of metastases, similar to the findings in CRCs.

Interestingly, relative hypermethylation of the ET_B gene has been reported in prostate, bladder, and colon cancer cell lines. Furthermore, this has also been found to correlate with transcriptional down-regulation, providing a plausible mechanism for reduced ET_B receptor expression in malignant tissue.

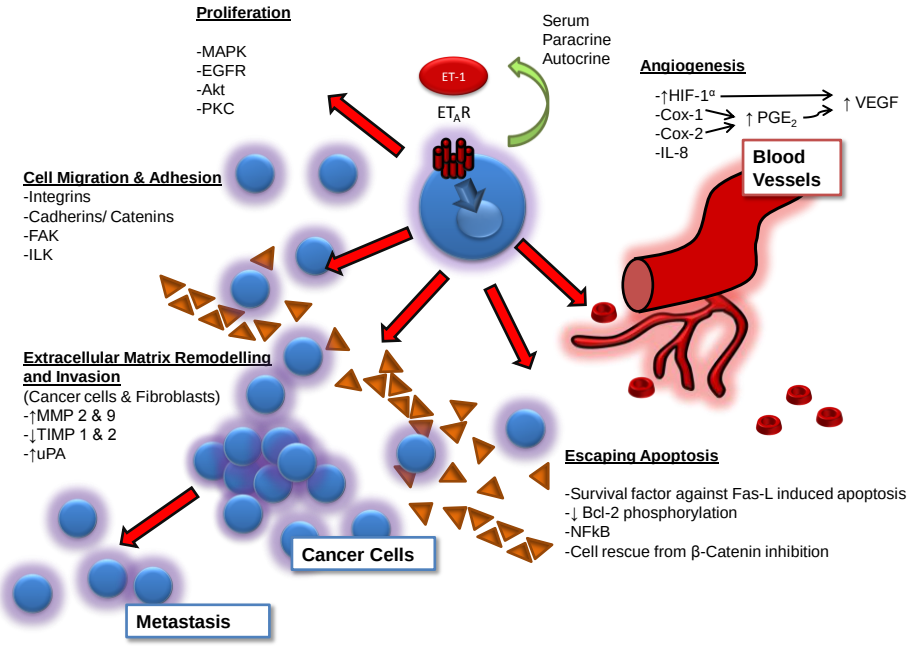


Figure 1.2. *Summary of the role of ET-1 in cancer spread.*

1.3.4 ET-1 as a Mitogen

Effects on mitogenesis are the most intensely studied amongst all the different tumourigenic actions of ET-1 (figure 1.2); they are driven through a complex network of multiple pathways which have been presented earlier and involve the MAPK cascade. For example in ovarian cancer cell lines, ET_A receptor antagonism reduced EGFR transactivation, implicating this receptor in mitogenesis. ET-1 stimulates growth of several human cancer cell lines. In CRC experimental models, ET_A receptor antagonism significantly reduced proliferation not only in cancer cell lines but also in colonic fibroblasts (Ali *et al*., 2000; Knowles *et al*., 2011). In ovarian cancers, the use of ET_A receptor antagonists ABT-627 and ZD4054 inhibited cell

proliferation and potentiated paclitaxel induced apoptosis (Rosano *et al*., 2007). The same receptor was associated with prostate and cervical cancer proliferation (Nelson *et al*., 2003; Nelson *et al*., 2008). In the later it was shown that atrasentan, an ET_A receptor antagonist, inhibited growth in xenografts. Other tumours that demonstrate ET-1 mitogenic activity via the ET_A receptor include lung, bladder and nasopharyngeal carcinomas. In breast and Kaposi's sarcomas, both ET_A and ET_B receptors were shown to be over-expressed. The use of dual receptor antagonists, bosentan in breast and A-182086 in Kaposi's sarcomas, significantly inhibited tumour growth and vascularity in *in vivo* models (Dreau *et al*., 2006). Studies on human melanoma cells have demonstrated that the mitogenic effect of ET-1 is purely ET_B receptor dependent. This has also been confirmed *in vivo*, where the specific ET_B antagonist (BQ788) slowed down significantly melanoma tumour growth in nude mice (Lahav *et al*., 1999).

The role of ET-1 as an autocrine growth factor has been demonstrated in human ovarian and colon cancer cell lines (Ali *et al*., 2000). A paracrine role for ET-1 has also been elucidated in ovarian cancer, where ET-1 production by human ovarian cancer cells stimulated growth of carcinoma associated fibroblasts in co-culture, an effect which was partially inhibited by both ET_A and ET_B antagonism.

1.3.5 ET-1 and Apoptosis

ET-1 also modulates apoptosis. Within rat colon carcinomas, Peduto-Eberl *et al*. demonstrated that ET-1 acted as a survival factor for rat colon carcinoma cells against FasL-induced apoptosis (Peduto-Eberl *et al*., 2003). They also showed that dual endothelial receptor antagonism with bosentan resulted in human CRC sensitization to FasL-induced caspase mediated apoptosis. Protection from FasL induced apoptosis, through suppressed Bcl-2 phosphorylation and PI3K mediated Akt activation, was also observed in ovarian cancers (Peduto-Eberl *et al*., 2003). Furthermore in CRC, ET-1 (which is a direct target of β-Catenin) has been shown to rescue cells from growth arrest and apoptosis which were originally caused by beta-Catenin inhibition (Kim *et al*., 2005). This suggests ET-1 having an oncogenic effect by interfering with the beta-Catenin pathway.

In both melanocytes and melanoma cell lines, ET-1 has been shown to decrease basic apoptotic rates. Like the proliferative action of ET-1 in these cells, the effect on apoptosis is ET_B dependent.

1.3.6 ET-1 and Angiogenesis

Additional to the mitogenic actions of ET-1 on epithelial cancer cells, it stimulates growth of endothelial cells, vascular smooth muscle cells (VSMC), fibroblasts and pericytes. Furthermore, ET-1 potentiates the effect of several pro-angiogenic factors in vitro, including PDGF and VEGF. ET-1 promotes cancer neovascularisation via ET_A receptor stimulation on vascular smooth muscle cells and ET_B receptor stimulation on endothelial cells. ET-1 has also been shown to stimulate various stages of neovascularisation, including protease production, tubular formation, endothelial cell proliferation, migration and invasion.

There is significant correlation between microvascular density, ET-1 and VEGF expression in ovarian cancer specimens. From the in vitro studies described, both in an ovarian cancer cell line and vascular smooth muscle cells, ET-1 was able to promote transcription and protein expression of VEGF to a level similar to that stimulated following hypoxia. This effect was mediated through ET-1 binding to ET_A receptors, stimulation of hypoxia inducible factor (HIF-1) production (or its stabilization in normoxic conditions), which then bound to hypoxia responsive elements and in turn increased VEGF production (Spinella *et al*., 2002). It has also been shown a reciprocal relationship exists where HIF-1 itself also stimulates ET-1 production. Furthermore ET-1 acting via the ET_A receptor, increased expression of COX-1 and COX-2 in ovarian cancer cells. This resulted in increased prostaglandin signalling, particularly PGE_2, and stimulation of several MAPK dependent signalling pathways, including p38 MAPK and p42/22 MAPK. This resulted in production of VEGF, MMP activation and angiogenic cellular invasion (Spinella *et al*., 2004).

Invasive tumour cells, including melanomas, prostate, breast and ovarian carcinomas have been shown to form de novo extracellular matrix rich vascular channels expressing vascular associated molecules. Both MMP2 and MT1-MMP, which are expressed by invasive ovarian cancer cells, play a key role in developing vasculogenic-like networks and matrix remodelling. Interestingly, ET_A receptor antagonism of these cells inhibits the formation of tumour like vascular channels.

In vivo studies looking at subcutaneous implants of matrigel impregnated with either ET-1 or VEGF confirmed increased vascular effects and vascularity above that of other angiogenic factors, e.g. bFGF.

In addition to an effect on tumour cells, ET-1 is known to protect human endothelial cells and VSMC's from serum deprivation induced apoptosis in vitro. This suggests that ET-1 could have a pro-angiogenic effect by acting as a survival factor for newly formed blood vessels.

1.3.7 ET-1 and Tumour Progression/Metastases

Further to its pro-angiogenic role, ET-1 may also influence tumour invasion and metastases by stimulating secretion of matrix remodelling proteins. In colonic fibroblasts, our group reported that ET-1 increased TIMP-1 and MMP-2 protein expression which are known to be involved in key tumourigenic processes: matrix degradation, activation of growth factors and degrading enzymes and modulation of angiogenesis (Knowles *et al*., 2011). Expression of both these molecules was inhibited by predominantly ET_A but also ET_B receptor antagonism. Furthermore, ET-1 stimulated expression of CTGF and Collagen Type XI mainly via the ET_A receptor. CTGF is known to activate intracellular pathways resulting in increased migration, proliferation and MMP production, whilst Collagen Type XI is associated with colonic diseases. Our group conducted proliferation and desmoplastic activation studies in colonic fibroblast strains using the specific ET_A receptor antagonist zibotentan: ET_A receptor blockade inhibited growth, contraction, migration and MMP production by these cells.

In ovarian cancer, ET-1 stimulated expression of several MMPs, particularly MMP-2 and MMP-9, and down-regulated tissue inhibitors of matrix metalloproteinases, TIMP1 and 2. Similar up-regulation of MMPs has also been shown in Kaposi's sarcoma cells. Furthermore, ET-1 can contribute to the creation of a 'reactive' tumour stroma, by stimulating myofibroblast induction (via ET_A), and the expression of matrix remodelling genes by these cells (Xu *et al*., 2004). Interestingly, tumour adherence to collagen is enhanced by integrin-linked kinase (ILK), a multi-domain focal adhesion protein. Blockade of ET-1/ET_A receptor induced ILK resulted in inhibition of MMP activation, as well as cell motility and invasiveness, indicating that the ET-1 axis is involved in aggressive cellular behaviour.

In prostate cancer ET-1 may contribute to the growth of bony metastases. In vitro, ET-1 production by prostate cancer cells is enhanced by bone contact, which in turn blocks osteoclastic bone reabsorption. Similarly, in an in vivo osteoblastic tumour model, tumours transfected to over-express ET-1 produced significantly more bone growth in nude mice compared with vector only controls (Nelson *et al*., 1999).

Furthermore, we have demonstrated increased ET-1 immunoreactivity in endothelial cells within colorectal liver metastases compared with surrounding vessels (Shankar *et al.*, 1998), suggesting that ET-1 may be involved in modulation of tumour blood flow.

1.3.8 Endothelin Antagonism *in Vivo*

Several in vivo models have been used to assess the role of endothelin antagonism in tumorigenesis. Work originating from our department using intraportally injected syngeneic MC28 cells in rats demonstrated that ET_A antagonism with BQ123 significantly reduced hepatic tumour load compared with controls (Asham *et al*., 2001).

The effect of bosentan, a dual receptor antagonist, on growth of peritoneal tumours derived from a syngeneic rat colonic adenocarcinoma cell line has been investigated. Although bosentan was not able to control tumour progression, tumours were generally of lower grade, and there were fewer spontaneous deaths in the treated versus the untreated groups. Egidy and colleagues used the same tumour model to assess histological differences between tumours of bosentan treated animals and controls (Egidy *et al*., 2000). They demonstrated that tumour cells were less densely packed, and there was less collagen matrix around tumour nodules in the treated compared to the untreated group.

Using an osteoblastic tumour model in nude mice Nelson and colleagues have shown that ET_A antagonism with A127722 significantly reduced the growth of new bone compared with vehicle treated controls (Nelson *et al*., 1999).

Finally, Rosano and colleagues have combined targeting of the ET_A and EGF receptors with promising results. They demonstrated co-administration of ZD4054 and gefitinib lead to a partial (82%) or complete tumour regression on HEY ovarian carcinoma xenografts. This also resulted in decreased vascularisation, MMP2, VEGF, MAPK and EGFR, and enhanced E-Cadherin expression (Rosano *et al*., 2007).

To date results from in vivo models are encouraging and warrant further investigation.

1.4 Clinical Trials

The availability of orally active bio-available endothelin receptor antagonists offers potential opportunities in adjuvant cancer treatment. One example is the drug atrasentan which has a 1000-fold greater affinity for the ET_A receptor then the ET_B receptor. A phase II randomized, placebo-controlled trial in 288 patients with asymptomatic hormone-resistant metastatic prostate cancer evaluated three groups; placebo, 2.5mg, or 10mg atrasentan. Delayed time to progression (TTP) was observed in the 10mg group, and stabilization of biochemical markers, including prostate specific antigen and lactate dehydrogenase compared with controls. In

phase III clinical trials involving 809 prostate cancer patients, 10mg of atrasentan daily was shown to delay time to disease progression when compared to placebo, albeit statistically non-significant, in men with metastatic hormone resistant prostate cancer (Carducci *et al*., 2007). However, secondary endpoint analysis demonstrated significantly delayed progression of bone acid phosphatase levels, and preserved prostate cancer-specific quality of life, particularly in terms of pain-related symptoms. Another phase III trial in non-metastatic prostate cancer once again showed statistically non-significant delay in disease progression (93 days) when compared to placebo with the same dosing of this drug (Nelson *et al*., 2008). It has been suggested that the future use of atrasentan may lie in combining it with other drugs. This was demonstrated by a phase I-II trial in patients with resistant prostate cancer, where results from this drug combined with docetaxel were comparable to results produced by docetaxel and prednisolone (Armstrong *et al*., 2008).

Another orally active ET receptor antagonist, currently used in pulmonary hypertension, is Bosentan. This is a non-selective ET_A and ET_B receptor antagonist. Promising preclinical results demonstrating inhibition of tumour progression were shown in in vitro and in vivo models of melanoma (Lahav *et al*., 1999). However, a phase II clinical trial found that patients with stage 4 metastatic melanoma developed progressive disease despite receiving 500mg BD of Bosentan (Kefford *et al*., 2007). More recently the novel specific ET_A receptor antagonist Zibotentan (trans, trans-2(4-methoxydhenyl)-4-(1-3-benzodiazol-5-yl)-1-(dibutylaminocarbonylmethyl)-pyrrolidine-3-carboxylic acid (ZD4054)), has been investigated in a number of clinical trials. In the clinical arena the original focus was within the field of prostate cancer. A phase II double blinded clinical trial allocated a total of 312 patients with pain free or mildly symptomatic hormone resistant prostate cancer patients with bony metastases to either receive daily doses of ZD4054 (10mg-15mg) or placebo. There was no significant difference seen for progression-free survival although there was a difference in the overall survival of these patients compared to the placebo group. Importantly this drug had an acceptable safety and tolerability profile (James *et al*., 2009). A recent Phase III clinical trial looked at ZD4054 in non-metastatic hormone resistant prostate cancer. This was terminated early in 2011 as it was unlikely that the trial would meet its primary efficacy end point of progression free survival and overall survival benefits. Due to the disappointment of monotherapy, interest has grown in its use as adjuvant therapy, for example the latest on-going phase II clinical trial (FOLFERA) which combines ZD4054 with chemotherapy agents irinotecan, fluorouracil and folinic acid (FOLFIRI) in patients with advanced colorectal cancer.

The endothelin axis is altered in cancer, aiding tumour growth and progression. It seems that selective ET_A antagonism provides the most effective method of endothelin system inhibition in cancer. With generally acceptable side effects, and suggested anti-tumour activity, further clinical evaluation of these agents is warranted to determine possible therapeutic potential as an adjuvant anti-cancer strategy.

1.5 AIMS

The overall aim of this thesis was to investigate the effects of ET-1 and its ET_A and ET_B receptors on a number of cellular and molecular effects in colonic fibroblasts and cancer cell lines. Throughout this thesis the potential role of the specific ET_A receptor antagonist ZD4054 (Zibotentan) was evaluated to determine its suitability for clinical use in the setting of colorectal cancers. Specific objectives were to:

1. To determine the cellular response to ET-1 and effects of receptor antagonism on proliferation, migration and contraction of colonic fibroblasts and cancer cell lines.

2. Identify novel genes that are regulated by ET-1 and determine whether antagonists including ZD4054 have potentially beneficial effects by blocking expression of these genes.

3. Determine ET-1 binding distribution by autoradiography in patient tumour sections and delineate binding characteristics of ET-1 and its receptor antagonists (Bmax, Kd and IC_{50}).

Chapter 2

General Materials & Methods

2 GENERAL MATERIALS AND METHODS

This chapter describes general techniques used throughout the thesis. Experimental protocols specific to particular studies are outlined within each chapter.

2.1 Cell Lines

Primary cell strains from submucosal colonic fibroblasts were used throughout. Fibroblast isolation work was previously carried out by Dr. Felicity Savage, UCL Division of Surgery and Interventional Science (cell isolation carried out pre-2003, with patient consent and UCH ethical approval). In brief, fibroblasts were obtained from surgical specimens of colons from patients undergoing colorectal cancer resection between 2001 and 2003. Once specimens were received, fibroblasts were extracted from macerated tissue adjacent to tumour, but macroscopically not part of the cancer itself. Cell-type specific antibody coated magnetic beads were then used to remove epithelial and endothelial cells, with the remaining cells grown in fibroblast selective medium. Fibroblast phenotype (β-actin positive) was previously confirmed by IHC. Cell strains (untransformed fibroblasts propagated in culture) were labelled CF (Colorectal Fibroblast) along with the patient sample number. Those used in this thesis were CF35, CF36, CF56, CF65, CF75 and CF78. Fibroblasts were used routinely between passages 4-14 and thereafter discarded due to loss of fibroblast phenotype (Kernochan *et al*., 2002).

Four colorectal cancer cell lines were used throughout. They were: HT29 (moderately differentiated colorectal adenocarinomas); SW480 (Primary Dukes B adenocarcinoma) and the line derived from its poorly differentiated Dukes C metastasis resected 18 months later from a lymph node, SW620 (all from ECACC, Salisbury, UK); and LIM1215, an immortalised line grown from an Hereditary Non Polyposis Colon Cancer (HNPCC) cancer (kindly donated from Professor Mike O'Hare, the Ludwig Institute, UK).

2.2 Cell Maintenance

Cells were routinely cultured in a humidified atmosphere, at 37^{o}C, 5% CO_2/air in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Basel, Switzerland) which was supplemented with 10% Foetal Calf Serum, L-Glutamine (2mM) and Gentamycin (1mg/ml). Once 80-90% confluence was reached, cells were disaggregated with trypsin (1mg/ml in 0.02% EDTA

PBS/PBS, Lonza) and passaged 1:2 or 1:3 into flasks for further stocks or for experimentation.

2.3 Materials

Experimental concentrations used for fibroblast and colorectal cancer cell protocols were previously determined by our laboratory and by collaborators within the Centre of Rheumatology, UCL Medical School (Ali *et al*., 2000; Grant *et al*., 2007, Shi-Wen *et al*., 2004). Dilutions were generally made in serum free DMEM containing gentomycin; as appropriate bovine serum albumin was used in the serum free DMEM, particularly for fibroblast culture.

For fibroblasts, ET-1 (Bachem Ltd, St Helens, Merseyside, UK) was used at a concentration of 10^{-7}M. The ET_A receptor specific antagonist BQ123 and the ET_B receptor specific antagonist BQ788 were used at 10^{-6}M. For colorectal cancer cells, ET-1 was used at 10^{-8}M, whilst BQ123 and BQ788 were used at 10^{-7}M. Similarly, the specific ET_A receptor antagonist ZD4054 (kindly donated by Astra Zeneca, Stanhope Gate, London, UK) was used at 10^{-6}M for fibroblast and 10^{-7}M for colorectal cancer experimentation. These concentrations were in keeping with relevant publications which used receptor antagonist at a 10-fold lower levels then ET-1 ligands (Davenport, 2002). All other reagents were purchased from Sigma-Aldrich Co, Irvine, Ayrshire, UK, unless otherwise stated.

2.4 Statistics

Data are presented as means with standard deviations or percentage of control values, as appropriate. Statistical testing was performed on the original values using one-way ANOVA followed by post–hoc analysis using Tukey's honestly significant difference test. As 6 or more independent repeats were used for each cell type or fibroblast strain, data was analysed as parametric data. Where $n<6$, the data was analysed as if they were non-parametric: Kruskal-Wallis analysis was used with Dunn's multiple comparison test performed on selected data sets and the data presented as a median with a range. Further details of statistical analysis used are given at the beginning of each chapter.

Chapter 3

Studies on Colonic Fibroblasts & Colorectal Cancer Cell Lines:

Proliferation, Migration & Contraction Studies

3.1 INTRODUCTION

This chapter describes the effects on ET-1 and ET receptor antagonism on the cellular behaviour of both colorectal cancer (CRC) cell lines and fibroblasts isolated from human colons. Specifically, the processes investigated were proliferation, migration and contraction.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture and Pharmacological Agents

Colorectal cancer cell lines SW620, HT29 and colonic fibroblast strains CF35, CF65, CF75 and CF78 were used. For fibroblasts, ET-1 was used at 10^{-7}M and receptor antagonists (for ET_A: BQ123, ZD4054; for ET_B: BQ788) were used at 10^{-6}M. For colorectal cancer cells, ET-1 was used at 10^{-8}M and receptor antagonists were used at 10^{-7}M. Cells were maintained routinely in culture and appropriate cellular concentrations were prepared for the experiments described below.

3.2.2 Proliferation Assay with ET-1 and/or ET_A and ET_B Receptor Antagonists

Cell proliferation in response to ET-1 and receptor antagonists was measured by the colourimetric Methylene Blue (MB) assay. Mechanistically the MB dye is positively charged at pH 8.5 and therefore chelates to negatively charged nucleic acids within cells. Briefly, cells were formalin-fixed, and then incubated with 1% MB (1g in 100ml 0.01M Borate Buffer) for at least 30 minutes. MB that was bound to nucleic acids was then eluted with 0.1M HCl and absorbance was read at 630nm (plate reader, Anthos 2010, Biochrom Ltd, Cambridge, UK). Measurements in the linear scale are proportional to cell numbers (Oliver *et al*, 1989; optimization experiments previously carried out). The initial proliferation technique used was the Alamar Blue Assay, based on the substrate reagent Resazurin metabolised by cellular mitochondria to produce a fluorescent signal. Readings correlate with cell growth. Unfortunately this assay produced variable readings (later transpired to be a plate reader error) and was abandoned (appendix 1 – both proliferation assays).

Depending upon cell type, 96-well flat microtitre plates (Nunc A/S Kamstrupvej, Denmark) were seeded with 10,000 – 15,000 cells/well containing fully supplemented medium. Plates were incubated routinely for 24 hours (CRC cells) or 24-36 hours (fibroblasts) to reach 60-70% confluence. Medium was changed to serum free medium for another 18-24 hours (BSA

containing media for fibroblasts). Medium was then discarded and wells washed twice with PBS. Wells were emptied using a pipette suction vacuum pump. Solutions were made in serum free medium for CRC cell lines or DMEM with 0.5% BSA for fibroblasts. ET-1 was used at 10^{-7}M or 10^{-8}M and receptor antagonists at 10^{-6}M or 10^{-7}M, for fibroblasts and CRC cells respectively. Control wells contained media only. Two plates were used for each individual repeat to accommodate all combinations of agents and solutions were added as shown below for 48 hours and growth assayed by MB (Figure 3.1).

Control	ET-1	ET-1 & BQ123	BQ123	ET-1 & BQ788	BQ788

Control	ET-1	ET-1 & BQ123	BQ123	ET-1 & ZD4054	ZD4054

Figure 3.1. *Schematic of 96 well plate rows used for proliferation experiments*

3.2.3 Migration Assay with ET-1 and/or ET_A and ET_B Receptor Antagonists

The technique employed was based upon a modified 'scratch wound' migration assay (Kernochan *et al*, 2002; Tangkijvanich *et al*, 2001).

Fibroblasts in fully supplemented DMEM were seeded at 100,000 cells/well into 12 well plates (Nunc). Upon achieving near confluence, wells were emptied by vacuum pipetting and pre-incubated with the relevant antagonists in serum free medium for 1 hour. A scratch wound was made across the centre of the well using a 100µl sterile pipette. The medium was replaced with serum-free medium (containing mitomycin C, Sigma-Aldrich) to inhibit proliferation - 1µg/ml for fibroblasts; 0.5 µg/ml for colorectal cancer cells) and ET-1 or ET receptor antagonists (BQ123, ZD4054, BQ788) (Shi-Wen *et al*, 2004) were added to the wells according to the diagram below (figure 3.2) for 24 hours, with the control wells receiving fresh media alone.

Control	Control	ET-1	ET-1
ET-1 & BQ123	ET-1 & BQ123	ET-1 & BQ788	ET-1 & BQ788
ET-1 & ZD4054	ET-1 & ZD4054	ET-1 & ZD4054 & BQ788	ET-1 & ZD4054 & BQ788

Figure 3.2. *Schematic for migration experiment*

Confocal microscopy was employed to photograph wells at 0 hours then at 6 hourly intervals up to 24 hours. This study was repeated for the colorectal cancer cell lines, with 100,000 cells seeded per well and with only 0.5 µg/ml mitomycin C in the media.

Once the photographs were taken, the mean of 3 scratch-width measurements of each well were used to calculate migration. Migrational response was shown in the form a percentage using the following formula:

$$= \left(1 - \frac{sample\ mean\ scratch\ width}{Control\ mean\ scratch\ width\ at\ 0\ hours}\right) X\ 100$$

3.2.4 Contraction Assay with ET-1 and/or ET_A and ET_B Receptor Antagonists

This technique involved growing cultured colonic fibroblast cells in three-dimensionally populated collagen type I lattices (FPCL; collagen from First Link, Birmingham, UK) (Ivarsson *et al*., 1993) (appendix 3).

Each FPCL was impregnated with 100,000 cells and placed into 24 well plates (Nunc). The gel lattices were then pre-incubated for 1 hour with the relevant ET receptor antagonist for wells that were marked to receive either antagonist alone or ET-1 plus antagonist (BQ123, BQ788 and/or ZD4054). Each FPCL was then floated within the well with 1ml of serum free DMEM medium containing ET-1. The control gel was floated in DMEM medium alone. Following 72 hours of incubation at 37^{o}C, they were fixed in 1ml of 10% formaldehyde. Gels were removed and scanned using a flat-bed scanner and weighed in order to determine weight loss corresponding to degree of contraction.

3.2.5 Statistical Analysis

Results are shown diagrammatically either as means and standard deviations of primary measurements (e.g., for fibroblast proliferation, means of 4 independent repeats for each cell strain are shown, n=4) or as percentages of control (100%), for ease of presentation.

Analysis of cell proliferation experiments was carried out using a one-way ANOVA test. If the results were significant, post-hoc analysis was undertaken using Tukey's honestly significant difference test, set at $p<0.05$. Where 4 or less independent repeats were used, Kruskall-Wallis analysis was performed with Dunn's multiple comparison for post-hoc analysis. All graphs are shown with standard deviations.

Floating collagen gel contraction experiments and migration studies were also analysed using a one-way ANOVA test with Tukey's honest significant difference test if n=6 or more. The data is presented as a mean with standard deviations. In cases where migration/contraction was less than n<6, the data was analysed as if they were non-parametric: Kruskal-Wallis analysis was again used (with Dunn's multiple comparison test performed on selected data sets) and the data presented as a median with a range.

3.3 RESULTS

3.3.1 Colonic Fibroblast Proliferation

The effect of ET-1 on cell growth was investigated in four colonic fibroblast strains (CF35, CF65, CF75 & CF78) whilst that of ET receptor antagonists was investigated in two (CF35 & CF78). Fibroblast strains were exposed to ET-1 alone, ET-1 and ET_A or ET_B receptor antagonists (BQ123/ZD4054, BQ788), and each receptor antagonist alone. Fibroblasts exposed to 0.5% BSA medium were used as controls. All strains showed a similar pattern of response with varying degrees of significance depending upon the receptor used.

All individual strains showed a significant increase in growth, as determined by the MB assay, when exposed to ET-1 compared to controls ($p<0.5$, figure 3.3). Growth was stimulated to between 28 – 52% above control values, with an average increase of ~35%. Results (Figure 3.3) are presented as percentage changes from controls (table), or as normalized data with controls set at the value of 1 (graph) for ease of presentation and comparison.

Cell Strain	Control vs ET-1 (10^{-7})	
	% Change	P value(<0.05)
CF35	51.9	Yes
CF65	26.9	Yes
CF75	45.4	Yes
CF78	28.8	Yes
Mean	34.8	Yes

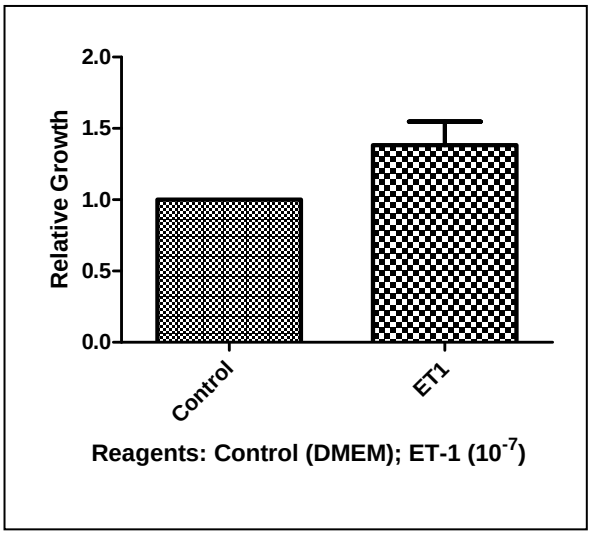


Figure 3.3. *Relative change in colonic fibroblast proliferation on addition of ET-1. Cells were incubated for 48 hours with ET-1 (10^{-7}M) and proliferation determined by the MB assay (absorbance 630nm). Results shown as* ***A:*** *percentage increase from controls (table) or* ***B:*** *normalised (graph); Statistical analysis carried out by 1-way ANOVA with post hoc analysis of raw absorbance readings. N=4 fibroblast strains; 4 independent repeats per strain.*

The cell proliferative effect of ET-1 is demonstrated once again following the use of ET receptor antagonists in two fibroblast strains. The contribution of receptor to the cell proliferative effect of ET-1 was investigated via the use of ET_A and ET_B antagonists. The specific ET_A receptor antagonist BQ123 resulted in significant reduction of ET-1 stimulated fibroblast proliferation for both strains of between 20.3-49.3% (mean values generated by 4-8 repeats for each strain; table 3.1). The addition of the specific ET_A receptor antagonist ZD4054 to ET-1 resulted in a slightly greater inhibitory effect between 20.0-52.9%. Combining the specific ET_B receptor antagonist with ET-1 resulted in a much smaller non-significant reduction in cell proliferation compared to BQ123 or ZD4054. Interestingly, CF35 was much more sensitive to both ET-1 stimulation and ET receptor antagonism than the other one (table 3.1). Results for each treatment are also shown in figure 3.4-3.5.

Receptor Antagonism

Plate 1

Cells	Control	ET-1	ET-1 +BQ123	ET-1 +BQ788	ET-1 vs ET-1 + BQ123		ET-1 vs ET-1 + BQ788	
	Mean Normalised Absorbance (nm)				% Change	P Value	% Change	P Value
CF35	1	1.437	1.222	1.323	-49.3%	p<0.05	-26.1%	NS
CF78	1	1.311	1.231	1.287	-25.6%	NS	-7.7%	NS
Average	**1**	**1.374**	**1.227**	**1.305**	**-37.5%**		**-16.9%**	

Plate 2

Cells	Control	ET-1	ET-1 +BQ123	ET-1 +ZD4054	ET-1 vs ET-1 + BQ123		ET-1 vs ET-1 + ZD4054	
	Mean Normalised Absorbance (nm)				% Change	P Value	% Change	P Value
CF35	1	1.519	1.318	1.245	-38.7%	NS	-52.9%	<0.05
CF78	1	1.288	1.230	1.231	-20.3%	<0.05	-20.0%	<0.05
Average	**1**	**1.404**	**1.274**	**1.238**	**-29.5%**		**-36.5%**	

Table 3.1. *Relative change in ET-1 stimulated colonic fibroblast proliferation on addition of receptor antagonists. Fibroblasts were exposed to ET-1 (10^{-7}M) alone or ET-1 (10^{-7}M) and either ET_A (BQ123/ZD4054; 10^{-6}M) or ET_B (BQ788; 10^{-6}M) receptor antagonists for 48 hours. Cell numbers were determined by the MB assay and compared to untreated controls. Mean normalised absorbance is equivalent to relative cell growth. With controls set at 1. Percentage change reflects the inhibition by ET receptor antagonists of ET-1 stimulated growth. Significant decreases in cell proliferation were observed on the addition of ET_A receptor antagonists (ZD4054> BQ123), but not the ET_B receptor antagonist BQ788. Statistics are based on absorbance values of 4 independent repeats per strain. NS = Non Significant.*

In addition fibroblasts were incubated with specific ET_A or ET_B receptor antagonists alone. Interestingly all the fibroblasts that were incubated with receptor antagonists alone showed a small but non-significant increase in proliferation above untreated controls (figure 3.4 & 3.5). Overall these experiments show that ET_A receptor blockade inhibited significantly the stimulatory effect on fibroblast proliferation of exogenous ET-1. However, there were some strain-specific responses to both type of receptor antagonists, demonstrating the existence of individual responses.

Figures 3.4 & 3.5. *ET-1 induced fibroblast proliferation for each strain. Fibroblasts were exposed to ET-1(10^{-7}M), ET-1 & ET_A receptor antagonists (BQ123 or ZD4054) or ET_B receptor antagonist (BQ788) (10^{-6}M), or antagonists alone for 48 hours. Control groups were exposed to 0.5% BSA medium alone. Cell number was determined by the colorimetric MB assay. Absorbance values (y-axis), corresponding to cell numbers, was measured at 630nm and compared to the untreated controls (100%). Both fibroblast strains showed a similar response to ET-1 and its antagonists. N=4 for each strain. Significance is indicated by the following:*

- *Control versus ET-1* ★
- *ET-1 versus ET1 + BQ123* ★
- *ET1 versus ET1 + BQ788* ★
- *ET1 versus ET1 + ZD4054* ★

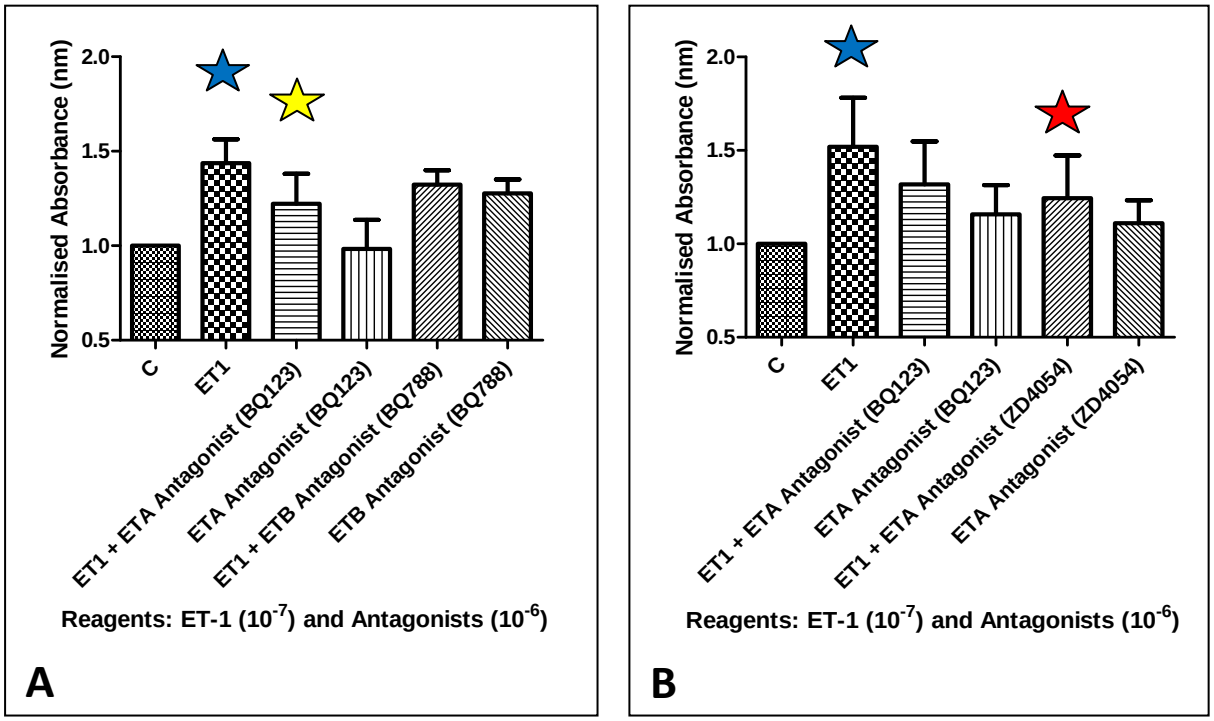


Figure 3.4. *Proliferation of CF35 cells incubated with ET-1 and/or $ET_{A/B}$ receptor antagonists.* ***A:*** *ET-1 vs ET-1+ET_A antagonist BQ123 showed a 49.26% growth reduction ($p<0.05$) whilst ET-1 vs ET-1+ET_B antagonist BQ788 showed a 26.07% reduction (NS).* ***B:*** *ET-1 vs ET-1 +ET_A antagonist BQ123: 38.65% reduction in proliferation (NS). ET-1 vs ET-1+ET_A antagonist ZD4054: 52.91% reduction ($p<0.05$).*

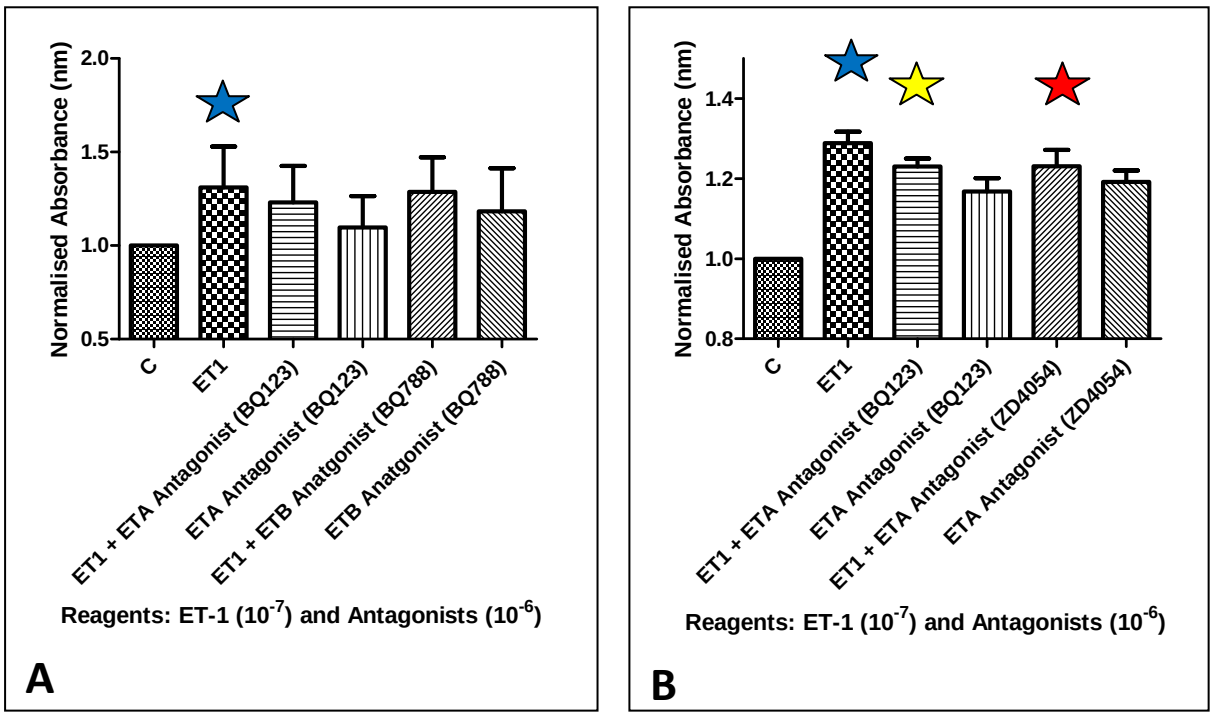


Figure 3.5. *Proliferation of CF78 cells incubated with ET-1 and/or $ET_{A/B}$ receptor antagonists.* ***A:*** *ET-1 vs ET-1 + ET_A antagonist BQ123 showed a 25.65% growth reduction (NS) whilst ET-1 vs ET-1 + ET_B antagonist BQ788 showed a 7.76% reduction (NS).* ***B:*** *ET-1 vs ET-1 + ET_A antagonist BQ123 caused a 20.24% reduction in proliferation ($p<0.05$). ET-1 vs ET-1 + ET_A antagonist ZD4054 caused a 19.98% reduction ($p<0.05$).*

3.3.2 Colorectal Cancer Cell Proliferation

The effect of ET-1 and specific ET receptor blockade on proliferation was also investigated in colorectal cancer cell lines: HT29 and SW620. In these experiments, cells were exposed to ET-1 (10^{-8}M) alone, ET-1 combined with receptor antagonists (ET_A receptor antagonists BQ123 or ZD4054; ET_B receptor antagonist BQ788) (10^{-7}M) or receptor antagonists alone. Controls were exposed to serum free medium alone.

Both cell lines demonstrated a similar pattern of response. The addition of ET-1 (10^{-8}M) resulted in an increase in cell proliferation in both cell lines after 48 hours (figure 3.6). This mean increase ranged from 26-29% compared to control groups and was significant in both cell lines ($p<0.05$).

Cell Line	Control vs ET-1 (10^{-8})	
	% Change	P value(<0.05)
HT29	26.2	Yes
SW620	29.3	Yes
Average	27.75	Yes

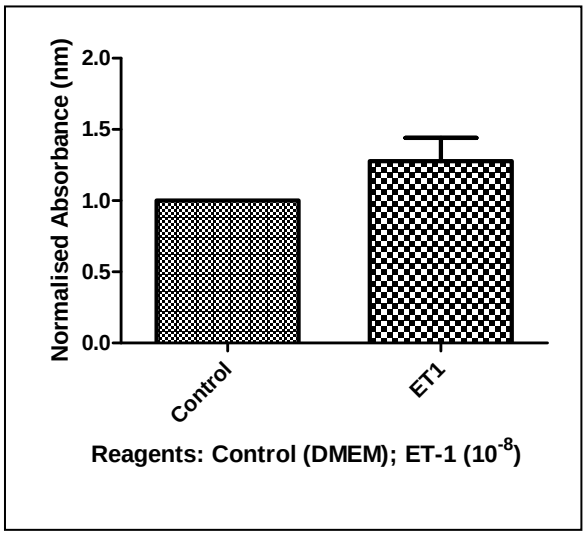


Figure 3.6.

A: *Relative change in CRC cell line proliferation resulting from addition of ET-1. Cells were incubated for 48 hours with ET-1 (10^{-8}) and proliferation determined by MB assay (absorbance 630nm). Results shown as percentage increase above control. Statistical analysis carried out by 1-way ANOVA with post hoc analysis of raw absorbance readings.*

B: *Relative change in proliferation of CRC cell lines depicted as histograms. Control values do not have error bars as numbers were normalised to 1. (N=2 CRC cell lines; 4 independent repeats).*

The receptor contribution to the cell proliferative effect of ET-1 is demonstrated following the use of ET receptor antagonists. Antagonism of ET-1 with the addition of the specific ET_A receptor antagonist BQ123 resulted in significant reduction of ET-1 stimulated proliferation in both cell lines of between 60.9-92.6% (mean values generated by 4-8 repeats for each strain; table 3.2). The addition of the specific ET_A receptor antagonist ZD4054 to ET-1 resulted in a similar inhibitory effect on cell proliferation of between 56.7-71.3%, being significant in both HT29 and SW620 cell lines. Combining the specific ET_B receptor antagonist resulted in a non-significant reduction in cell proliferation which was less then that seen by BQ123 or ZD4054 (table 3.2).

Receptor Antagonism

Plate 1

Cells	Control	ET-1	ET-1 +BQ123	ET-1 +BQ788	ET-1 vs ET-1 + BQ123		ET-1 vs ET-1 + BQ788	
	Mean Normalised Absorbance (nm)				% Change	P Value	% Change	P Value
HT29	1	1.197	1.015	1.127	-92.6%	p<0.05	-35.3%	NS
SW620	1	1.111	1.035	1.084	-68.1%	p<0.05	-23.9%	NS
Average	**1**	**1.154**	**1.025**	**1.106**	**-80.3%**		**-29.6%**	

Plate 2

Cells	Control	ET-1	ET-1 +BQ123	ET-1 +ZD4054	ET-1 vs ET-1 + BQ123		ET-1 vs ET-1 + ZD4054	
	Mean Normalised Absorbance (nm)				% Change	P Value	% Change	P Value
HT29	1	1.171	1.079	1.049	-60.9%	P<0.05	-71.3%	p<0.05
SW620	1	1.441	1.170	1.191	-61.3%	p<0.05	-56.7%	p<0.05
Average	**1**	**1.306**	**1.125**	**1.120**	**-61.1%**		**-64%**	

Table 3.2. *Relative change in ET-1 stimulated colorectal cancer cell line proliferation on addition of receptor antagonists. Cells were exposed to ET-1 (10^{-8}M) alone or ET-1 (10^{-8}M) and either ET_A (BQ123/ZD4054; 10^{-7}M) or ET_B (BQ788; 10^{-7}M) receptor antagonists for 48 hours. Cell numbers were determined by the MB assay and compared to untreated controls. Mean normalised absorbance is equivalent to relative cell growth. With controls set at 1. Percentage change reflects the inhibition by ET receptor antagonists of ET-1 stimulated growth. Significant decreases in cell proliferation were observed on the addition of ET_A receptor antagonists (ZD4054> BQ123), but not the ET_B receptor antagonist BQ788. Statistics are based on absorbance values of 3 independent repeats per cell line. NS = Non Significant.*

Overall these experiments show that ET_A receptor blockade significantly reduced cell proliferation of cancer cell lines which were stimulated by exogenous ET-1. Results for individual cancer cell lines are shown in figure 3.5-3.6.

Figures 3.7 & 3.8. *ET-1 induced proliferation for each cancer cell line. Cancer cell lines were exposed to ET-1(10^{-8}M), ET-1 & ET_A receptor antagonists (BQ123 or ZD4054) or ET_B receptor antagonist (BQ788) (10^{-7}M), or antagonists alone for 48 hours. Control groups were exposed to serum free medium alone. Cell number was determined by colorimetric MB assay. Absorbance values (y-axis), corresponding to cell numbers, was measured at 630nm and compared to the untreated controls (100%). Both cell lines showed a similar response to ET-1 and its antagonists. N=5 for HT29 ET_A receptor antagonists; N=3 for SW620 ET_A receptor and N=3 for each HT29 and SW620 when comparing ET_A and ET_B receptor antagonists. Significance is indicated by the following:*

- *Control versus ET-1* ★
- *ET-1 versus ET1 + BQ123* ★
- *ET1 versus ET1 + BQ788* ★
- *ET1 versus ET1 + ZD4054* ★

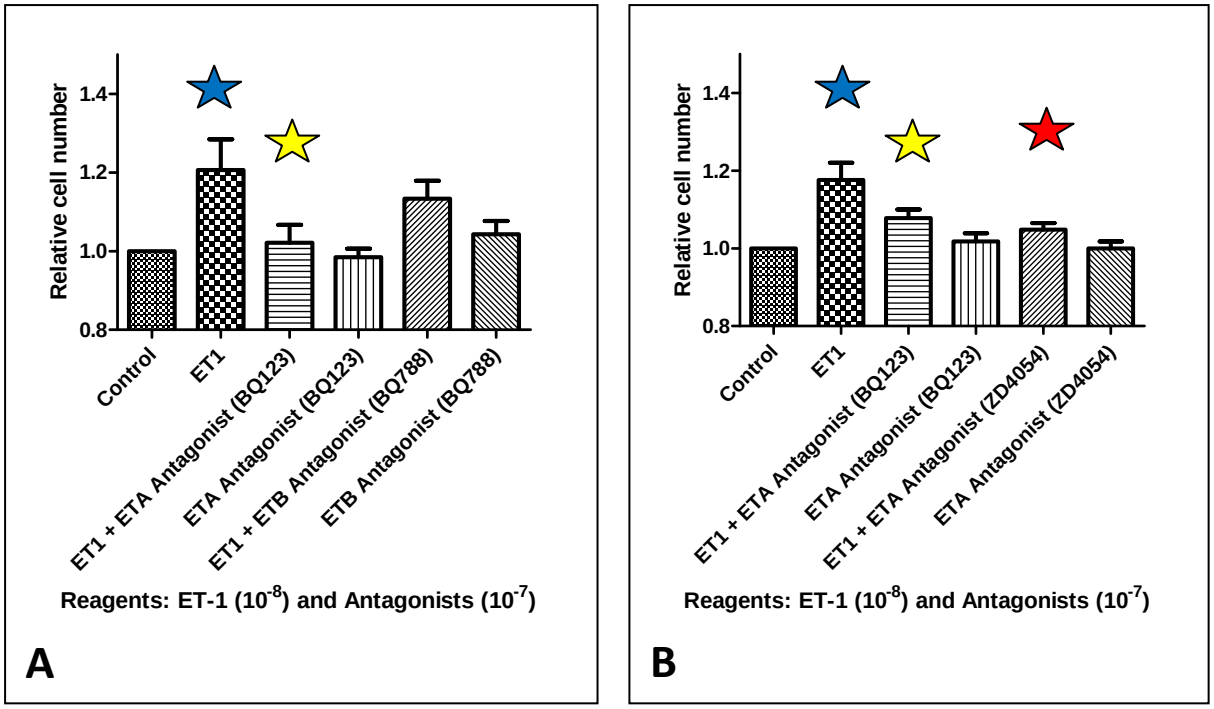


Figure 3.7. *Proliferation of HT29 cells incubated with ET-1 and/or $ET_{A/B}$ receptor antagonists. This graphical representation is produced by five and three independent repeats of this cell line using Methylene Blue assay (graphs A&B respectively). Values are represented using ratios to control.* ***A:*** *ET-1 vs ET-1+ET_A antagonist BQ123 showed a 92% growth reduction ($p<0.05$) whilst ET-1 vs ET-1+ET_B antagonist BQ788 showed a 35% reduction compared to ET-1 induced growth (NS).* ***B:*** *ET-1 vs ET-1 +ET_A antagonist BQ123: 46% reduction in proliferation ($p<0.05$). ET-1 vs ET-1+ET_A antagonist ZD4054: 57% reduction ($p<0.05$).*

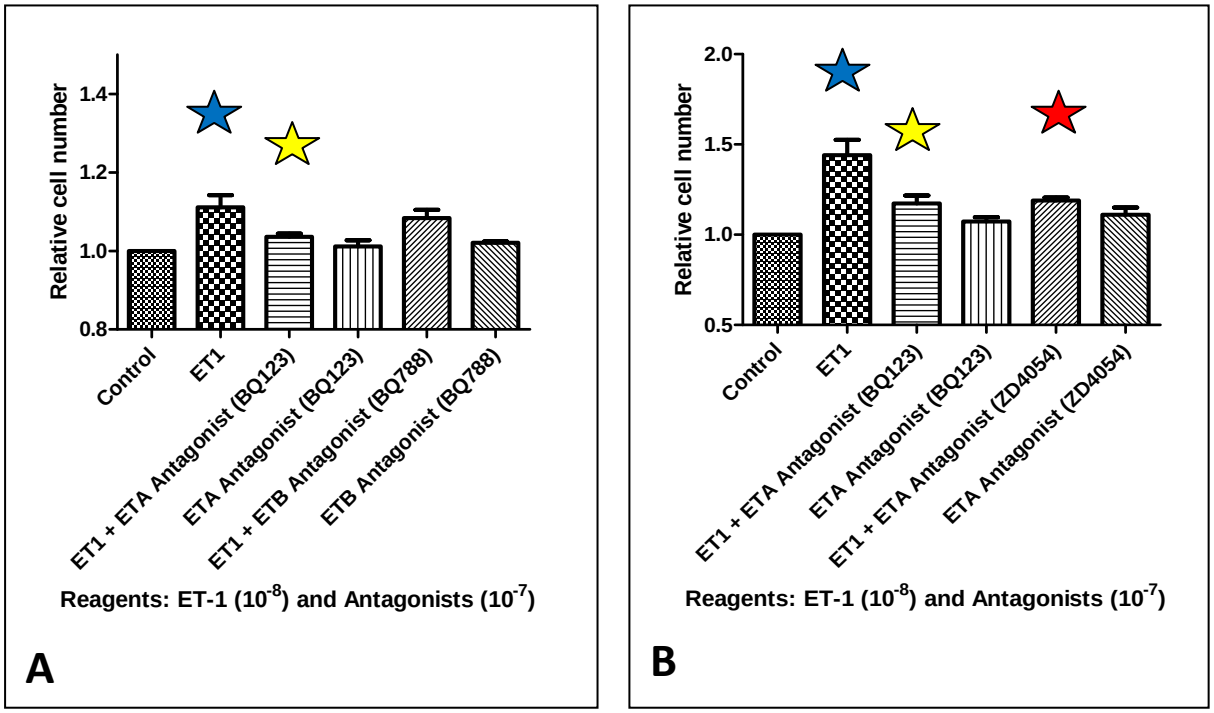


Figure 3.8. *Proliferation of SW620 cells incubated with ET-1 and/or $ET_{A/B}$ receptor antagonists. This graphical representation is produced by three independent repeats of this cancer cell lines using Methylene Blue assay. Values are represented using ratios to control.* ***A:*** *ET-1 vs ET-1 + ET_A antagonist BQ123 showed a 68% growth reduction ($p<0.05$) whilst ET-1 vs ET-1 + ET_B antagonist BQ788 showed 23% reduction (NS).* ***B:*** *ET-1 vs ET-1 + ET_A antagonist BQ123 caused a 61% reduction in proliferation ($p<0.05$). ET-1 vs ET-1 + ET_A antagonist ZD4054 caused a 57% reduction ($p<0.05$).*

3.3.3 Fibroblast and CRC Cell Migration

The effect of ET-1 and ET receptor blockade with antagonists was investigated in five fibroblast strains and two colorectal cancer cell lines. Migration was recorded by light microscopy up to 24 hours after the initial scratch was created through confluent wells (two of five fibroblasts shown in Figure 3.9; combined results in figure 3.10). For fibroblasts, the migration pattern was similar in all of the fibroblast strains: the scratch wound was completely obscured with fibroblasts by 18-24 hours in wells incubated with ET-1 (100% migration), whilst untreated cells only migrated by 13%. ET_B blockade with BQ788 produced the greatest inhibition of ET-1 induced migration (of 71%), with cells migrating into less than a third of the initial scratch width. ET-1 induced migration was partially inhibited by ET_A blockade with ZD4054 and BQ123 by 47-51%, resulting in the cells migrating across half the initial scratch width. However, combined blockade of ET_A and ET_B receptors with ZD4054 and BQ788 produced an effect in-between that seen with either antagonists individually (inhibition by 58.8%, resulting in 41.2% migration). Migration experiments with colorectal cancer cells were negative, since after 24 hours, only minimal migration could be seen in the wells incubated with ET-1. Compared to fibroblasts, colorectal cancer cell lines displayed incredibly limited response to ET-1.

Graphical representation of individual fibroblast strain response to ET-1 and $ET_{A/B}$ receptor antagonism is shown in figures 3.9, with combined results shown in figure 3.10. Photographs showing migratory response in two of the fibroblast strains (CF35 & CF78) and the HT29 cancer cell line are shown in figures 3.11 – 3.13. SW620 behaved very similarly to HT29 cells and results are not shown.

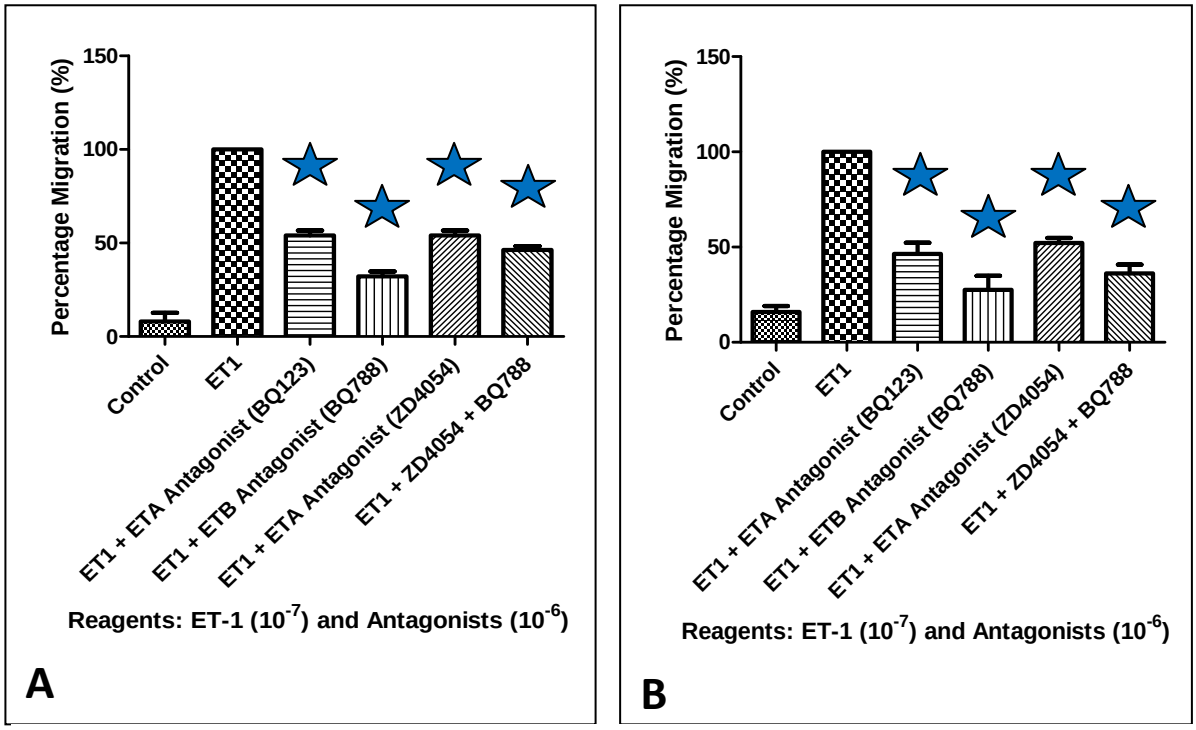
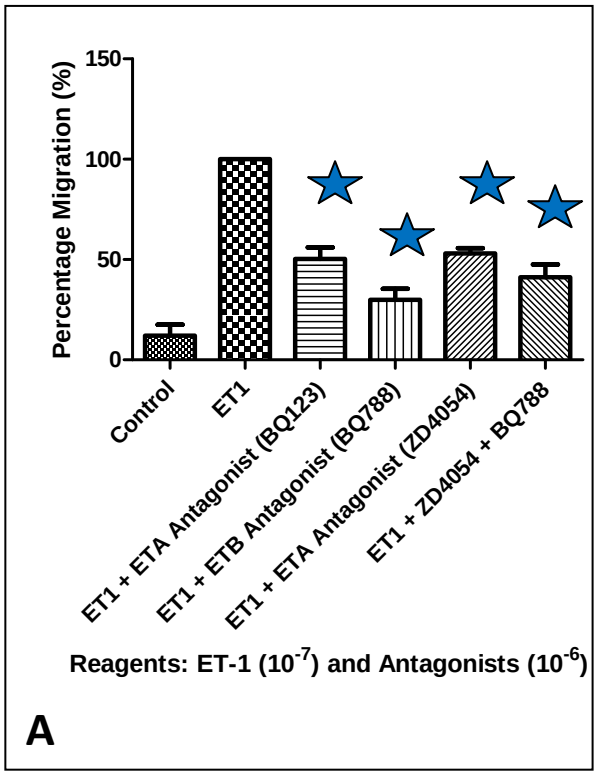


Figure 3.9. *Fibroblast migration following 24 hour exposure to ET-1 and/or $ET_{A/B}$ receptor antagonists.* ***A:*** *CF35 cells.* ***B:*** *CF78 cells. Each graphical representation is for 6 independent repeats of each fibroblast strain. Twenty-four hours of ET-1 exposure resulted in 100% migration of fibroblasts. All receptor antagonists, alone or in combination, resulted in statistically significant reduction in migration ($p<0.05$). The greatest decrease in migration was seen with BQ788 antagonism.* ★ *=significance versus ET-1*



Antagonist	Migrational Reduction c.f. ET-1 alone	
	% Decrease	Significance
BQ123	49.8	$p<0.05$
BQ788	70.1	$p<0.05$
ZD4054	46.9	$p<0.05$
ZD4054+BQ788	58.8	$p<0.05$

B

Figure 3.10. ***A:*** *Graphical representation of migration for all five fibroblast strains combined, each with 6 independent repeats, following 24 hours of exposure to ET-1 (10^{-7}M) or ET-1 + receptor antagonists (BQ123, BQ788 and ZD4054, 10^{-6}M).* ***B:*** *Table showing percentage decrease in migration from ET-1 induced migration set at 100%. ET_A blockade with BQ123 and ZD4054 produced a 50% and 47% decrease in migration respectively ($p<0.05$).*

Most inhibition was produced with ET_B blockade of 70% with BQ788 ($p<0.05$). Combined ET_A and ET_B blockade with ZD4054 and BQ788 resulted in a 59% reduction in migration ($p<0.05$). n=6. ★ *=significance versus ET-1*

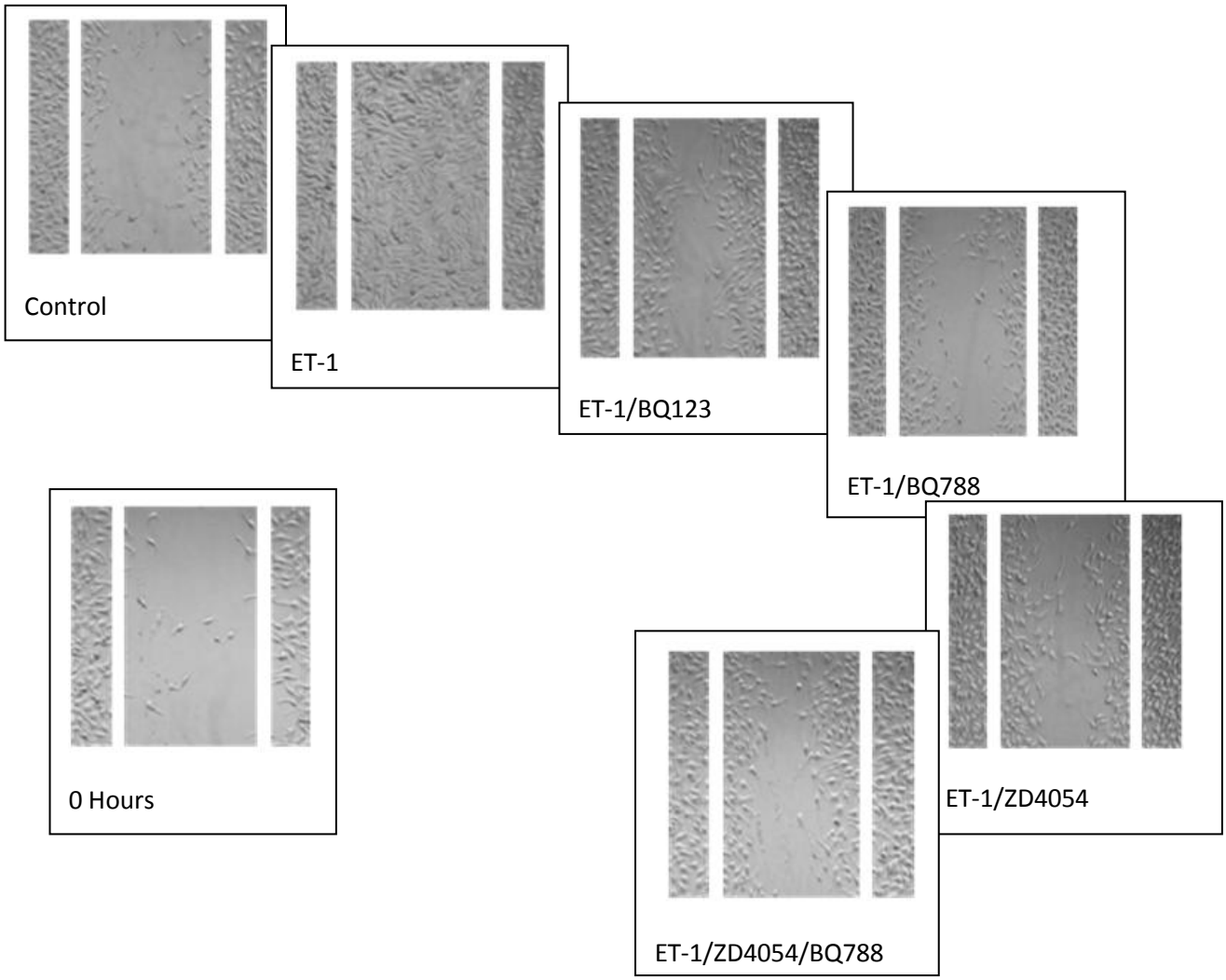


Figure 3.11. *Migration of CF35 fibroblast strains in response to ET-1 and ET receptor antagonists (BQ123, BQ788 and ZD4054) following 24 hours of exposure. Fibroblasts were grown to confluence then scratch wounds made through the wells. Cells were then exposed to ET-1 (10^{-7}M) alone or ET-1 and $ET_{A/B}$ receptor antagonists (BQ123, ZD4054,BQ788; 10^{-6}M). Photographs were taken at 0 and 24 hours. Complete fibroblast migration was seen in ET-1 exposed cells alone. Marked inhibition was observed through ET_B receptor blockade, with a lesser extent of blockade through ET_A and combined receptor blockade.*

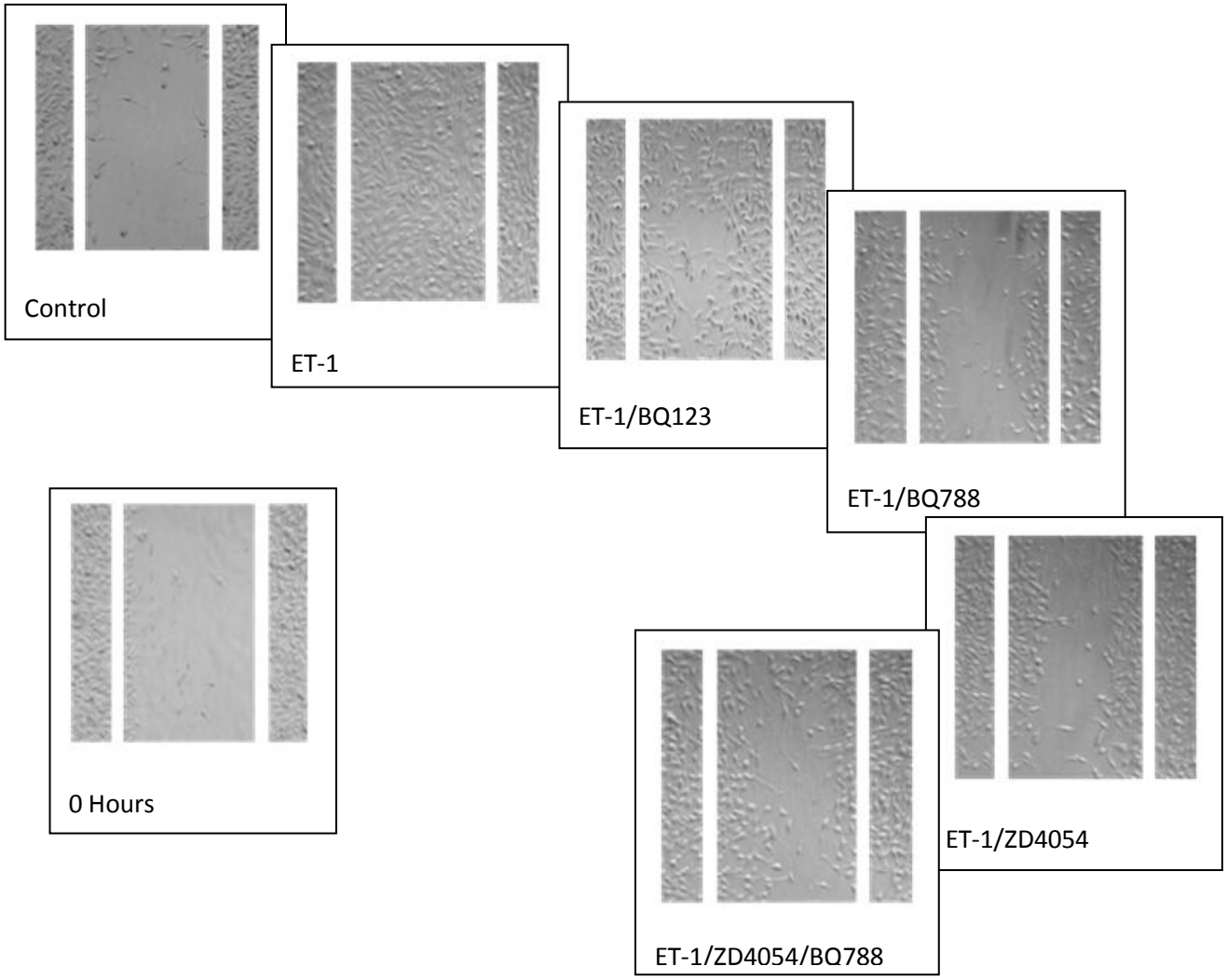


Figure 3.12. *Migration of CF78 fibroblast strains in response to ET-1 and ET receptor antagonists (BQ123, BQ788 and ZD4054) following 24 hours of exposure. Fibroblasts were grown to confluence then scratch wounds made through the wells. Cells were then exposed to ET-1 (10^{-7}M) alone or ET-1 and $ET_{A/B}$ receptor antagonists (BQ123, ZD4054,BQ788; 10^{-6}M). Photographs were taken at 0 and 24 hours. Complete fibroblast migration was seen in ET-1 exposed cells alone. Marked inhibition was observed through ET_B receptor blockade, with a lesser extent of blockade through ET_A and combined receptor blockade.*

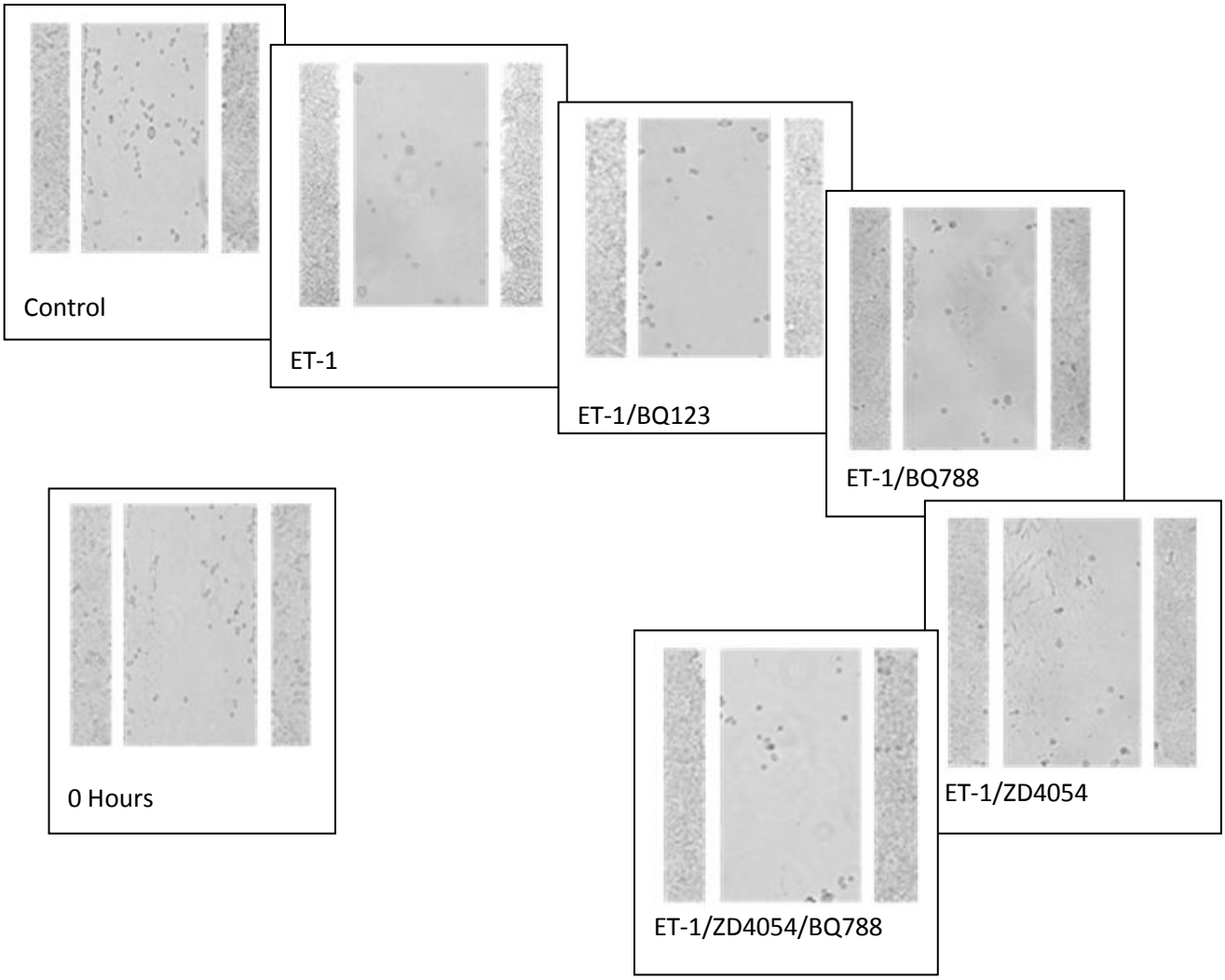


Figure 3.13. *Migration of* HT29 colorectal cancer cell line *in response to ET-1 and ET receptor antagonists (BQ123, BQ788 and ZD4054) following 24 hours of exposure. Cancer cell lines were grown to confluence then scratch wounds made through the wells. Cells were then exposed to ET-1 (10^{-8}M) alone or ET-1 and $ET_{A/B}$ receptor antagonists (BQ123, ZD4054,BQ788; 10^{-7}M). Photographs were taken at 0 and 24 hours. Following 24 hours of exposure, minimal migration was observed in all wells.*

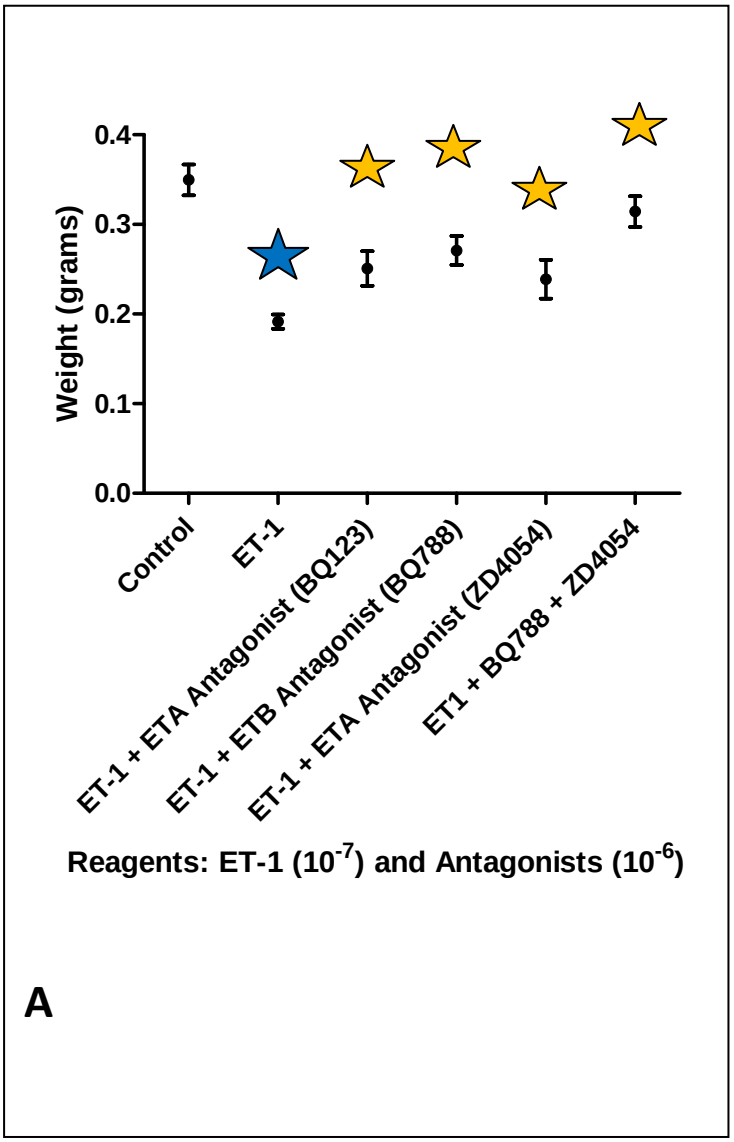
3.3.4 Colonic Fibroblast and CRC Cell Contraction

The effect of ET-1 and its receptor antagonists on contraction was investigated in rat collagen gels containing either colonic fibroblasts or HT29 colorectal cancer cells. Contraction over 72 hours was recorded photographically and concomitant reduction in gel weight was measured with control gels set at 100%. ET-1 caused gel contraction in both fibroblast strains investigated, with a relative gel weight of 56.55%. Therefore ET-1 reduced gel weights by ~43% (mean range varying from 41-45%) (Table 3.3).

Cell strain	**Control vs ET-1 10^{-7} M**	
	% Decrease	P Value
CF65	45.3	<0.05
CF75	41.8	<0.05
Average	43.5	

Table 3.3. *Contraction of fibroblast-impregnated collagen gels in response to ET-1 (10^{-7}M) over 24-72 hours. Extent of contraction was determined by reduction in gel weight when compared to controls (taken as 100%).*

The addition of receptor antagonists, either against ET_A or ET_B, opposed ET-1 induced contraction to varying degrees. All three antagonists separately reversed contraction significantly. The ET_B receptor antagonist (BQ788) was most effective at reversing ET-1 induced contraction in the CF65 fibroblasts, whilst the ET_A receptor antagonist (BQ123) was most effective in the CF75 fibroblasts (77.49% and 80.58% respectively). ZD4054 showed the least inhibition of contraction following ET-1 exposure with a relative weight from control of 68.33% and 68.11% in CF65 and CF75 fibroblasts respectively. In both fibroblast strains, combined ET_A and ET_B receptor antagonism (ZD4054 plus BQ788) resulted in the most marked blockade of gel contraction, returning gel weights to 89.27%. Contraction studies were also performed using HT29 colorectal cancer cells. ET-1 did not stimulate gel contraction and no significant changes in gel weight were seen with addition of individual antagonists or in combination.



All with ET-1 present	%Contraction of control size gel* (*Control gel set at 100%)	
	%	Significance
ET-1	54.7	P<0.05**
BQ123	71.7	P<0.05*
BQ788	77.5	P<0.05*
ZD4054	68.3	P<0.05*
BQ788+ZD4054	89.9	P<0.05*

*** = contraction compared to control*

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

B

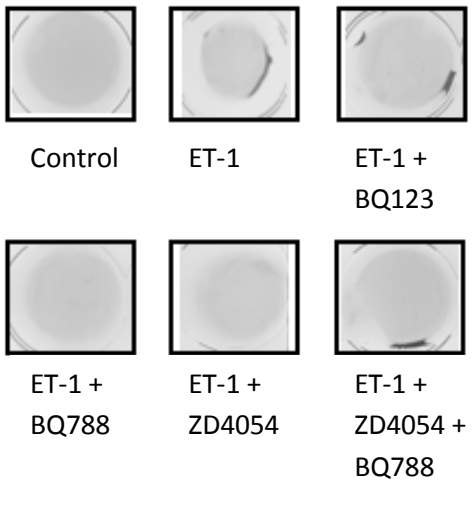
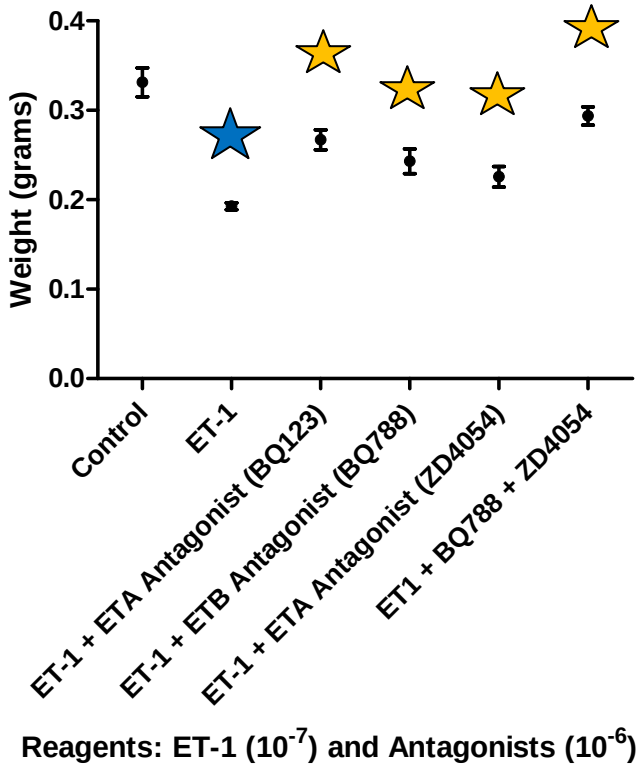


Figure 3.14. *Contraction of CF65 fibroblast impregnated gels in response to ET-1 and ET receptor antagonists (BQ123, BQ788 and ZD4054). Following fibroblast impregnation and 1 hour preconditioning with relevant antagonists, they were exposed to ET-1 (10^{-7}M) alone or combined with ET_A (BQ123/ZD4054) or ET_B (BQ788) receptor antagonists (10^{-6}M). Controls were left untreated. Following 72 hours, collagen lattice gels were removed from wells and weighed.* ***A:*** *Weight of each of the collagen gels following exposure to their relevant reagents and correspond to level of contraction.* ***B:*** *Percentage contraction of collagen lattices compared to control following exposure to ET-1 and relevant antagonists.* ***C:*** *Photographs of fibroblast* impregnated *collagen lattices following 48 hours of exposure, placed on glass slides. ET-1 cause marked contraction of collagen gels resulting in reduced gel diameter, which was significantly inhibited by all antagonists. N=3.* ★ *= significant vs. control values* ☆ *= significant vs. ET-1 values*



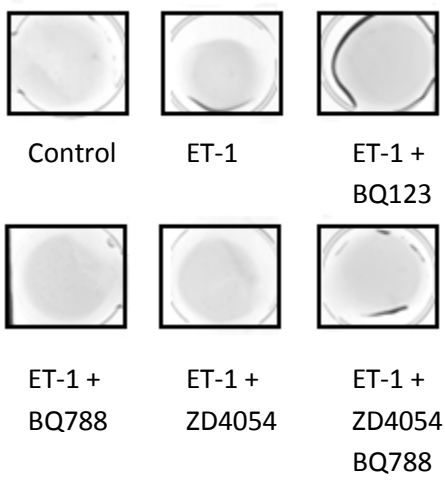
A

All with ET-1 present	%Contraction of control size gel* (*Control gel set at 100%)	
	%	Significance
ET-1	58.2	P<0.05**
BQ123	80.6	P<0.05*
BQ788	73.3	P<0.05*
ZD4054	68.1	P<0.05*
BQ788+ZD4054	88.6	P<0.05*

*** = contraction compared to control*

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

B



C

Figure 3.15. *Contraction of CF75 fibroblast impregnated gels in response to ET-1 and ET receptor antagonists (BQ123, BQ788 and ZD4054). Following fibroblast impregnation and 1 hour preconditioning with relevant antagonists, they were exposed to ET-1 (10^{-7}M) alone or combined with ET_A (BQ123/ZD4054) or ET_B (BQ788) receptor antagonists (10^{-6}M). Controls were left untreated. Following 72 hours, collagen lattice gels were removed from wells and weighted.* ***A:*** *Weight of each of the collagen gels following exposure to their relevant reagents and correspond to level of contraction.* ***B:*** *Percentage contraction of collagen lattices compared to control following exposure to ET-1 and relevant antagonists.* ***C:*** *Photographs of fibroblast impregnated collagen lattices following 48 hours of exposure, placed on glass slides. ET-1 cause marked contraction of collagen gels resulting in reduced gel diameter, which was significantly inhibited by all antagonists. N=3.* ★ *= significant vs. control values* ☆ *= significant vs. ET-1 values*

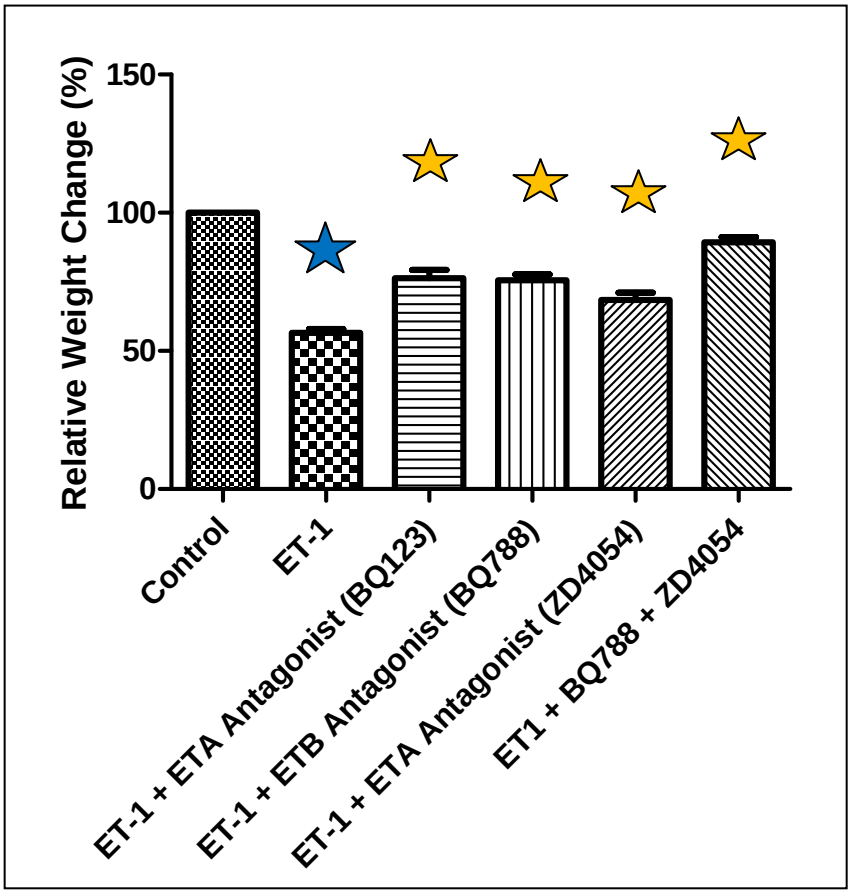


Figure 3.16. *Combined CF65 and CF75 fibroblast strain data for response to ET-1 and its receptor antagonists. Relative weight change compared to controls (100% weight) were calculated for each strain then combined for the above graph (y-axis). ET-1 stimulation induced a 44% reduction in gel weight ($p<0.05$). This effect was significantly inhibited by both ET_A and ET_B receptor antagonists (BQ123, ZD4054, BQ788; $p<0.05$), with the most marked effect seen with combined receptor blockade ($p<0.05$).*

★ *= significant vs. control values* ☆ *= significant vs. ET-1 values*

3.4 DISCUSSION

3.4.1 Proliferation Studies

In the *in vitro* studies presented, both colonic fibroblasts and colorectal cell lines demonstrated significant growth compared to control groups when treated with ET-1. Previous work from our group has shown similar results with fibroblast strains, but only one out of six strains demonstrated a significant increase in growth compared to controls (Knowles *et al*, 2011). In this study only two of the previously tested fibroblasts strains were used – the strain that responded to ET-1 and one other. Also, the protocol was changed to include a 24 hour period of starvation of cells prior to addition of exogenous ET-1, to maximize any potential stimulus. This resulted in a significant growth effect. The only other study that examined growth of colonic fibroblasts in response to ET-1 found small non-significant increases in cell numbers compared to control groups (Kernochan *et al*., 2002). Differences may be explained both by the different protocols employed and also by the nature of the fibroblasts: ours where isolated from adult colons, while the previous study employed a cell line (No Co18) isolated from a 2.5 month old (Kernochan *et al*., 2002). The effect of ET-1 on fibroblasts taken from patients with different tumours has also been investigated, although there has only been two published study to date. In the first, ET-1 stimulated significant growth increases of three ovarian fibroblast cell lines at concentrations ranging from 10^{-12} M to 10^{-7} M (Moraitis *et al*, 1999). The most recent study demonstrated ET-1 induced proliferation in primary oral fibroblasts (Hinsley *et al.,* 2012). Furthermore, fibroblasts isolated from non-tumour sites such as human dermal and rat cardiac fibroblasts also demonstrated a proliferative effect from ET-1 via the ET_A receptor (Piacentini *et al*., 2000; Xu *et al*., 1998).

The mitogenic role of ET-1 on colorectal cancer cell lines has been well documented and the present results using HT29 and SW620 cells are consistent with previous work by our group which also published similar results with the LIM1215 cell line (Ali *et al*., 2000a; Grant *et al*., 2007). ET-1 was reported to stimulate growth in various different cell lines including those from ovarian and prostate cancer, via activation of the ET_A receptor (Bagnato *et al.,* 1999; Nelson 1996). Furthermore, ET-1 can propagate its signal via EGFR transactivation when the receptor is present on colorectal cancer cells (Grant *et al.*, 2007). The striking exception is within melanomas, where ET-1-stimulated growth of melanocytes occurs via activation of the ET_B receptor (Lahav *et al*., 2004).

Interestingly the observed extent of proliferation, although significant, varied between cell lines. This may be related to varying endogenous levels of ET-1 in different cell lines

affecting their response to exogenous stimulation. In colorectal cancer cell lines, HT29 cells produced the highest levels of endogenous ET-1 with 41.7 fmol/ml/10^6 cells, whilst SW620 had moderate expression levels at 8.4 fmol/ml/10^6 cells (Ali *et al*., 2000a). Same order of magnitude figures were reported in other publications with HT29 producing 23 fmol/ml/10^6 cells (Kusahara *et al.,* 1990) and ovarian cell lines around 56 – 74 fmol/ml/10^6 cells (Bagnato *et al*., 1995). However, a systematic investigation to determine response to ET-1 should include both determination of endogenous ET-1 production and also quantification of the ET receptors on the cell surface.

Cell growth was reduced by the ET_A receptor antagonists BQ123 and ZD4054 in both fibroblast strains. Also when combined, both BQ123 and ZD4054 demonstrated significant reductions in cell growth compared to ET-1 treated cells. Overall, the effects of these two ET_A antagonists were similar, indicating that ZD4054 is as efficacious in blocking ET_A-receptor mediated-growth of colonic fibroblasts. The ET_B receptor antagonist BQ788 produced a lesser reduction in cell growth, which was not significant in individual strains or when combined. Other studies from our department have shown occasionally that the ET_B receptor antagonist does produce a significant inhibitory effect on proliferation (unpublished; Bhalla, 2008; Heetun, 2009). This may be explained by some ET_A receptor blockade by BQ788. The affinity of BQ788 for ET_B receptors is only tenfold greater than its affinity for ET_A receptors and therefore it cannot be considered a highly specific ET_B antagonist. In contrast, the ET_A receptor antagonist BQ123 has 1000-10,000 times greater affinity for the ET_A receptor than the ET_B receptor (Davenport, 2002). No studies have been published documenting the effect of ZD4054 on colonic fibroblasts, but in the Moraitis study (1999) ET-1-induced growth of fibroblasts from patients with ovarian cancer was inhibited by the ET_A receptor antagonist BQ123 and ET_B receptor antagonist BQ788. Interestingly, this suggests that ET-1 stimulates the growth of these cells via both receptors.

A similar pattern of results was seen with the colorectal cancer cell lines. Both individual and combined results from HT29 and SW620 cells, demonstrated ET_A receptor antagonists BQ123 and ZD4054 significantly reduced cell growth compared to those cells incubated with ET-1 only. Other cell lines such as prostate and ovarian have been shown to be similarly affected by ET_A antagonism. Furthermore, some of the ET-1 proliferative signal appears to be promoted through transactivation of EGF receptors (Grant *et al*., 2007; Rosano *et al*., 2007).

Both fibroblasts and colorectal cancer cell lines were also incubated with receptor antagonists alone. The rationale for this was based on previous work in the department,

which found that endogenously produced ET-1 was an important mitogenic stimulus for fibroblast growth that was not greatly increased by the addition of exogenous ET-1. In this study (Knowles *et al.*, 2011), the use of the ET_A receptor antagonist BQ123 significantly reduced fibroblast growth compared to controls. The author suggested that this showed that there were a finite number of receptors on the fibroblasts which appeared to be almost totally occupied by endogenous ET-1. The results in this thesis appear to oppose this as addition of ET-1 to both fibroblasts and colorectal cell lines significantly increased cell growth above control levels. Furthermore, use of ET_A receptor antagonists did not reduce cell growth below those of the control group, as would be expected if endogenous ET-1 was the more important mitogenic stimulus. Consequently, although these cells continued to express ET receptors, exogenous ET-1 appeared to be more important in stimulating the growth of both these cell groups. Most likely this discrepancy is related to the altered protocol of serum starving the cells prior to adding exogenous ET-1, unlike in the previous publication. Another plausible explanation may be related to the higher passage numbers of cells used in this experiment which may have had an effect on endogenous ET-1 expression, but with little effect on ET receptor expression. Indeed, primary cell strains are known to change morphology and biochemistry with increased passage (Valentich *et al*., 1997) and unpublished data from our laboratory shows a similar pattern.

Overall, the cell growth studies suggest that ZD4054 is as efficacious at antagonising the ET_A receptor in both fibroblasts and colorectal cancer cells as BQ123. To date, there have been no studies investigating the effect of ZD4054 on colonic fibroblasts and colorectal cancer cells. One group reported on the inhibition of ET-1-induced cell growth of ovarian cancer cell lines by ZD4054 with a reduction in ET_A-mediated angiogenesis and the generation of invasive mediators such as VEGF and MMPs. (Rosano *et al*., 2005, 2007). ZD4054 has also been shown to enhance the pro-apoptotic activity of existing chemotherapeutic agents such as Paclitaxel (in an *in vitro* and *in vivo* model of ovarian cancer (Rosano *et al*., 2007), indicating a possible role for combination therapy with other agents.

3.4.2 Migration Studies

Migration of fibroblasts is thought to be a defining characteristic of this cell type and also an integral part of a number of physiological and pathological responses, mostly on wound healing. However, in laboratory settings, migration is not always observed. Fibroblast migration is a complex process involving alterations in cell shape and adhesion, which are

differentially controlled at the leading and trailing edge of the cell (Lauffenburger and Horwitz, 1996). Here, five fibroblast strains were investigated in terms of their migratory response to ET-1 and its receptor antagonists. All strains migrated rapidly when incubated with ET-1 alone and this effect was blocked by all three receptor antagonists to varying degrees. The most marked inhibition of migration was caused by the addition of the ET_B receptor antagonist BQ788. Combined ET_A and ET_B receptor blockade with ZD4054 and BQ788 produced a migratory effect in-between that seen for the ET_A and ET_B receptor antagonists individually. Taken together, these results suggest that ET-1-induced fibroblast migration is mediated via both receptors, but perhaps the greatest effect occurs at the ET_B receptor. When the results are combined from each strain, it can be seen that ZD4054 produces a very similar inhibitory effect on fibroblast migration to BQ123 overall.

The only previous study involving human colonic fibroblasts showed that ET-1 stimulated migration predominantly via the ET_B receptor and this was blocked significantly by the ET_B antagonist BQ788; in contrast, ET_A blockade resulted in minimal effects (Kernochan *et al*., 2002). Previous unpublished work in the department showed a similar pattern of response with ET_B receptor antagonism generating the greatest inhibition of migration followed by ET_A receptor antagonism, but combined receptor blockade inhibited migration more markedly (Knowles *et al*., 2006; Bhalla *et al*., 2008). This is consistent with both ET_A and ET_B receptors being important in the migratory response, with the authors suggesting that perhaps different aspects of the migration process are controlled by the different ET receptors. The work presented here is generally consistent with these previous findings, except that combined receptor blockade did not elicit the greatest inhibitory effect on migration as would have been expected if both receptors affected different aspects of the migration process.

Work has been done to look at different fibroblast responses to other stimuli. Jiang *et al*., (2008) looked at dermal foreskin fibroblasts and the response to PDGF, serum lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P) and endothelin-1. They found that PDGF promoted a strong migratory effect through activation of Rac causing protrusion of fibroblast dendritic extensions. The latter three peptides, including ET-1, were Rho activators which resulted in retraction of dendritic extensions and contraction. Similarly, Kinnman *et al*., (2000) found that PDGF had a 3 fold increase in hepatic stellate cell migration while ET-1 only had a 1.7 fold increase. They found that anti-PDGF antibodies and PDGF receptor tyrosine kinase inhibitors resulting in significant migratory inhibition (60% and 80% respectively), whilst dual receptor antagonism with Bosentan had non-significant inhibitory effects. Of interest, in chapter 4 of this thesis, gene array data shows that ET-1

stimulated PDGF expression by over 1.5 fold. This may lead us to hypothesis that the migratory response may be a result of ET-1, second messengers and other regulated peptides such as PDGF. In keeping with this hypothesis, Shlyonsky *et al*., (2011) showed that ET-1 increased α-smooth muscle actin and cell migration in lung fibroblasts. They also found that BMP-2, which inhibits PKC in a dose dependent manner, inhibited ET-1 ability to increase α-smooth muscle actin and migration.

The HT29 cancer cell line did not display any migratory response. No research group had specifically published on ET-1 effects on HT29 cells. However, these and other CRC cell lines have been extensively studied in other settings. Colorectal cancer cell lines are known to express increased levels of CXCR4 that are required for migration, whilst decreasing expression of CXCL12. Rubie *et al*., (2011) examined the response of HT29, SW480 and CaCo2 cell lines in response to increased CXCL12. They found that migration was increased in all cell lines examined however to a much lesser extent in HT-29 cell lines as CXCR4 expression was less. This may be one reason why migration was not observed in this particular cell line. HT29 cell lines also express MMP7 and LN5 (Laminin-5/Laminin-332), the latter which causes firm adhesion and hemi-desmosome formation. Remy *et al*., (2006) found that increasing MMP7 concentrations cleaved LN5 and therefore increased cell motility. In chapter 4, MMP7 was seen to be significantly up-regulated in cancer cell lines on gene arrays and time point induction experiments that were carried out. However, significant increased levels were only seen at 24 hours which was the maximum time point observed in these migration studies. It is possible that extending the time of these experiments may have yielded better migratory responses. In keeping with this Banning *et al*., (2008) described how Glutathione Peroxidase 2 which is up-regulated in CRC cell lines inhibited COX2 (which ET-1 also acts via down-stream) mediated HT29 migration. Silencing of this selenoprotein resulted in increased HT29 migration on scratch wound assays. Most of this was observed after 24 hours and up to 72 hours. Other experimental factors may have accounted for these observations. For example some other research groups that looked specifically at cancer cell lines used 1% BSA whereas we used 10% FCS, and where we used Mitomycin C some other groups used Cycloheximide to inhibit cell proliferation.

3.4.3 Contraction Studies

In the Kernochan study (2002), ET-1 was shown to stimulate colonic subepithelial fibroblast contraction via both ET_A and ET_B receptors and this was associated with an increase in cytosolic calcium and myosin phosphorylation. The authors used the same receptor

antagonists (BQ123, BQ788) and found they both significantly inhibited ET-1 stimulated contraction by similar levels, whilst incubation with both antagonists together completely abolished ET-1 stimulated contraction. Here, similar results were achieved with the fibroblasts used. Incubation with ET-1 resulted in significant contraction, with both ET_A and ET_B antagonists inhibiting contraction similarly. Combined ET_A and ET_B receptor blockade produced the greatest inhibition of contraction compared to those cells incubated with antagonists alone, although the contractile response was not completely abolished. These findings support previous reports from within our department. Although ZD4054 was able to inhibit ET_A-mediated fibroblast contraction individually, it did so to a lesser degree than both the ET_A receptor antagonist BQ123 and the ET_B receptor antagonist BQ788. One possible explanation is that contraction may be a response to stimulating both receptors. BQ123 and BQ788 do partially act by blocking both receptors whilst ZD4054 is so highly specific that it only blocks the ET_A receptor. In other tissues, ET-1 has been reported to cause contraction of primary lung fibroblasts, although this response appeared to be mediated by the ET_A receptor predominantly, rather than both receptors (Shi-Wen., 2004).

A large volume of publications exist on the contractile abilities and stimuli of fibroblasts, since contraction is an integral step of connective tissue diseases and cancer invasion. Several growth factors have been shown to promote matrix contraction, namely TGF-β (Tingstrom *et al*., 1992), Sphingosine-1-phosphate (S1P; Cooke *et al*., 2000), EGF (Smith *et al*., 2006), bFGF (Abe *et al*., 2007) and ET-1 (Guidry *et al*.,1991). ET-1 and ET-2 have been shown to bind to high affinity receptors (mostly ET_A) on human fibroblast cell surfaces rapidly increasing free calcium and stimulating PLC and PKC (Takuwa *et al*., 1989). Endothelins also act synergistically to amplify fibroblast response to growth factors such as PDGF, bFGF and insulin like growth factors (Takuwa *et al*., 1989). Mechanistically, Guidry *et al*., (1991) used cycloheximide to inhibit ^{35}S-methionine to establish that ET-1 was a type B contraction promoter therefore requiring protein synthesis to establish cell contraction. This group also suggested that the delay in contraction may have indicated that ET-1, PDGF and TGF-β may also act via second messengers or multiple systems. Furthermore withdrawal of ET-1 did not reverse collagen gel contraction immediately but took over 1 hour, indicating that it promotes contraction via an effector protein or ET-1 acts as a co-factor. ET-1 is also over-expressed in fibrotic fibroblasts isolated from patients with connective tissue diseases where it enhanced adhesion to and contraction of matrix by this cell type (Shi-Wen *et al*., 2004, 2006, 2007).

3.5 Studies to date looking at ET-1 and its effect on ET receptors

Cancer Type	ET-1 Effect	Receptor Involvement	Receptor Antagonism Effect & Clinical Effects	Reference
Prostate	Growth, inhibits apoptosis	High ET_AR, decreased/absent ET_BR. Frequent methylation of ET_BR gene	Pain relief via ET_AR & clinical and biochemical progression.	Nelson *et al*. Antonarakis *et al*. 2010 James *et al*. 2010
Ovarian	Cell proliferation, survival, angiogenesis, invasion & EMT via ET_AR	ET_AR mRNA in 84% of Ca. & ET_BR in only 40%. ET_AR mediates all effects	Antitumor effect in xenografts via ET_AR; Zibotentan+cisplatinum+paclitaxel effective for epithelial ovarian cancers	Bagnato *et al*. 2005 Rosano *et al*. 2007a, 2007b
Melanoma	ET-1 (& ET-3) promotes proliferation and invasion	ET_AR decrease & ET_BR increase compared to benign nevi	ET_BR inhibited growth in cell lines & reduced tumour growth in nude mice; ET-1 inhibits prolyl hydroxylase domain 2 to activate HIF-1α in melanoma cells. Bosentan has benefits in disease stable disease.	Spinella *et al*. 2010 Guise *et al*. 2004 Kefford *et al*. 2007
Osteosarcoma/ Bone malignancies	Increased osteocalcin & new bone formation; MMP induction via NFkB	Mostly ET_AR expression but also ET_BR	ET_AR antagonists block ET-1 effects & MMP; inhibits progression of prostate cancer metastasis	Grimshaw *et al*. 2007 Guise *et al*. 2004, 2006
Breast	Increased invasiveness; levels inversely correlate with tumour cell differentiation	Elevated ET_AR correlates with invasiveness	Blocks invasive effect; MMP/cytokine cross-talk; ZD4054 exhibit additive effects with aromatase inhibitors & fulvestrant in cancer therapy. Improved *in vivo* efficacy of anastrozole	Grimshaw *et al*. 2005 Binder *et al*. 2009 Smollich *et al*. 2010 Smollich and Wulfing 2007
Renal	Opposes paclitaxel induced apoptosis in renal carcinoma cell lines	All express ET_AR	Blocks invasive effect	Pflug *et al*. 2007 Antonarakis *et al*. 2010
Lung	Detected in most squamous cell & adenocarcinomas	ET_AR & ET_BR are expressed	ET_AR antagonists combined with EGFR show promise in treatment of non-small cell lung cancer	Boldrini *et al*. 2005 Campbell *et al*. 2010
Cervical	Proliferation of HPV positive	Express both receptors; increase in	Inhibition of tumour growth in xenografts	Bagnato *et al*. 2002

	cervical cancer cell lines	ET_AR on HPV- positive cells	and additive effect when combined with taxanes;	Guise *et al*. 2004
Kaposi's Sarcoma	ET-1 (& ET-3) induces proliferation, migration and invasion	Both $ET_{A/B}R$ expression	Dual antagonists block proliferation & invasion; inhibits tumour growth in nude mice	Guise *et al*. 2004 Rosano *et al*. 2003
Glioblastoma	Proliferation	$ET_{A/B}R$ expression	ET_BR antagonists block proliferation and induce apoptosis in glioma cells. Bosentan induce apoptosis in glioblastoma cell lines	Paolillo *et al*. 2010 Egidy *et al*. 2000
Neuroblastoma	Proliferation	$ET_{A/B}R$ expression	Cells express ECE-1; BQ123 blocks proliferation *in vitro*; statins induce Bcl-2 via ET-1 and NFATc3 in SH-SY5Y cells	Grimshaw *et al*. 2007
Nasopharyngeal		Three quarters over-express ET_ARs	ET_AR antagonist inhibits tumour growth and metastasis, showing synergic effects with cytotoxic drugs	Mai *et al*. 2006 Guise *et al*. 2004
Colorectal	Proliferation, anti-apoptosis, & invasion. Stromal tissue invasion and migration	Increased ET_AR (80% primary CRCs and majority of metastasis); decreased ET_BR expression	Blocks proliferation & tumourogenic and neovascularisation effects on adjacent stroma. Altered MAPK pathway, EGFR signalling & FasL induced apoptosis. Pre-clinical studies: ET_AR and ZD4054 blocks desmoplastic activation of colorectal fibroblasts and CRC proliferation.	Asham *et al*. 2001 Shankar *et al*. 1998 Ali *et al*. 2000a, 2000b Hoosein *et al*. 2007 Eberl *et al*. 2000, 2003 Knowles *et al*. 2011 Haque *et al*. 2009a, 2009b, 2012

Table 3.4 *This table outlines the effects of ET-1 in different cancers along with the receptors involved, the effect of antagonising them and relevant publications to date in each field.*

Chapter 4

Studies on Colonic Fibroblasts & Cancer Cell Lines:

Gene Arrays, Conventional PCR, Quantitative Real Time qRT-PCR, Western Blotting & Gene Silencing with SiRNA

4.1 INTRODUCTION

This chapter considers novel genes regulated by ET-1 that were identified by gene array. Time point inductions confirmed their induced expression at the mRNA and protein levels. The effects of ET receptor antagonists on selected genes were evaluated at the mRNA and protein level whilst silencing with siRNA confirmed each receptor's role.

4.2 MATERIALS AND METHODS

4.2.1 Gene Arrays and Time Point Inductions

Fibroblasts or CRC cell lines were seeded at 200,000 – 300,000 cells/35mm cell culture dish containing fully supplemented DMEM medium. Plates were incubated routinely for 24 hours (CRC cells) or 24-36 hours (fibroblasts) to reach 60-70% confluence. Media were then changed to serum free for another 18-24 hours (0.5% BSA containing medium for fibroblasts) to growth-arrest cells. Wells were emptied using a pipette suction vacuum pump. Solutions were made in serum free medium for CRC cell lines or DMEM with 0.5% BSA for fibroblasts. ET-1 was used at 10^{-7}M or 10^{-8}M and receptor antagonists at 10^{-6}M or 10^{-7}M, for fibroblasts and CRC cells respectively. Control wells contained medium only. Total RNA was extracted at 4 hours for gene array analysis (Illumina Ref 8, Cambridge) and at time points 0, 30 minutes, 1, 2, 4, 8 and 24 hours for mRNA gene analysis following ET-1 exposure. Total RNA was extracted from control dishes at 0 and 24 hours.

4.2.2 RNA & Protein Assay with ET-1 and/or ET_A and ET_B Receptor Antagonists

Six-well plates (Nunc A/S Kamstrupvej, Denmark) were seeded with fibroblasts or CRC cell lines at 200,000 – 300,000 cells/well containing fully supplemented DMEM medium. Plates were incubated routinely for 24-48 hours until 60-70% confluent. Medium was changed to serum free medium for another 18-24 hours (0.5% BSA containing media for fibroblasts). Medium was then discarded and wells washed twice with PBS. Media were removed by aspiration and replaced by solutions containing ET-1 and receptor antagonists as previously described above. Control wells contained medium only. For the first hour, cells were

exposed to relevant antagonists alone with control and ET-1 wells incubated with serum free media only. Wells were then aspirated and treatment wells replaced with antagonists and/or ET-1. RNA was extracted at 4 hours and protein extracted following 24 hours of treatment. Plates were laid out as follows:

A

Plate 1

Control	BQ123	BQ788
ET-1	ET-1 + BQ123	ET-1 + BQ788

Plate 2

Control	ZD4054	ET-1 + BQ123 + BQ788
	ET-1 + ZD4054	ET-1 + ZD4054 + BQ788

B

Control	ET-1	ET-1 + BQ123
ET-1 + ZD4054	ET-1 + BQ788	ET-1 + ZD4054 + BQ788

Figure 4.1. *Layout A (top) and Layout B (bottom). Schematic for layout of 6 well plates to investigate RNA and protein expression following ET-1 and* $ET_{A/B}$ *receptor antagonists.*

Expression Analysis Following RNAi (RNA Interference):

4.2.3 RNA & Protein Assays with ET-1 and/or ET_A and ET_B SiRNA (Small Interfering RNA Transfections/Gene Silencing)

Following methodological development optimal conditions and concentrations were established. RNA interference (RNAi) was accomplished by transfecting cells with ON-TARGET plus SMART pool small interfering RNA from Dharmacon Inc. (Lafayette, CO). Silencing RNA for human ET-1 (EDN;NM_001955), ET_A receptors (EDNRA;NM_001957) and ET_B receptors (EDNRB;NM_003991) were used. Transfections were performed using oligofectamine reagent (Invitrogen). Briefly, six well plates (Nunc) were seeded with fibroblasts or CRC cell lines at 50,000 – 200,000 cells/well with antibiotic free DMEM. When 30-40% confluent, medium was changed to serum free DMEM for 24 hours, cell were then transfected with 4µl Oligofectamine and siRNA at a final concentration of 25nM. Medium was then discarded and cells re-incubated with fully supplemented 10% FCS DMEM for a further 24 hours followed by serum starvation for 12-18 hours to arrest growth. Fibroblasts and CRC cell lines were then stimulated with ET-1 at 10^{-7}M or 10^{-8}M respectively. Controls and ET-1 only stimulated cells were transfected with scrambled non-targeting pooled SiRNA (*siControl*) (Dharmacon Inc.). Total RNA was extracted and expression levels of specific genes of interest were examined following 4 hours of ET-1 stimulation. Protein expression levels were examined at 24 hours. Plates were laid out as follows:

Scrambled	Scrambled + ET-1	
ETAR SiRNA + ET-1	ETBR SiRNA + ET-1	ETAR SiRNA + ETBR SiRNA + ET-1

Figure 4.2. *Schematic for layout of 6 well plates for silencing experiments.*

4.2.4 Protein Expression Analysis

Plates were transferred onto ice: cells washed twice with PBS and lysed with 200µl RIPA Buffer (Sigma-Aldrich, Dorset, UK). The resulting lysate was gently scraped from wells, centrifuged at 4℃ for 5 minutes and the protein supernatant removed and stored at -70℃

until required for dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). (Shi-Wen *et al*., 2004).

When required, each supernatant was thawed and the protein concentration determined by Lowry assay. 50µg of protein concentration was loaded on each lane. Following method development, for each 20µl sample, 5µl Laemmli Buffer (x4), 2µl β-Mecaptoethanol and 5µl Urea (8M; for CTGF) was added. Samples were heated to 95℃ (Tectine, Dri-Block DB.2A) for 5 minutes. Polyacrylamide electrophoresis gels were made (appendix 7) or SDS-PAGE gels were used for protein electrophoresis. 15µl protein ladder (SeeBlue Plus, Invitrogen Ltd) and 25µl of protein were added to wells (appendix 8). Gels were run at 125V for 90 minutes and proteins transferred to nitrocellulose membrane (Hybond-C, Amersham, UK) at 35V for 90 minutes (appendix 8).

Membranes were blocked with 5% milk (Marvel) for 1 hour, washed in PBS three times for 10 minutes each, then incubated with primary antibodies [ET_A receptors (dilution 1:400); ET_B receptor (1:400); CTGF (1:500); COL11A1 (1:250); AML-1 (1:250); GAPDH (1:1000)]. Following further PBS washes, secondary antibodies [anti-mouse/anti-rabbit/anti-goat (1:1000)] were added for 1–2 hours, washed again in PBS then visualised by adding ECL and exposing to film (appendix 8) or by exposure to super-signal West (Thermo-Scientific, UK) and viewed using Chemi-Doc System and software (Bio-Rad, Hertfordshire, UK). Molecular weights were confirmed with reference to the protein ladder. Equal loading of protein concentration was confirmed using GAPDH as the internal control.

4.2.5 RNA Extraction

Following treatment, cells were washed twice with PBS then total RNA extracted by one of two methods, either: the RNeasy 'mini' kit (Qiagen, Crawley, UK) or TRIzol, (Invitrogen^{TM}) according to the manufacturer's guidelines (appendix 9). Once extracted, samples were stored at -20℃. Purity and concentration were assessed using a NanoDrop 8000 8-Sample Micro-Volume UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA).

4.2.6 Conventional RT-PCR

'Conventional' RT-PCR was carried out using OneStep (Qiagen™) (appendix 11). To reduce pipetting errors and contamination, three separate 'master mixes' were prepared: briefly, the first aliquot contained 0.9µl of sense primer (10mM), 0.9µl of antisense primer (10mM) and 3.2µl of Amplification Grade water (Promega, Madison, USA) for each reaction. The Mastermix aliquot contained 3µl of OneStep® RT-PCR Buffer (Qiagen), 0.6µl of dNTP mix (Qiagen), 0.6µl of OneStep® RT-PCR Enzyme Mix (Qiagen) and 0.8µl of molecular grade water (Promega) per reaction. The enzyme mix was the last component to be added. The third aliquot contained a final concentration of 100ng of RNA template made up to a volume of 5µl with molecular grade water. Reaction mixtures were pipetted into thin walled 0.2ml PCR tubes and loaded into a Flexigene Thermal cycler (Techne, Staffordshire, UK). Primers (Table 4.1) were as follows: CTGF, ADM, STC-1, MT1X, MMP7, PPP2R5D, EGFR, ET-1, EDNAR, EDNBR and GAPDH.

Products were visualised using 2% agrose gels (made as per Appendix 10). Samples were supplemented with 2µl of Blue/orange 6x loading dye (Promega, Madison, USA) and loaded into gels within a Horizon 11.14 Horizontal Gel Electrophoresis Apparatus (Life Technologies, California, USA). Samples were run for approximately 30-60 minutes at 50-125 volts to create sufficiently separated bandings. Gels were visualised using Gel Doc 2000 transilluminator (BioRad, Philadelphia, USA) and accompanying program Quantity One (BioRad).

4.2.7 Real Time Quantitative qRT-PCR

Real time quantitative RT-PCR (qRT-PCR) was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). This included a ready-made mastermix (with polymerase), buffer dNTPs and SYBR green dye. Detailed volumes and calculations are described in appendix 12. Briefly, 10µl of Mastermix, 2µl of sense and 2µl of anti-sense primers (final concentration 1µM), 0.2µl of enzyme and 100ng of total RNA template made to a volume of 5.8µl were used per reaction. Samples were loaded into glass capillaries then into the LightCycler® 1.5 instrument and analysed using LightCycler® Software 3.5.3 (Both Roche Diagnostics Ltd, Burgess Hill, UK). GAPDH was used as a standard. Primers were

designed and through method development optimum conditions were obtained (appendix 13). Primers for genes whose expressions were examined are as follows: CTGF, ADM, STC-1, MT1X, MMP7, PPP2R5D, EGFR, ET-1, EDNAR, EDNBR and GAPDH. Gene expression is shown as a fold change in transcript expression in treated fibroblasts and CRC cell lines compared to untreated, or ET-1 induced cells only, using the ΔCt method, per manufacturer's instructions (Applied Biosystems).

Primer/Oligo Name	Sense Sequence (5'→3')	Anti-Sense Sequence(3'→5')
ET-1 (QETs/QETas)	GCTCGTCCCTGATGGATAAAG	CCATACGGAACAACGTGCT
ET_AR (QETAs/QETAas)	CTCAACCTCTGCGCTCTTAGT	AGCCAATCGCTTCAGGAATGG
ET_BR (TBR1s/TBR1as)	GCTTTAAGGCTGGGCCA	GCTCTTTCTTTCTGGCCACA
CTGF (CT1s/CT1as)	GTTCCAAGACCTGTGGGAT	GCCCTTCTTAATGTTCTCTTCC
ADM (ADM1s/ADM1as)	GAGGGAACTGCGGATGT	GCTGTCTTCGGGGCTT
STC-1 (STC1s/STC1as)	GGAGCAGAATGACTCTGTGA	CGAACCACTTCAGCTGAGTT
MT1X (MT1Xs/MT1Xas)	TCTCCTTGCCTCGAAATGGAC	GGGCACACTTGGCACAGC
MMP7 (MMP7s/MMP7as)	GGGAGGCATGAGTGAGCTAC	TCTCCTTGAGTTTGGCTTCTAAA
PPP2R5D (PPP2s/PPP2as)	GCTGCCCGAGTCCTCCCCATCAT	AACCGGCCCTTCTGCTTCTCTGC
EGFR (EGR2s/EGR2as)	CATGCAGAAGGAGGCAAAGT	CAAAGGTCATCAACTCCCAA
GAPDH	AGATCATCAGCAATGCCTCC	AGTGATGGCATGGACTGTGGT

Table 4.1. *This table shows the primer sequences that were designed to investigate mRNA expression levels for conventional and real-time PCR.*

4.3 RESULTS SECTION

SECTION A:

Genes Regulated by Endothelin-1 in Colorectal Fibroblasts and Cancer Cell Lines & the Effect of Receptor Antagonism on Expression of Selected Genes

4.3.1 Colonic Fibroblasts:

Genes Regulated in Fibroblasts by Endothelin-1

The effect of ET-1 on the regulation of genes in fibroblasts was investigated in four fibroblast strains (CF35, CF56, CF65 & CF75). Fibroblasts were grown to 60% confluence; then following exposure to ET-1 (10^{-7}M) for 4 hours, mRNA was extracted using the Mini-RNeasy Kit (Quigen). Samples were analysed using Illumina gene array analysis. Fibroblasts exposed to 0.5% BSA containing medium were used as controls.

All fibroblasts strains behaved in a similar manner but with a slight variation in fold change between control and treatment groups. Illumina Gene Array resulted in data for 11,193 genes, of which 70 genes were up-regulated by over 1.5 fold when combining all fibroblast results. Of these, 13 genes were up-regulated by over 2 fold and showed significance ($p<0.05$). There were only 5 genes that were down regulated by over 1.5 fold, of which 4 were significant ($p<0.05$). The full dataset is included in a supplementary file (see supplementary data CD).

Two of the significantly up-regulated genes following ET-1 exposure belong to the same CCN family of genes. Both CYR61 (cystein rich 61; CCN1) and CTGF (connective tissue growth factor; CCN2) are growth factors with known roles in cell proliferation, adhesion, migration and angiogenesis in the setting of fibroblast actions and cancer progression. Another significantly up-regulated gene was Adrenomedullin (ADM/AM), a vasodilator peptide linked to tumourigenic roles of cell proliferation, angiogenesis and inhibition of apoptosis. Thus CTGF and ADM were chosen for further investigation to look at their regulation and possible roles in response to ET-1.

The most significantly down regulated gene was Stanniocalcin-1 (STC-1). It is a glycoprotein involved in calcium/phosphate homeostasis and normally induced by BRCA1 tumour suppressor gene in breast tissue. It has both positive and negative correlations with a number of malignancies, and therefore was a gene chosen to be investigated as significantly down regulated by ET-1.

In an earlier study within the Department of Surgery, UCL, a gene array study (Affymatrix) was carried out with different colonic fibroblast strains. Two significantly regulated genes identified in this study were Collagen Type XI (COL11A1) and Acute Myeloid Leukaemia-1 (AML-1). Both these genes were up-regulated on the gene arrays in this study, however non-significantly. These genes were therefore investigated at the protein expression level in addition to the chosen genes above.

A. Fibroblast Up-Regulated Genes

Gene Name	Accession No.	CF36	CF56	CF65	CF75	Combined	P Value	
Genes up-regulated over 2 fold								
ANKRD1	NM_014391.2	3.6142	2.7002	1.6994	5.7644	3.423187	3E-08	Transcription Factor
CYR61	NM_001554.3	2.7574	3.1903	1.9979	4.5921	3.283528	5E-08	Growth Factor
TNFRSF12A	NM_016639.1	2.9545	3.1154	2.7227	4.1563	3.265402	0.0003	TNF Receptor (↑IL8/MCP-1)
CTGF	NM_001901.2	2.5737	2.8009	2.6684	3.3257	**2.768337**	0.0087	Growth Factor
ADM	NM_001124.1	2.0292	2.5114	2.3809	3.4979	**2.680695**	0.0042	Angiogenesis/Mitogen/Hypoxic survival (↑HIF/VEGF)
MGC4677	NM_052871.3	2.2045	2.0136	2.2273	2.4342	2.265645	0.0215	
SHRM	NM_020859.1	2.9439	2.4214	1.5035	2.1243	2.227831	0.002	
PTRF	NM_012232.3	2.2069	1.5385	2.2973	2.7358	2.201301	0.0017	
SLN	NM_003063.2	1.8826	1.8557	2.1725	3.5994	2.174202	0.1794	
EMP1	NM_001423.1	2.502	1.5433	2.7389	1.5897	2.101444	0.0709	Associated myc signalling
CCL2	NM_002982.3	2.0298	1.644	2.789	2.2631	2.099299	0.0023	Survival Cytokine
ANXA1	NM_000700.1	2.0336	1.495	2.3484	2.1608	2.021314	0.0039	Anti-inflam. Poor prognosis in CRC
TNNT2	NM_000364.2	1.6669	1.4507	1.9012	3.0091	1.981131	0.0131	Troponin type 2
TNNC1	NM_003280.1	1.8789	1.3594	1.8033	2.8157	1.950142	0.0156	Troponin type 1
Genes up-regulated over 1.5 fold with significant p-value								
AXUD1	NM_033027.2	1.7896	1.5944	1.9232	1.8811	**1.785487**	4E-05	Nuclear Protein
KLF2	NM_016270.2	1.7448	1.4246	1.6739	1.4538	**1.572457**	6E-05	Transcription Factor
STX11	NM_003764.2	1.4552	1.4514	1.4273	1.64	**1.501461**	4E-11	Cell invasion and metastasis??
Genes of significance on previous gene arrays								
COL11A1	NM_001854.3	1.1941	1.1019	1.4964	0.9013	**1.182965**	0.6608	Colorectal Pathology
AML1		1.1127	1.1618	1.3238	0.9641	**1.136460**	0.1086	

B. Fibroblast Down-Regulated Genes

Gene Name	Accession No.	CF36	CF56	CF65	CF75	Combined	P Value	
Genes down-regulated over 2 fold								
FGD3	NM_033086.2	1.7718	1.5325	1.2529	1.5693	1.497354	0.0147	
LMO4	NM_006769.2	1.4152	1.4972	1.6751	1.4653	1.512204	0.0003	
SYTL3	NM_001009991.2	1.5311	1.1817	1.6292	1.9389	1.526359	0.0042	
GPER	NM_001039966.1	1.3065	1.7889	1.5524	1.5549	1.535269	0.0557	
STC1	NM_003155.2	1.5651	1.3037	1.9398	1.9229	**1.630137**	0.0038	Death and prolif. Inhib. Resistance.

Figure 4.3. *Gene array data showing up and down regulation of selected genes in fibroblasts following ET-1 exposure. Cells were incubated with ET-1 (10^{-7}) for 4 hours then RNA extracted and prepared using the Mini-RNeasy Kit. Altered gene expression was examined with Illumina gene array analysis (Cambridge). Tables show significantly* ***A:*** *up-regulated genes and* ***B:*** *down-regulated genes with p-values <0.05 and either 1.5 or 2 fold changes in gene expression. Significantly altered genes previously investigated within the department are also shown. Green highlighted rows are genes that were selected for further evaluation.*

To investigate mRNA expression of these selected genes, primers were designed to each of the genes (appendix 13 & Table 4.1). Conventional PCR was carried out up to 35 cycles to confirm expression of these genes within fibroblasts. Colorectal cancer cell lines were also included in the panel of cells for completion (figure 4.4).

CTGF and ADM were expressed throughout all four fibroblasts and CRC cell lines. ADM seemed to be expressed to a slightly lower level throughout the panel of cells. STC-1 was specific for fibroblasts with an extremely low expression seen in the SW480 cell line. GAPDH confirmed that the template loading concentrations were the same throughout all cells that were investigated.

Confirming Expression of Genes of Interest

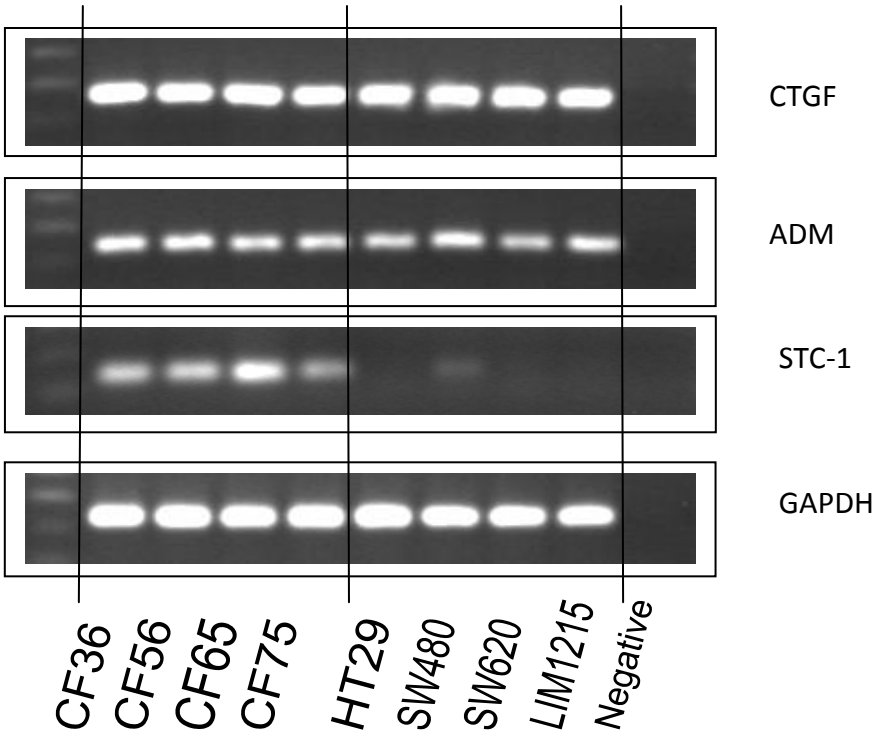


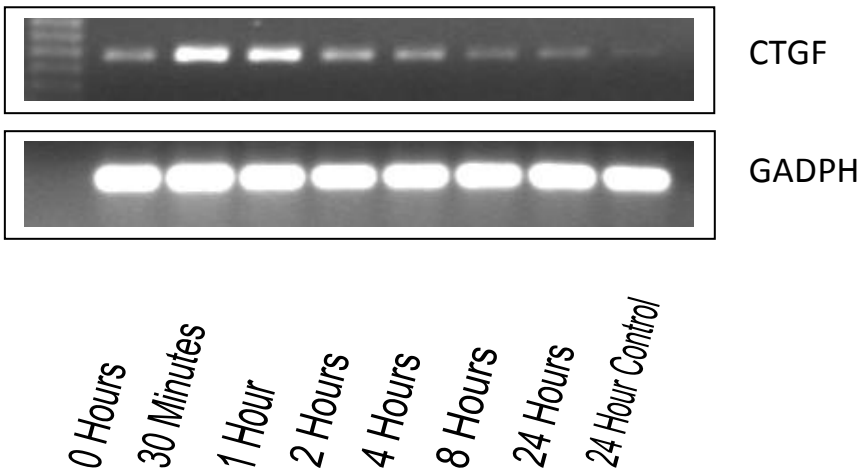
Figure 4.4. *RT-PCR showing expression levels of genes in both fibroblasts and colorectal cancer cell lines. Primers for selected genes from gene array data were designed with annealing temperatures of 58°C and flanking introns to be RNA specific. RT-PCR was carried out as per standard conventional-PCR protocol using a Mastercycler Gradient thermal cycler (Eppendorf) for 35 cycles. All genes were expressed in both fibroblasts and cancer cell lines except STC-1 which was expressed in fibroblasts alone.*

To confirm that gene induction or suppression resulted from ET-1 exposure, time point inductions were carried out using conventional and real-time RT-PCR.

Quantitative real-time RT-PCR of CTGF revealed an up-regulation reaching a peak at around 1-2 hours with a 3.5-3.8 fold increase. This then tapered off to below control levels up to 24 hours (figure 4.5B). Conventional PCR was carried out to demonstrate these findings on ethidium bromide gels, which showed a similar trend with a peak expression reached at 30 minutes to 1 hour before tapering off. GAPDH confirmed equal loading in all experiments (figure 4.5A).

Time Induction Assays: CTGF

A: Conventional RT-PCR



B: Real-Time RT-PCR

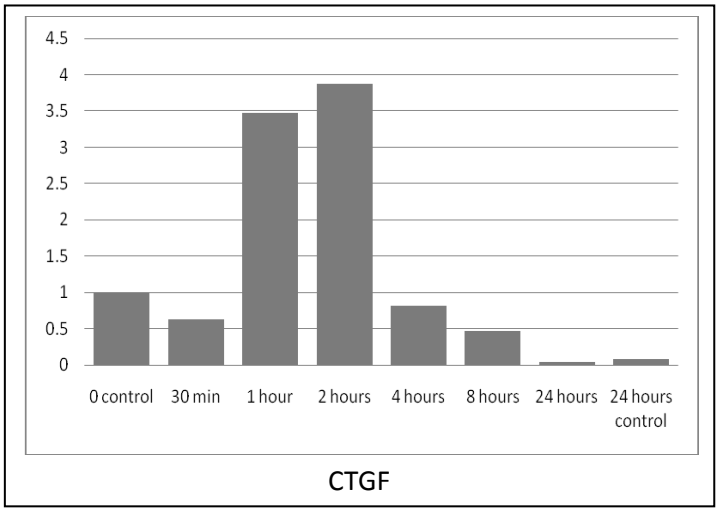
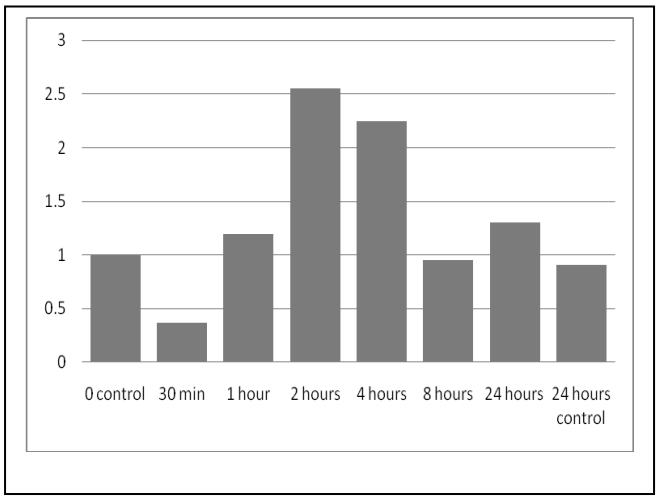


Figure 4.5. *Time point gene induction of CTGF demonstrated using* ***A:*** *conventional and* ***B:*** *qRT-PCR. CF75 fibroblasts were exposed to ET-1 (10^{-7}) for 0, 30 minutes, 1, 2, 4, 8 and 24 hours followed by RNA extraction using the Mini-RNeasy Kit. Conventional RT-PCR was carried out using the OneStep® RT-PCR kit (Qiagen) and qRT-PCR was carried out using a QuantiTect SYBR Green RT-PCR Kit (Qiagen). RT-PCR images are generated by stopping the PCR reaction at 23 cycles. Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated fibroblasts compared to untreated cells using the ΔCt method, as per manufacturer's instructions (Applied Biosystems). CTGF induction is seen at between 1-4 hours with a maximum fold change of 3.8. N=2 independent repeats.*

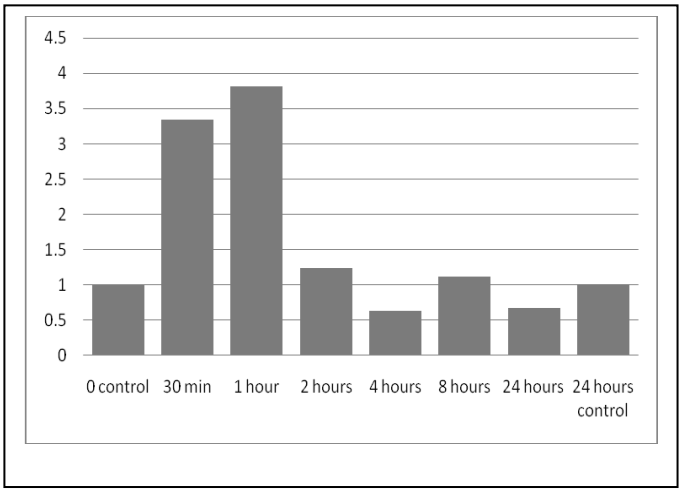
Same protocols were used to investigate ADM and STC-1 changes in gene expression following exposure to ET-1. Up-regulation of ADM was confirmed with a peak expression level seen at 2 to 4 hours with a maximum 2.6 fold increase (figure 4.6A). STC-1 had shown

a decreased expression level at 4 hours according to the gene array. However, time point inductions surprisingly showed an up-regulation at 30 minutes to 1 hour of between 3.4 and 3.8 fold above control expression levels (figure 4.6B). This indicates that STC-1 may act as an early response gene. As GAPDH was used to ensure equal loading and that template concentrations were used throughout experiments, a qRT-PCR was carried out to confirm that this gene was not affected by ET-1 exposure. Indeed a maximum fold increase of only 1.18 was observed indicating this to be a suitable marker to use. GAPDH was used to confirm equal loading in all experiments and used for normalisation when calculating delta CT values.

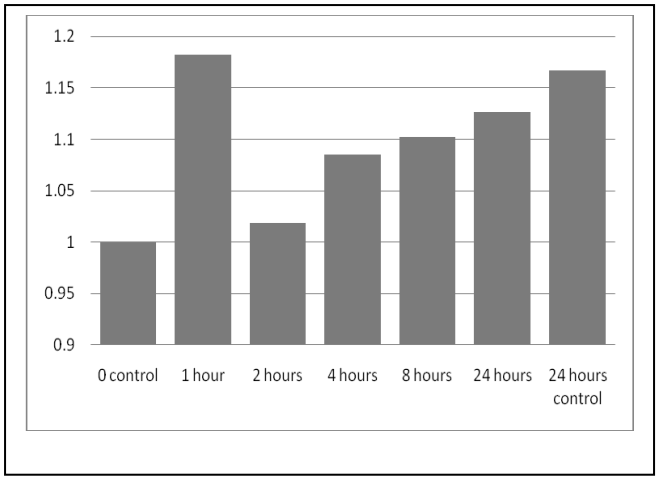
Real Time PCR Time Induction Assays in CF75 Fibroblasts



A: ADM



B: STC-1



C: GAPDH

Figure 4.6. *Real time RT-PCR showing time point inductions of* ***A:*** *ADM,* ***B:*** *STC-1 and* ***C:*** *GAPDH. CF75 fibroblasts were exposed to ET-1 (10^{-7}) for 0, 30 minutes, 1, 2, 4, 8 and 24 hours followed by RNA extraction using the Mini-RNeasy Kit. Real time RT-PCR was carried out using a QuantiTect SYBR Green RT-PCR Kit (Qiagen). Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated fibroblasts compared to untreated cells using the ΔCt method, as per manufacturer's instructions (Applied Biosystems). A 2.6 fold increase in ADM was seen at 2 hours, 3.8 fold increase in STC-1 at 1 hour and no significant change in GAPDH expression seen at any time point. N=2 independent repeats for each gene.*

The effect of receptor antagonism on the expression of selected genes was investigated in CF56, CF65 and CF75 cell strains. Fibroblasts were exposed to ET-1 alone, ET-1 and ET_A and/or ET_B receptor antagonists (BQ123/ZD4054, BQ788). Fibroblasts incubated with 0.5% BSA medium were used as controls (figure 4.7).

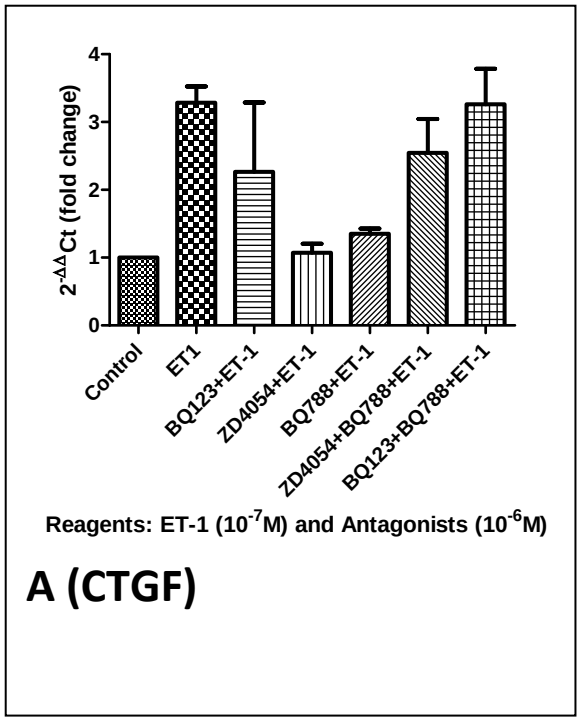
In general ET_A receptor blockade appeared to oppose ET-1 actions and of the three antagonists used, the specific ET_A receptor antagonist ZD4054 was the most effective in reversing ET-1 actions. Graphical results show trends for the three fibroblast strains combined (figure 4.7).

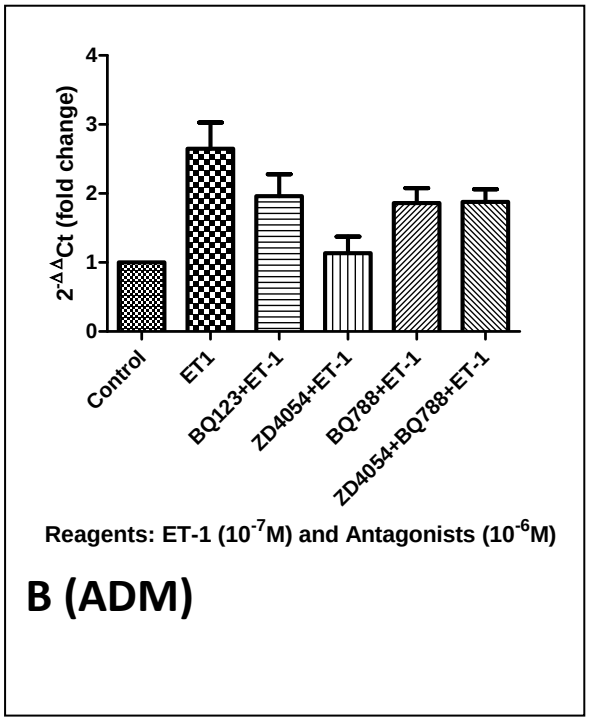
BQ123 did not result in significant alterations in gene expression levels (mean reduction of expression for CTGF: 44.6% & ADM: 41.7%; mean inhibition of STC-1: 31.2%; figure 4.7D). However, there was significant gene expression changes in two of the three fibroblast strains used ($p<0.05$). In comparison ZD4054 resulted in greater inhibition than that of BQ123 (CTFG: 96.8%; ADM: 91.9%; STC-1: 60.0%). This was significant for CTGF and ADM ($p<0.05$). Significant inhibition with ZD4054 was reached in two of the three fibroblast strains carried out for STC-1.

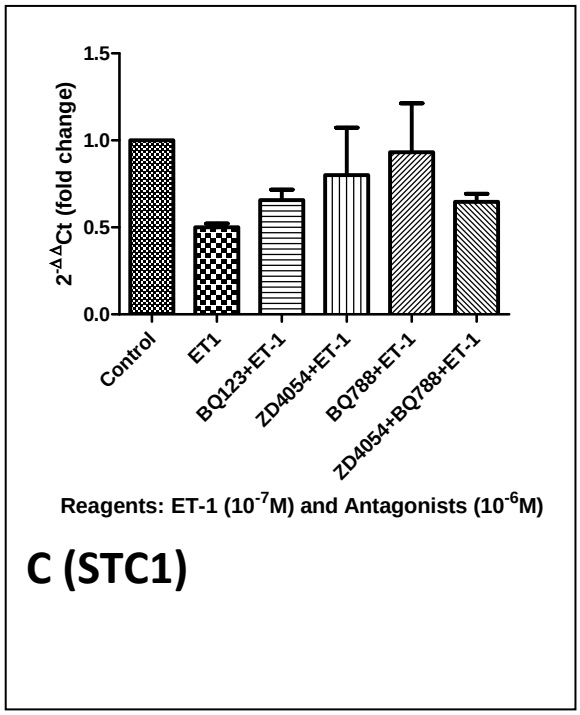
The ET_B receptor antagonist BQ788 resulted in significant inhibition of CTGF when data was combined for all fibroblast strains (84.6%; $p<0.05$). Despite significant inhibition with BQ788 being reached in 2 of the 3 fibroblast experiments for ADM and STC-1, when combined they lost this significance (inhibition of ADM: 47.7% and STC-1: 86.4%). Combined results are shown graphically in figure 4.7 A-C and as a table in D).

When combined, receptor antagonists gave mixed results. Generally combinations seem to partly reverse single agent action, suggesting complex actions and cross talk between the two receptors.

ET_A and ET_B Receptor Antagonist Effect on Gene Expression







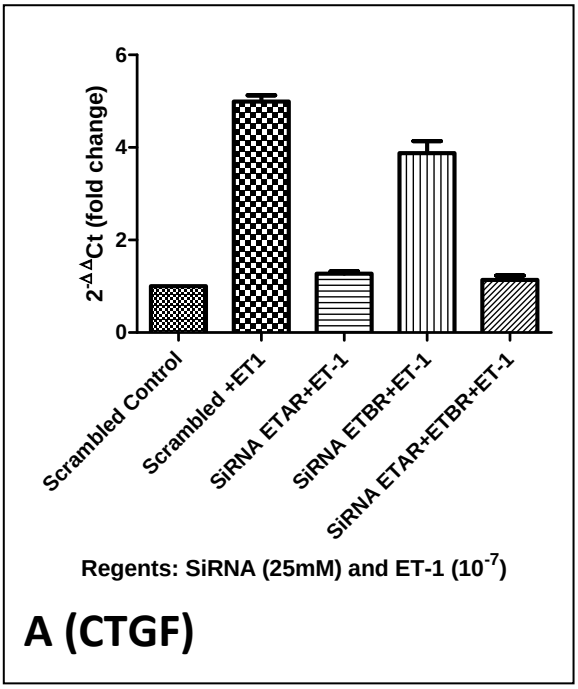
D	Antagonist	Change in CTGF expression	
		% Decrease	Significance
	BQ123	44.6	NS
	BQ788	84.6	p<0.05
	ZD4054	96.8	p<0.05
	ZD4054+BQ788	32.5	NS
	Antagonist	Change in ADM expression	
		% Decrease	Significance
	BQ123	41.7	NS
	BQ788	47.7	NS
	ZD4054	91.9	p<0.05
	ZD4054+BQ788	46.7	NS
	Antagonist	Change in STC1 expression	
		%Inhibition	Significance
	BQ123	31.2	NS
	BQ788	86.4	NS
	ZD4054	60.0	NS
	ZD4054+BQ788	29.2	NS

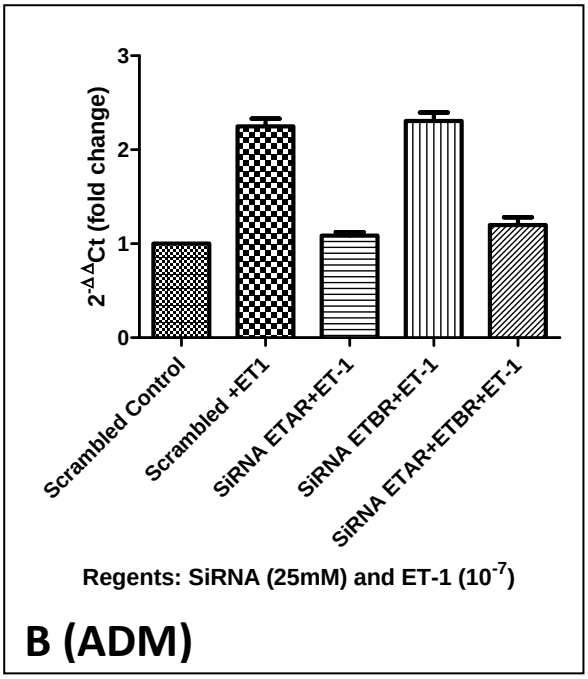
Figure 4.7. *mRNA expression levels of* ***A:*** *CTGF,* ***B:*** *ADM and* ***C:*** *STC-1 in the presence of ET-1(10^{-7}M) and/or $ET_{A/B}$ receptor antagonists (10^{-6}M) following 4 hours of exposure. This graphical representation is produced by three independent repeats. Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated fibroblasts compared to untreated cells.* ***D:*** *Table showing percentage decrease/inhibition of ET-1 induced gene expression.*

To further confirm specific receptor subtypes involved in altering expression of selected genes, we silenced individual receptors using ET_A and/or ET_B SiRNA in the presence of ET-1. Fibroblasts exposed to scrambled SiRNA alone were used as controls (figure 4.8).

The ET-1 induction or suppression of genes was clearly demonstrated once again. ET_A receptor silencing resulted in a near return to control levels in the presence of ET-1 for all three genes (inhibition of CTGF: 93.1%; ADM: 95.6%; STC-1: 98.7%; $p<0.05$). ET_B receptor silencing did not have a significant effect on altering gene expression of ADM and STC-1 (4.3% and 0.01% respectively). There was a smaller significant reduction in CTGF expression (23.3%). Combined ET_A and ET_B receptor silencing demonstrated similar results to that of ET_A receptor silencing alone (inhibition of CTGF: 95.3%; ADM: 89.6%; STC-1: 88.9%; $p<0.05$).

SiRNA of ET_A and ET_B Receptors





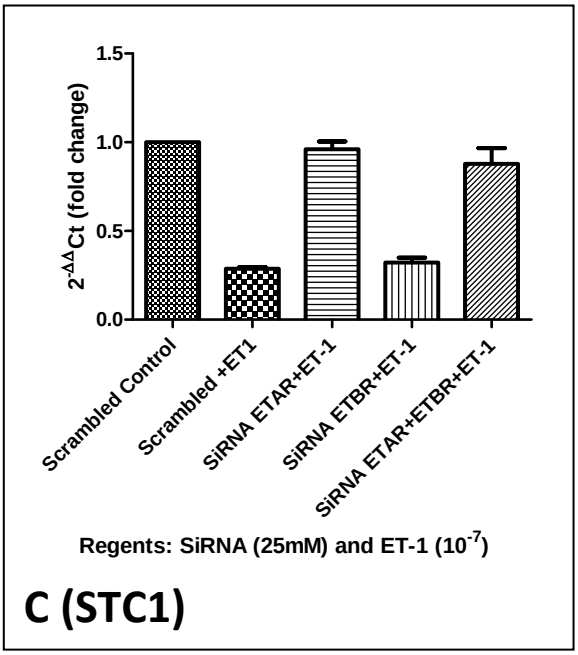


Figure 4.8. *mRNA expression levels of* ***A:*** *CTGF,* ***B:*** *ADM and* ***C:*** *STC-1 in the presence of scrambled SiRNA and/or ET-1 (10^{-7}M) and/or SiRNA $ET_{A/B}$ receptor (25nM) following 4 hours of exposure. This graphical representation is produced by three independent repeats. Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated fibroblasts compared to scrambled control. ET-1 significantly increased expression of all genes of interest.* ***A:*** *Silencing both ET_A and ET_B receptors significantly inhibited CTGF expression (ET_A>ET_B).* ***B & C:*** *ET_A receptor silencing inhibited ADM and STC-1 expression significantly.*

Most cell signalling occurs at the protein level, therefore CTFG was further investigated to see if ET-1 and its receptors had an influence on protein expression of this gene.

Fibroblasts were grown to 60% confluence then exposed to ET-1 (10^{-7}M) for 24 hours before protein was extracted. In the three colonic fibroblast strains examined (CF36, CF56 and CF65), all showed a significant increase in protein expression (figure 4.9).

The effect of receptor antagonism on protein expression of CTGF was investigated in CF35, CF56, CF75 and CF78 cell lines. Fibroblast strains were exposed to ET-1 alone, ET-1 and ET_A (BQ123/ZD4054) and/or ET_B (BQ788) receptor antagonists. Fibroblasts exposed to 0.5% BSA medium were used as controls (figure 4.10-4.11).

The greatest inhibitory effect on protein expression of CTGF was seen with the ET_A receptor antagonist ZD4054 where expression was returned back to control levels or below control levels. This was significant in all fibroblast strains investigated. Both ET_A receptor (BQ123) and ET_B receptor (BQ788) antagonists also resulted in a significant inhibition of CTGF protein expression in all fibroblast strains, with BQ788 being marginally better than BQ123. Combined ET_A (ZD4054) and ET_B (BQ788) receptor antagonists produced similar protein expression levels as ZD4054 alone. Interestingly, the CF78 fibroblast strain responded less to antagonists than the other fibroblast strains used. Figure 4.10 shows examples of 2 Western blots. Figure 4.11 (A-D) demonstrates graphically the CTGF protein expression levels of individual fibroblast strains. Combined results are shown as graphical representation in figure 4.12A and in a table as percentage decrease in figure 4.12B.

CTGF: Control versus ET-1

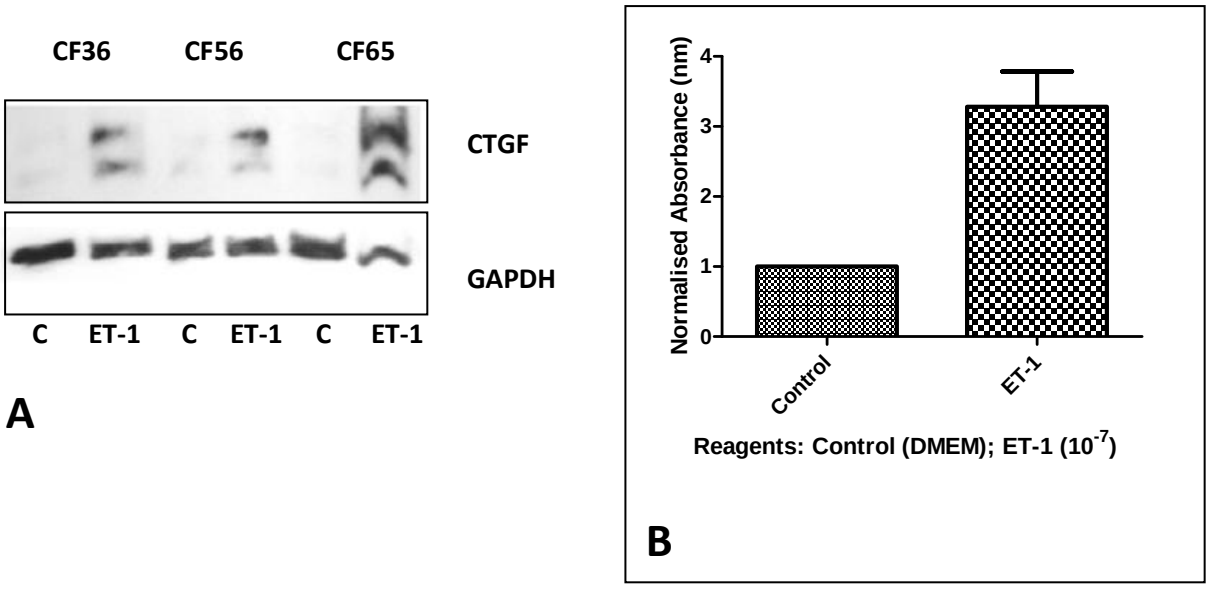
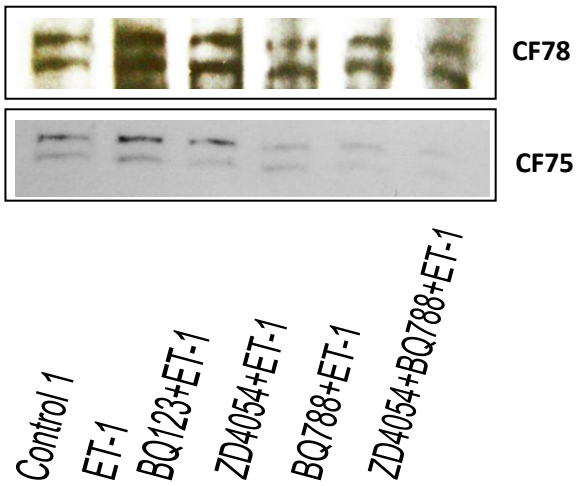


Figure 4.9. *CTGF protein expression on exposure to ET-1 in fibroblasts. Cells were exposed to control medium alone or ET-1 (10^{-7}M) then protein extracted after 24 hours.* ***A:*** *Western blotting images of three fibroblasts demonstrating ET-1 induced protein expression.* ***B:*** *Combined graphical representation of ET-1 induced protein expression in 3 independent repeats.*

Figure 4.10. *Western blotting showing protein expression of CTGF on exposure to ET-1 and/or antagonists. Fibroblasts were exposed to ET-1 (10^{-7}M) alone or ET-1 and $ET_{A/B}$ receptor antagonists (BQ123, ZD4054, BQ788; 10^{-6}M). Increased expression of CTGF is shown when exposed to ET-1 only. Both ET_A receptor antagonists inhibited expression (ZD4054>BQ123), but ET_B blockade with BQ788 produced greater inhibition. The most marked inhibition is seen with combined ET_A and ET_B receptor blockade.*



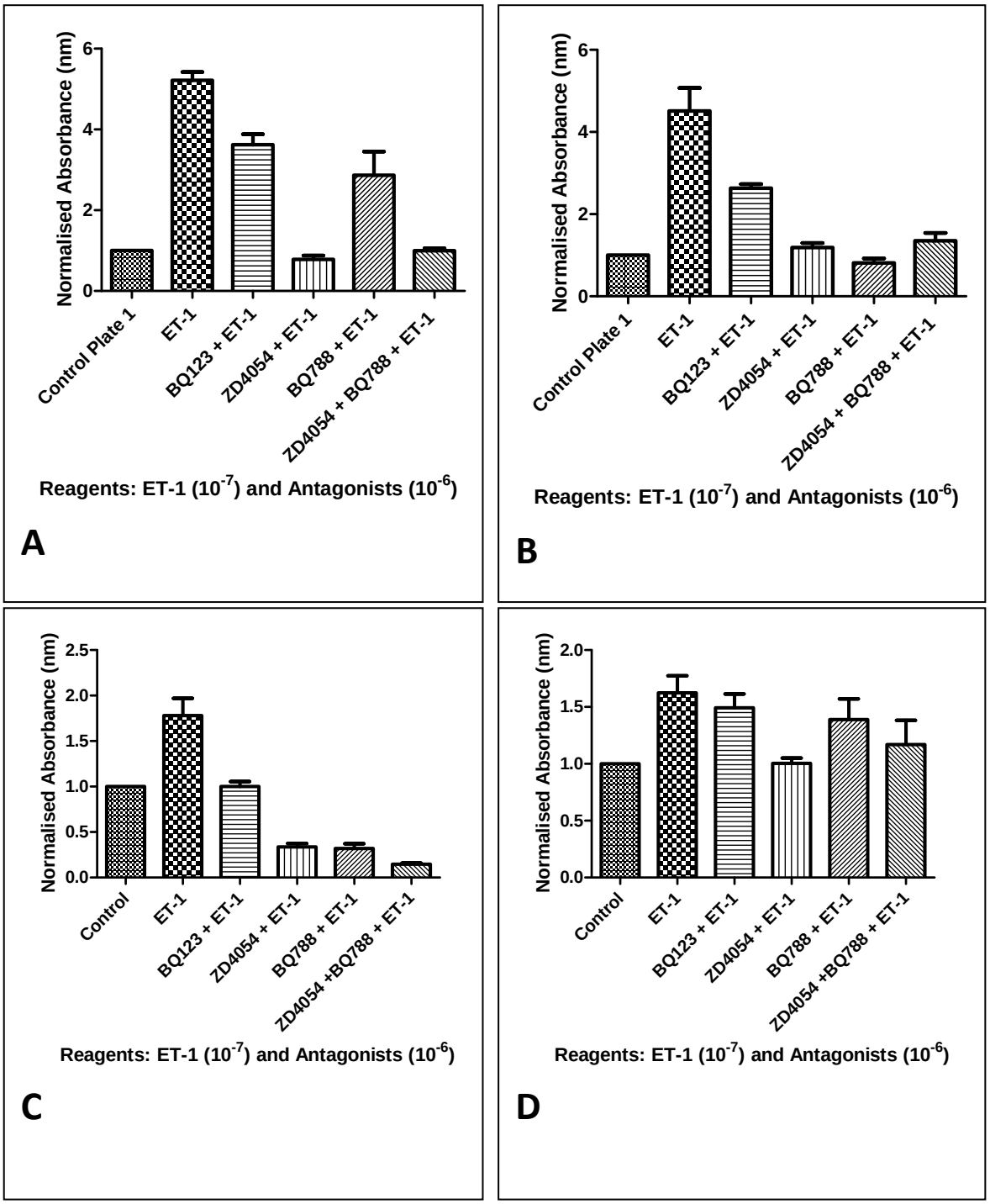
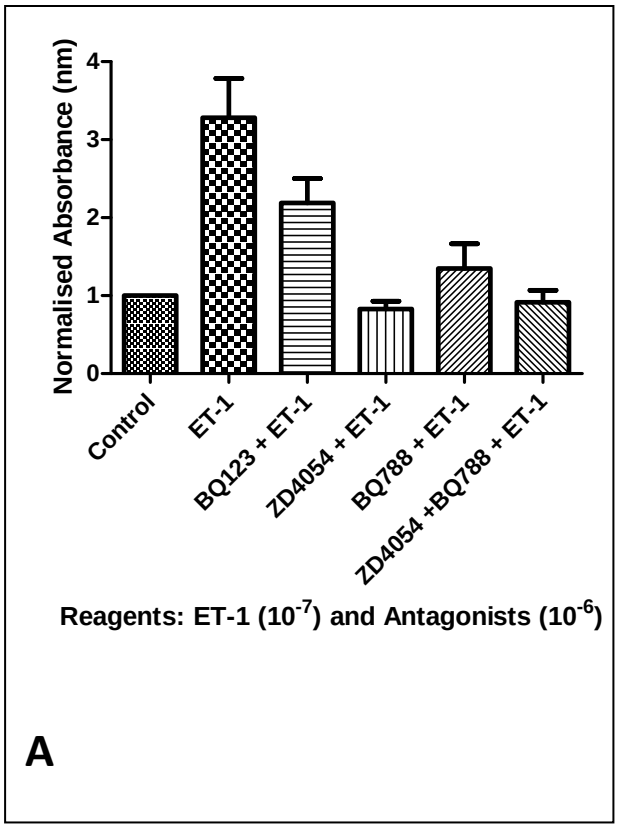


Figure 4.11. *CTGF protein expression in* ***A:*** *CF35,* ***B:*** *CF56,* ***C:*** *CF75 and* ***D:*** *CF78. Fibroblasts were incubated with ET-1 and/or $ET_{A/B}$ receptor antagonists. This graphical representation is produced by four independent repeats of each fibroblast strains using Western blotting with antibodies to CTGF. Values were normalised by using ratios to control to aid comparison of strains. In all strains both ET_A and ET_B receptor antagonists significantly reduced protein expression of CTGF. There was a less marked difference seen in the CF78 fibroblast strain.*



A

Reagents	% Decrease
BQ123 + ET-1	33.4
ZD4054 + ET-1	74.8
BQ788 + ET-1	59.0
ZD4054 + BQ788 + ET-1	72.1

B

Figure 4.12. *Combined results of CTGF protein expression for all colonic fibroblasts.* ***A:*** *Graphical representation of protein expression for all four fibroblast strains combined, each with 4 independent repeats, following 24 hours of exposure to ET-1 (10^{-7}M) or ET-1 + receptor antagonists (BQ123, BQ788 and ZD4054, 10^{-6}M).* ***B:*** *Table showing percentage decrease in protein expression with ET-1 induced expression set at 100%. ET_A blockade with BQ123 and ZD4054 produced a 33% and 75% decrease in protein expression respectively (ZD4054 being the most efficacious at reducing CTGF expression). ET_B blockade with BQ788 produced a 59% decrease.*

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4.3.2 Colorectal Cancer Cell Lines:

Genes regulated in cancer cell lines by Endothelin-1

The effect of ET-1 on the regulation of genes in CRC cells was investigated in four lines (HT29, SW480, SW620 & LIM1215). Cancer cell lines were grown to 60% confluence then following exposure to ET-1 (10^{-8}M) for 4 hours, mRNA was extracted. Samples were analysed using Illumina gene array analysis. Cells exposed to serum free medium were used as controls.

Three of the cell lines (HT29, SW480 & SW620) behaved in a similar manner but with a slight variation in fold change between control and treatment groups. Illumina gene array gave data on 11,505 genes, of which 6 genes were up-regulated by over 1.5 fold and 36 genes down regulated by over 1.5 fold when all cell line data were combined. The LIM1215 cell line behaved differently to the others, with a 2.8 fold maximum increase and 32 fold decrease in gene expression on exposure to ET-1. Because of this great difference in behaviour and sometimes opposite response to ET-1 (i.e. LIM1215 had a 32 fold decrease in MT1G gene expression whilst near maximum gene induction reaching a 4 fold increase was observed in the other three cancer cell lines) this cell line was omitted when combining gene fold changes to decide on genes to investigate. All further work was carried out excluding this cancer cell line.

The Metallothionein (MT) proteins were the most up-regulated genes when combining the fold change increases of the HT29, SW480 and SW620 cells (range 2.49 – 1.43 fold increases). These cysteine-rich physiological and xenobiotic heavy metal binding proteins have positive correlations with increased malignancy in a number of cancers including colorectal. Their expression is associated with poorer prognosis, increased migration, invasion and angiogenesis. As antibodies were readily available for MT1X, this was the gene chosen for further investigations.

PPP2R5D is a gene encoding a tumour suppressor serine/threonine kinase enzyme. This was down-regulated in all cancer cell lines with a combined 1.23 fold decrease (significance: $1x10^{-10}$; range: 1.14 – 1.29 fold decrease). Matrix Metalloproteinase (MMP7) was also down-regulated with a combined fold change of 1.43. However, this resulted from a significant down regulation of 1.52 fold in the HT29 cell line, whilst a small but insignificant up

regulation was seen in SW480 and SW620 cell lines. Decreased PPP2R5D and increased MMP7 expression are associated with increased malignancy in colorectal cancers.

Figure 4.13 shows genes that were up or down regulated after 4-hour exposure to ET (10^{-8} M).

A: Colorectal Cancer Cell Lines: Up-Regulated Genes

Gene Name	Accession No.	HT29	SW620	SW480	LIM1215	Combined		
Genes up-regulated over 2 fold								
MT1G	NM_005950.1	**1.9987**	**4.0312**	**1.043**	**0.0311**	**2.49161231**		Migration/ Invasion/ Angiogenesis Poor prognosis, advanced cancer Drug resistance
MT1X	NM_005952.2	**1.5474**	**3.3982**	**1.3503**	**0.0710**	**2.12512458**		Migration/ Invasion/ Angiogenesis Poor prognosis, advanced cancer Drug resistance
Genes up-regulated over 1.5 fold with significant p-value								
MT1H	NM_005951.2	1.5843	2.6793	1.0789	0.0975	1.79125883		
MT1E	NM_175617.3	1.3452	3.6994	1.3627	0.0332	1.73884663		
MT1A	NM_005946.2	1.5131	1.9369	1.1773	0.0748	1.62909643		
MTE	NM_175621.2	1.3987	2.2133	1.2478	0.0947	1.58343753		
MT2A	NM_005953.2	1.3078	1.8599	1.1802	0.0853	1.56506175		
MT1F	NM_005949.2	1.3591	2.3747	1.0208	0.2834	1.43490337		

B: Colorectal Cancer Cell Line: Down-Regulated Genes

Gene Name	Accession No.	HT29	SW620	SW480	LIM1215	Combined		
Genes down-regulated ~1.5 fold								
MMP7	NM_002423.3	1.5146	0.9237	0.8837	2.298	1.43832838		Invasion and Migration
PPP2R5D	NM_180977.1	0.8772	0.7848	0.7779	1.025	1.23346136		Tumour Suppressor , Neg. growth and division

Figure 4.13. *Gene array data showing up and down regulation of selected genes in CRC cell lines following ET-1 exposure. Cells were incubated with ET-1 (10^{-8}M) for 4 hours then RNA extracted and prepared using the Mini-RNeasy Kit. Altered gene expression was examined with Illumina gene array analysis (Cambridge). Tables show significantly* ***A:*** *up-regulated genes and* ***B:*** *down-regulated genes with p-values <0.05 and either 1.5 or 2 fold changes in gene expression. Green highlighted rows are genes that were selected for further evaluation.*

To investigate mRNA expression of these selected genes, primers were designed to each of the genes (appendix 13 & Table 4.1). Conventional RT-PCR was carried out up to 35 cycles to confirm expression of these genes within all cancer cell lines. Fibroblasts were also included within the panel of cells out of interest (figure 4.14).

MT1X and PPP2R5D appeared to be expressed throughout all four CRC cell lines and colonic fibroblasts. MMP7 was specific for CRC cell lines with no expression seen in the fibroblasts. GAPDH confirmed that the template loading concentrations were the same throughout all cells that were investigated.

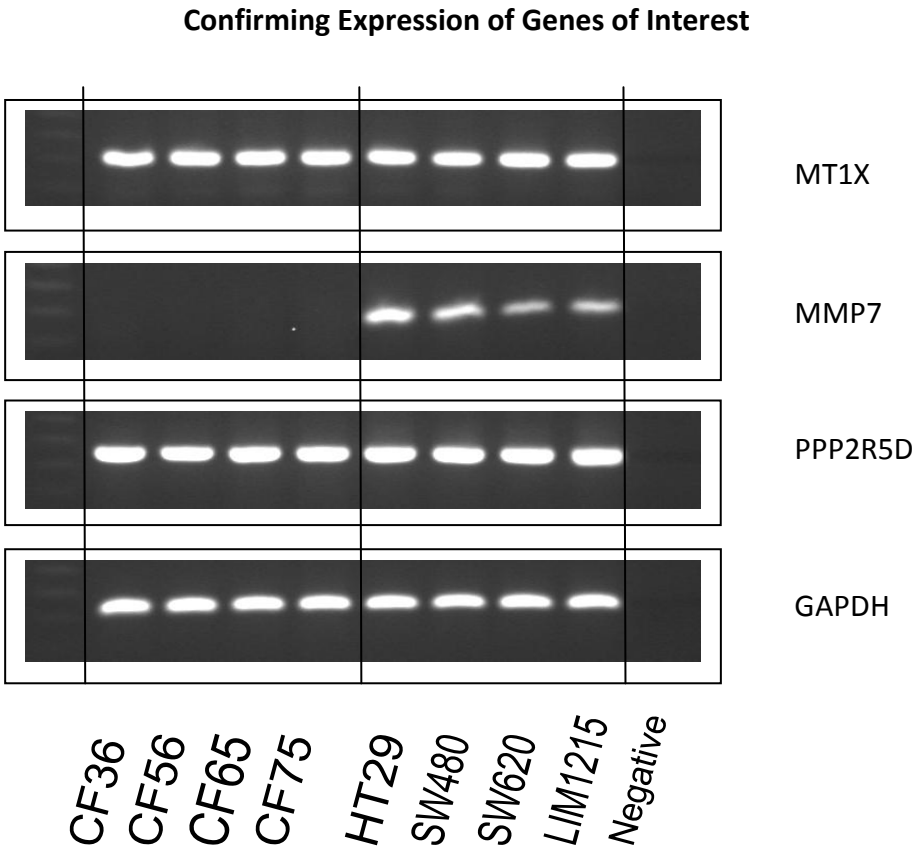
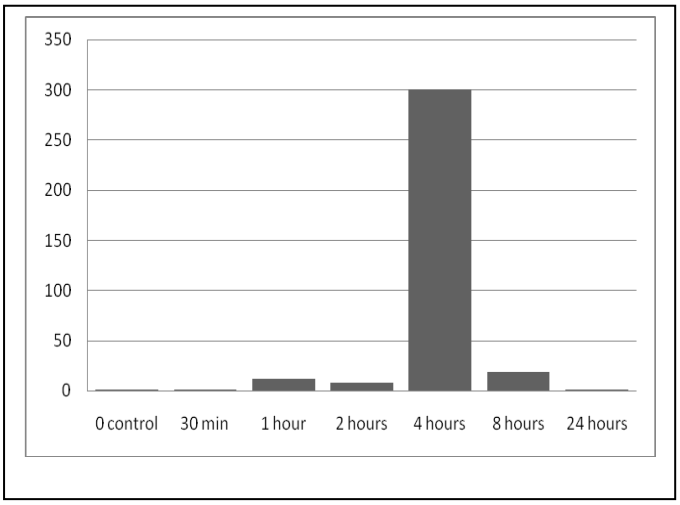


Figure 4.14. *RT-PCR showing expression levels of genes in both fibroblasts and colorectal cancer cell lines. Primers for selected genes from gene array data were designed with annealing temperatures of 58°C and that flanked introns to be RNA specific. RT-PCR was carried out as per standard conventional RT-PCR protocol using a Mastercycler Gradient thermal cycler (Eppendorf) up to 35 cycles. All genes were expressed in both fibroblasts and cancer cell lines except MMP7 which was expressed in cancer cell lines alone.*

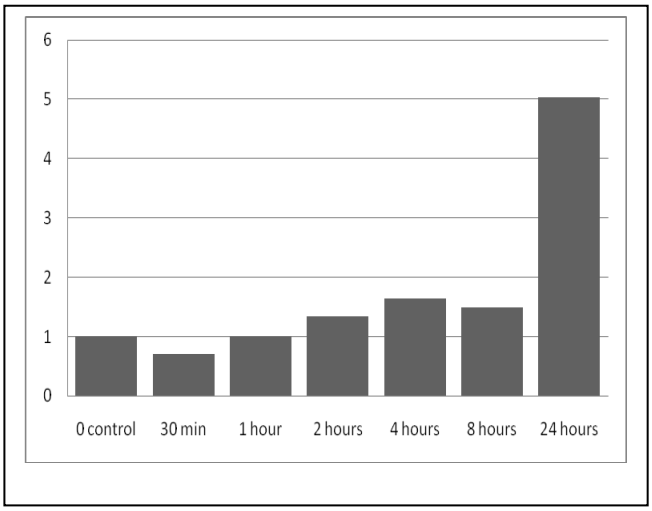
Time point inductions were similarly carried out to confirm changes in gene expression resulting from ET-1 exposure, time point inductions were carried out using quantitative real-time RT-PCR. The HT29 cancer cell line was grown to 50-60% confluence, starved in serum free medium, then exposed to ET-1 for 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours. At these time points, mRNA was extracted and conventional or quantitative real-time RT-PCR carried out for the relevant genes. Controls were incubated with serum free medium alone and expression levels of relevant genes examined at 0 and 24 hours (figure 4.15).

qRT-PCR of MT1X had shown an up regulation of 12 fold as early as 1 hour after ET-1 exposure, reaching a peak at 4 hours with a 300 fold increase. Contrary to gene array data findings, MMP7 showed a 1.6 fold increase at 4 hours, rising to 5 fold at 24 hours. No significant changes were observed following ET-1 exposure in regards to PPP2R5D expression. As GAPDH was used to ensure equal loading and template concentrations were used throughout experiments, a qRT-PCR was carried out to confirm that this gene was not affected by ET-1 exposure. GAPDH was used to confirm equal loading in all experiments and used for normalisation when calculating delta CT values.

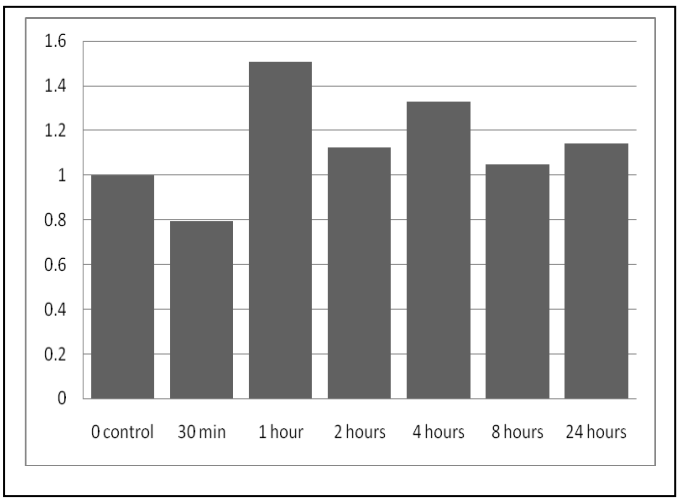
Quantitative Real Time RT-PCR Time Induction Assays in HT29 Cancer Cell Lines



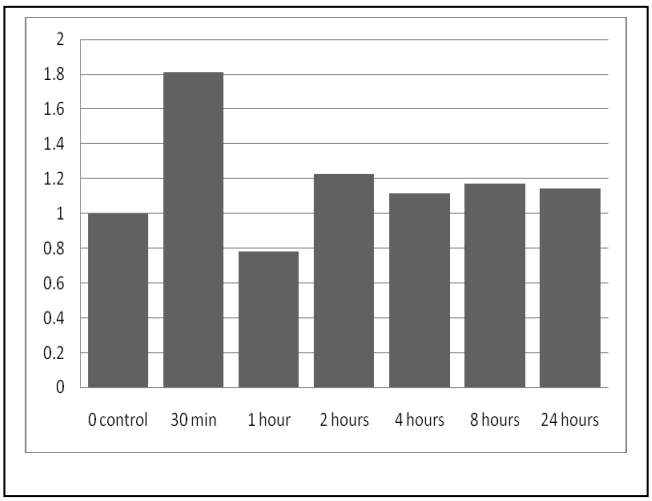
MT1X



MMP7



PPP2R5D



GAPDH

Figure 4.15. *Real-time RT-PCR showing time point inductions of* ***A:*** *MT1X,* ***B:*** *MMP7,* ***C:*** *PPP2R5D and* ***D:*** *GAPDH. Cancer cell lines were exposed to ET-1 (10^{-8}M) for 0, 30 minutes, 1, 2, 4, 8 and 24 hours followed by RNA extraction using the Mini-RNeasy Kit. Real time RT-PCR was carried out using a QuantiTect SYBR Green RT-PCR Kit (Qiagen). Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated cancer cell lines compared to untreated cells using the ΔCt method, as per manufacturer's instructions (Applied Biosystems). A 300 fold increase in MT1X was seen at 4 hours, 5 fold increase in MMP7 at 24 hours and no significant change in PPP2R5D expression seen at any time point although a fluctuating increase in expression between 0.8 – 1.5 fold was seen.*

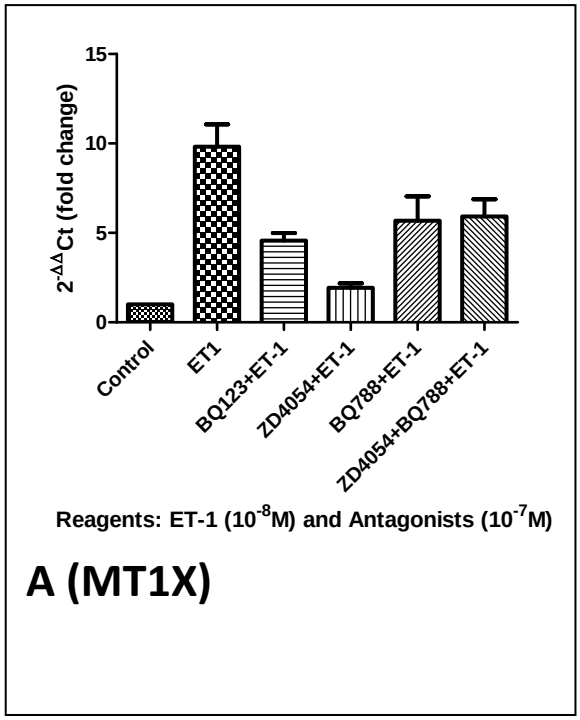
The effect of receptor antagonism on the expression of selected genes was investigated in HT29 and SW620 cell lines. Cells were exposed to ET-1 alone, ET-1 and ET_A and/or ET_B receptor antagonists (BQ123/ZD4054, BQ788) and compared with controls (figure 4.16).

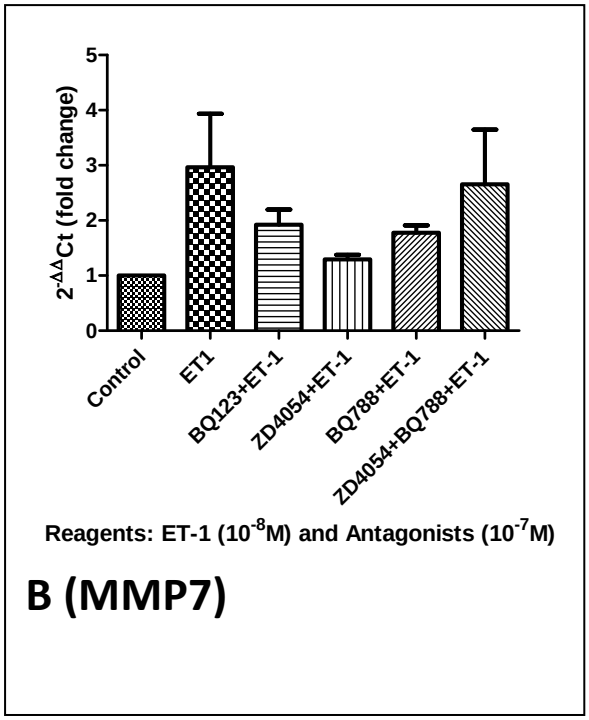
The effect of ET-1 on gene induction of MT1X was demonstrated with a 9.8 fold increase seen at 4 hours following ET-1 exposure (range: 7.4 to 11.5 fold). Interestingly ET-1 also induced the expression of MMP7 by an average of 3 fold (range: 1.8 to 4.9 fold) despite the observed down regulation on the gene array data for this cell line. Greater variability was seen with the PPP2R5D, showing an average 1.26 fold increase (HT29 showed 1.05 and 1.06 fold change whilst SW620 showed a 1.67 fold increase).

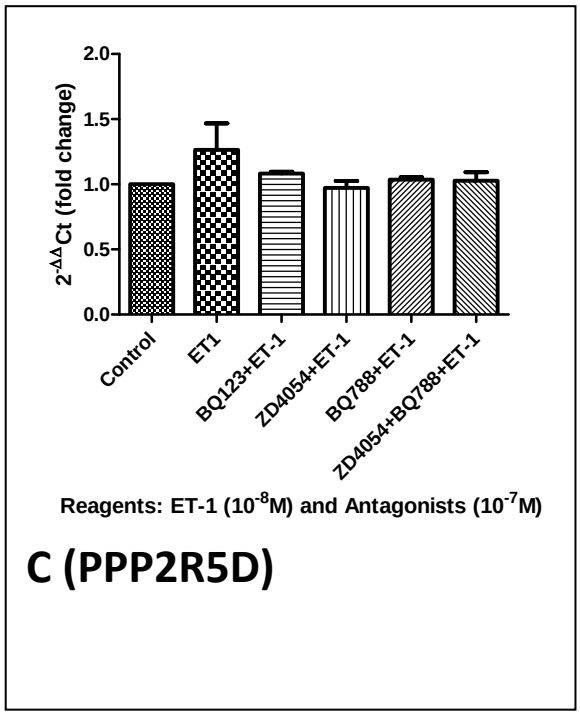
The contribution of receptors to gene regulation was investigated via the use of ET_A and ET_B antagonists. The ET_A receptor antagonist ZD4054 was most efficacious at inhibiting the ET-1 induced gene expression of MT1X and MMP7 (89.5% and 85.0% respectively; significance $p<0.05$). Both the ET_A (BQ123) and ET_B (BQ788) receptor antagonists inhibited expression of both MT1X and MMP7 to a similarly significant extent (BQ123: 59.5% and 53.0% respectively; BQ788: 47% and 60.5% respectively; all significantly with $p<0.05$). Combined receptor antagonism with ZD4054 and BQ788 had the least inhibitory effect (MT1X: 44.2%; MMP7: 15.7%) reaching significant inhibition of MT1X only.

PPP2R5D did not show any significant inhibition of ET-1 induced changes, using any of the receptor antagonists. Expression levels of this gene stayed around control and were non-significant. Combined results are shown graphically in figure 4.16 (A-C) and as a table in figure 4.16 (D).

ET_A and ET_B Receptor Antagonist Effect on Gene Expression







D

Antagonist	Change in MT1X expression	
	% Decrease	Significance
BQ123	59.5	p<0.05
BQ788	47.0	p<0.05
ZD4054	89.5	p<0.05
ZD4054+BQ788	44.2	P<0.05
Antagonist	Change in MMP7 expression	
	% Decrease	Significance
BQ123	53.0	P<0.05
BQ788	60.5	P<0.05
ZD4054	85.0	P<0.05
ZD4054+BQ788	15.7	NS
Antagonist	Change in PPP2R5D expression	
	%Inhibition	Significance
BQ123	69.0	NS
BQ788	86.5	NS
ZD4054	110.2	NS
ZD4054+BQ788	89.6	NS

Figure 4.16. *mRNA expression levels of* ***A:*** *MT1X,* ***B:*** *MMP7 and* ***C:*** *PPP2R5D in the presence of ET-1(10^{-8}M) and/or $ET_{A/B}$ receptor antagonists (10^{-7}M) following 4 hours of exposure. This graphical representation is produced by three independent repeats. Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated cell lines compared to untreated cells.* ***D:*** *Table showing percentage inhibition of ET-1 induced gene expression.*

Gene silencing was used to identify specific receptor subtypes involved in altering gene expression of selected genes (figure 4.17).

ET-1 induction of MT1X was also demonstrated but with limited response seen in MMP7 and PPP2R5D. In the MT1X experimental repeats, silencing the ET_A receptor appeared to increase MT1X gene expression above ET-1/scrambled exposure alone (mean increase of 68.7%), whilst ET_B receptor silencing demonstrated similar expression levels to ET-1/scrambled exposure alone (decrease of 5.1%). Combined silencing showed an increase in MT1X expression. All results were non-significant.

Both ET_A and ET_B receptor silencing resulted in a reduction of MMP7 expression. ET_A receptor and combined $ET_{A/B}$ receptor silencing resulted in significantly decreased expression of this gene (40.5% and 42.5% respectively; $p<0.05$). ET_B receptor silencing was non-significant (decrease of 31.8%).

No significant changes were seen with expression levels of PPP2R5D.

SiRNA Silencing of ET_A and ET_B Receptors

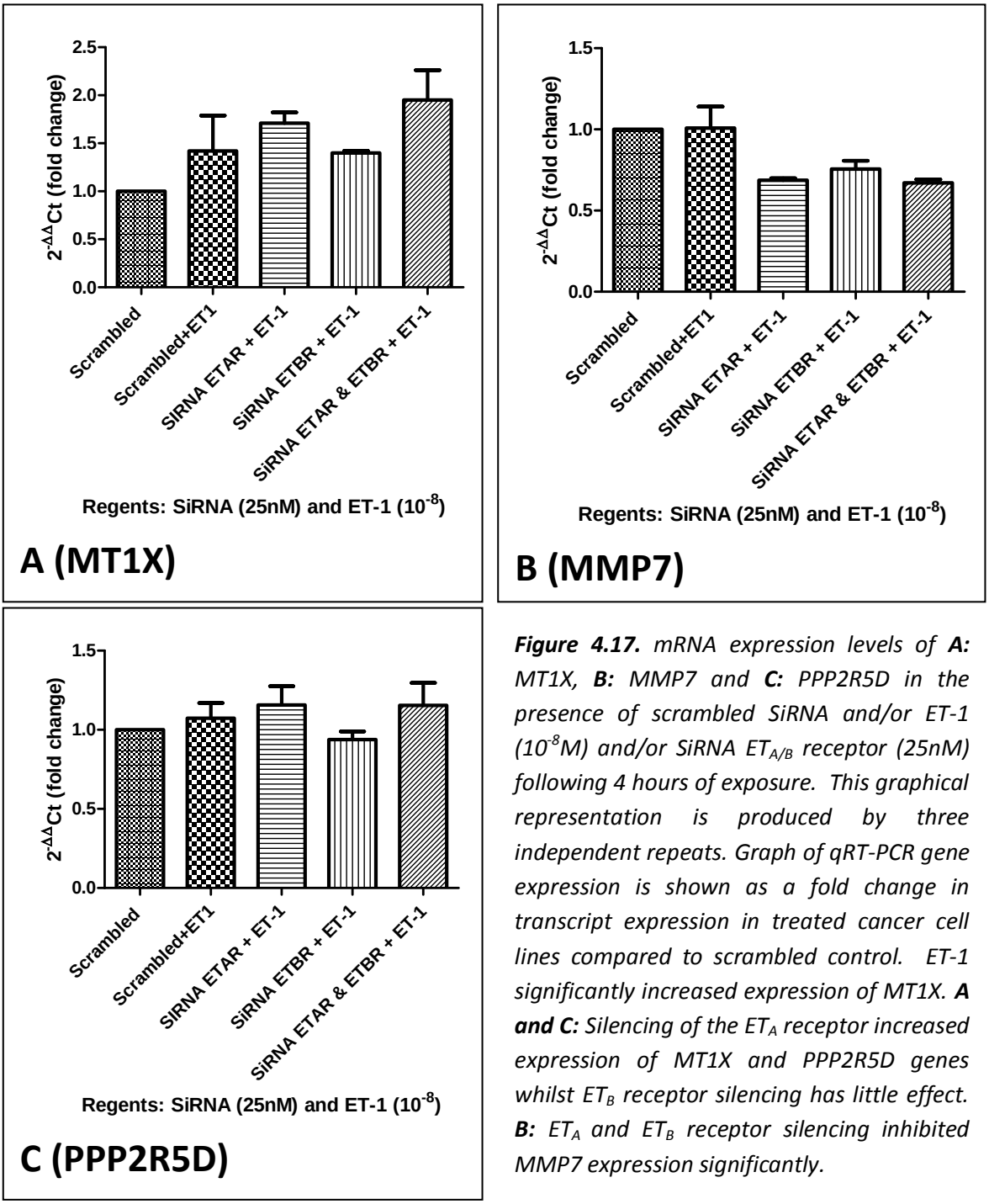


Figure 4.17. *mRNA expression levels of* ***A:*** *MT1X,* ***B:*** *MMP7 and* ***C:*** *PPP2R5D in the presence of scrambled SiRNA and/or ET-1 (10^{-8}M) and/or SiRNA $ET_{A/B}$ receptor (25nM) following 4 hours of exposure. This graphical representation is produced by three independent repeats. Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated cancer cell lines compared to scrambled control. ET-1 significantly increased expression of MT1X.* ***A and C:*** *Silencing of the ET_A receptor increased expression of MT1X and PPP2R5D genes whilst ET_B receptor silencing has little effect.* ***B:*** *ET_A and ET_B receptor silencing inhibited MMP7 expression significantly.*

4.4 RESULTS SECTION

SECTION B:

The Effect of Endothelin Receptor Antagonists at the Protein Level Of Previously Identified Genes Altered by ET-1

4.4.1 Collagen Type XI (COL11A1)

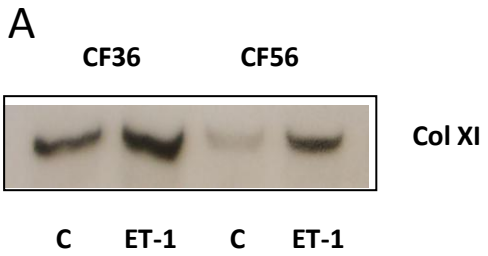
Collagen Type XI was shown to be significantly up-regulated in previous work done within the department. The present experimental conditions and fibroblast strains used demonstrated a similar up-regulation on gene arrays, although less marked (CF36: 1.2 fold; CF56: 1.1 fold; CF65: 1.5 fold; combined: 1.2 fold) (figure 4.3). This section investigated whether ET-1 and its receptors had an influence on protein expression of this gene.

Fibroblasts were grown to 60% confluence then exposed to ET-1 (10^{-7}M) for 24 hours before protein was extracted (appendix 5). All 4 colonic fibroblast strains examined (CF36, CF56, CF65 and CF75) showed a significant increase in protein expression (figure 4.18).

The effect of receptor antagonism on protein expression of Collagen Type XI was investigated in these same 4 fibroblast strains. Fibroblasts were exposed to ET-1 alone, ET-1 and ET_A (BQ123/ZD4054) and/or ET_B (BQ788) receptor antagonists. Fibroblasts incubated with 0.5% BSA medium were used as controls (figure 4.19 – 4.20).

The greatest inhibitory effect on protein expression of collagen Type XI was seen with the ET_A receptor antagonist ZD4054 where expression was reduced to control levels or below control levels ($p<0.05$ significance; combined decrease 65.1%). Combined ET_A (ZD4054) and ET_B (BQ788) receptor antagonists also demonstrated significant inhibition of expression (combined decrease 67.5%; $p<0.05$). This was significant in all fibroblast strains investigated except CF75 strains which demonstrated greater variability. Interestingly, the CF75 fibroblast strain responded less to antagonists than the other fibroblast strains used. Figure 4.19 presents examples of 2 Western blots used. Graphical representation of four independent repeats of these fibroblast strains is shown in figure 4.20 (A-D). Combined results are graphically represented in figure 4.21 (A) and in a table as percentage decrease in figure 4.21 (B). ★ *= significant vs. control values* ☆ *= significant vs. ET-1 values*

Collagen Type XI: Control versus ET-1



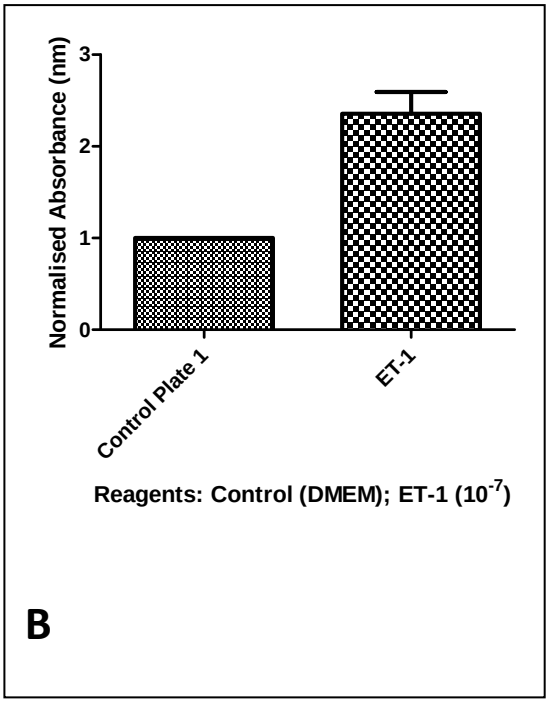


Figure 4.18. *Collagen Type XI protein expression on exposure to ET-1 in fibroblasts. Cells were exposed to control medium alone or ET-1 (10^{-7}M) then protein extracted after 24 hours.* ***A:*** *Western blotting images of two fibroblast strains demonstrating ET-1 induced protein expression.* ***B:*** *Combined graphical representation of ET-1 induced protein expression in 4 fibroblast strains.*

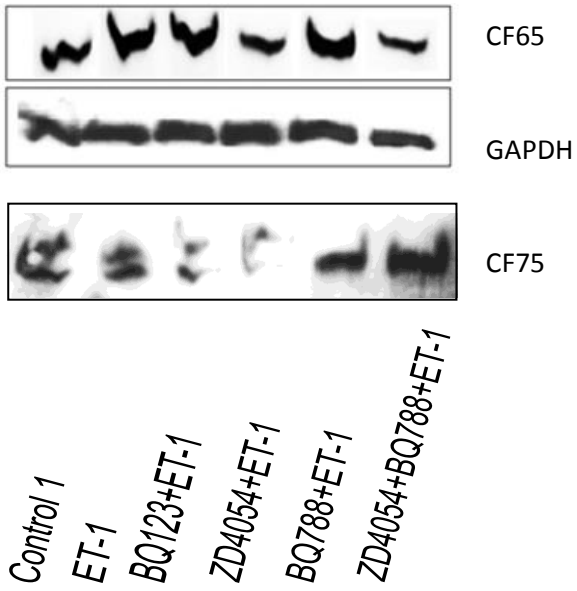


Figure 4.19. *Western blotting showing fibroblast protein expression of collagen type XI on exposure to ET-1 and/or antagonists. Cells were exposed to ET-1 (10^{-7}M) alone or ET-1 and $ET_{A/B}$ receptor antagonists (BQ123, ZD4054,BQ788; 10^{-6}M). Increased expression of collagen type XI is seen when exposed to ET-1 only. Both ET_A receptor antagonists inhibited expression (ZD4054>BQ123) with a less marked effect seen with ET_B blockade (BQ788) produced greater inhibition. Marked inhibition is seen with combined ET_A and ET_B receptor blockade.*

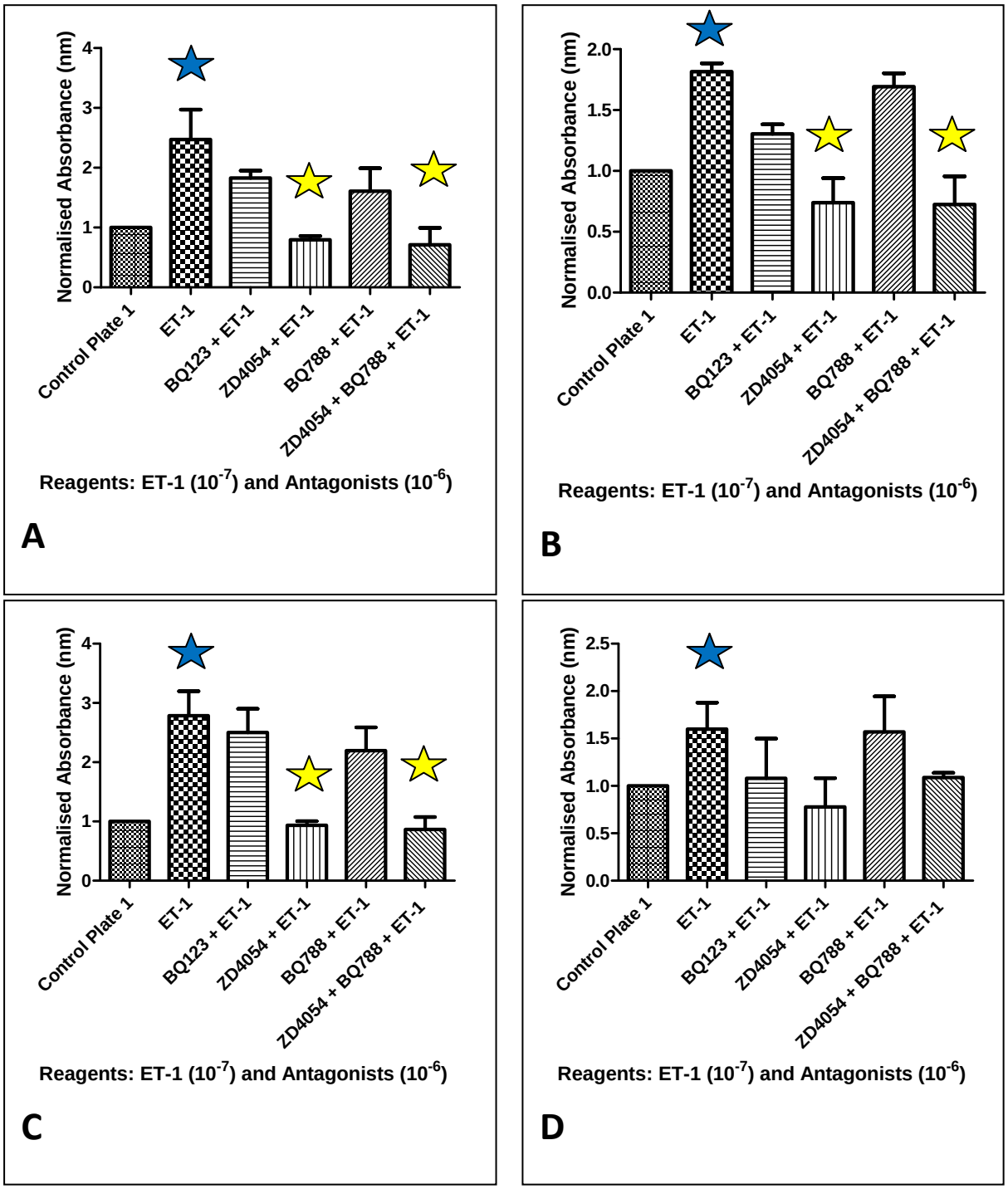
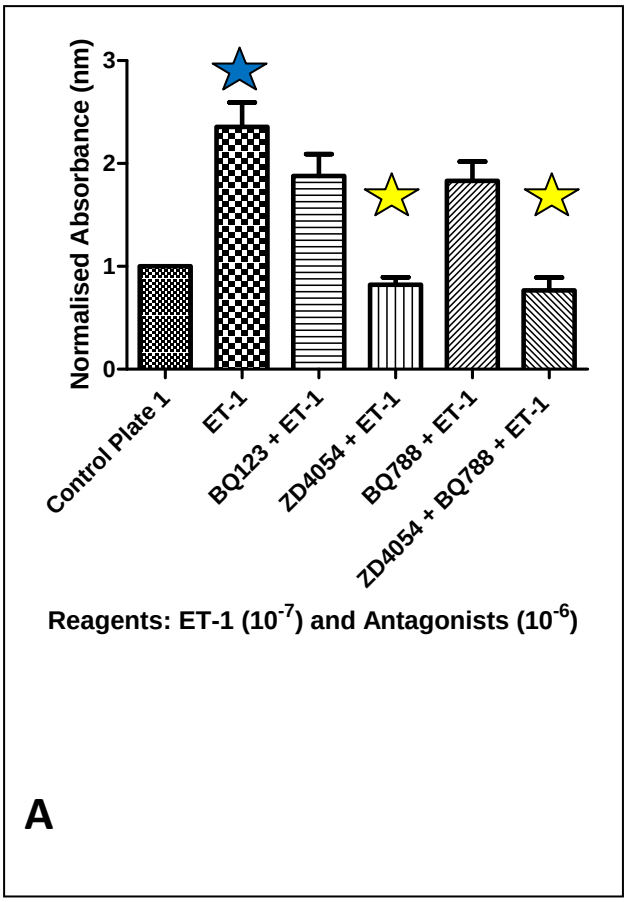


Figure 4.20. *Collagen type XI protein expression in* ***A:*** *CF35,* ***B:*** *CF56,* ***C:*** *CF65 and* ***D:*** *CF75. Cells were incubated with ET-1 and/or* $ET_{A/B}$ *receptor antagonists. This graphical representation is produced by four independent repeats of these fibroblast strains using Western Blotting with antibodies to collagen type XI. Values were normalised by using ratios to control for aiding comparison of strains. In all strains the* ET_A *receptor antagonist ZD4054 significantly reduced protein expression of collagen type XI.*



Reagents	% Decrease
BQ123 + ET-1	20.29
ZD4054 + ET-1	65.11
BQ788 + ET-1	22.30
ZD4054 + BQ788 + ET-1	67.50

B

Figure 4.21. *Combined results of collagen type XI protein expression for all colonic fibroblasts.* ***A:*** *Graphical representation of protein expression for all four fibroblast strains combined, each with 4 independent repeats, following 24 hours of exposure to ET-1 (10^{-7}M) or ET-1 + receptor antagonists (BQ123, BQ788 and ZD4054, 10^{-6}M).* ***B:*** *Table showing percentage decrease in protein expression from ET-1 induced expression set at 100%. ET_A blockade with BQ123 and ZD4054 produced a 20.3% and 65.1% decrease in protein expression respectively (ZD4054 being the most efficacious at reducing collagen type XI expression). ET_B blockade with BQ788 produced a 22.3% decrease.*

4.4.2 Acute Myeloid Leukaemia-1 (AML-1)

AML-1 was another significantly up-regulated gene, previously demonstrated within the department. Under present experimental conditions only slight up regulation was observed by gene array at 4 hours (CF36: 1.11 fold; CF56: 1.16 fold; CF65: 1.32 fold; combined: 1.14 fold) (figure 4.3).

Fibroblasts were grown to 60% confluence then exposed to ET-1 (10^{-7}M) for 24 hours before protein was extracted ET-1 resulted in a significant increase in AML-1 protein expression in all fibroblast strains following 24 hours of exposure. The effect of receptor antagonism on protein expression of AML-1 was investigated in 3 fibroblast strains (CF65, CF75 and CF78). They were exposed to ET-1 alone, ET-1 and ET_A (BQ123/ZD4054) and/or ET_B (BQ788) receptor antagonists. Fibroblasts exposed to 0.5% BSA medium were used as controls (figure 4.22).

The greatest inhibitory effect on protein expression was observed with both ET_A receptor antagonists BQ123 and ZD4054, where expression was reduced to control levels. This was significant in the CF65 and CF78 strains (combined decreased in all fibroblasts with BQ123: 75.1% and ZD4054 77.1%; $p<0.05$). Combined ZD4054 and BQ788 antagonists also demonstrated inhibition of AML-1 expression, reaching significance in the CF78 fibroblast strain (CF78: 56.9% decrease; combined decrease 63.3%; $p<0.05$). BQ788 did not induce significant inhibition in any of the fibroblast strains examined (combined inhibition of 16%).

Interestingly, the variability previously observed in the CF75 fibroblast strains was repeated here. The great variability of protein expression unfortunately meant that statistical significance in response to ET-1 exposure could not be achieved. Figure 4.22 (A) shows examples of 3 Western blots used whilst figure 4.22 (B-D) demonstrates graphically the protein expression in individual fibroblast strains following densitometry analysis. Combined results are shown graphically in figure 4.23 (A) and in a table as percentage decrease in figure 4.23 (B). ★ *= significant vs. control values* ☆ *= significant vs. ET-1 values*

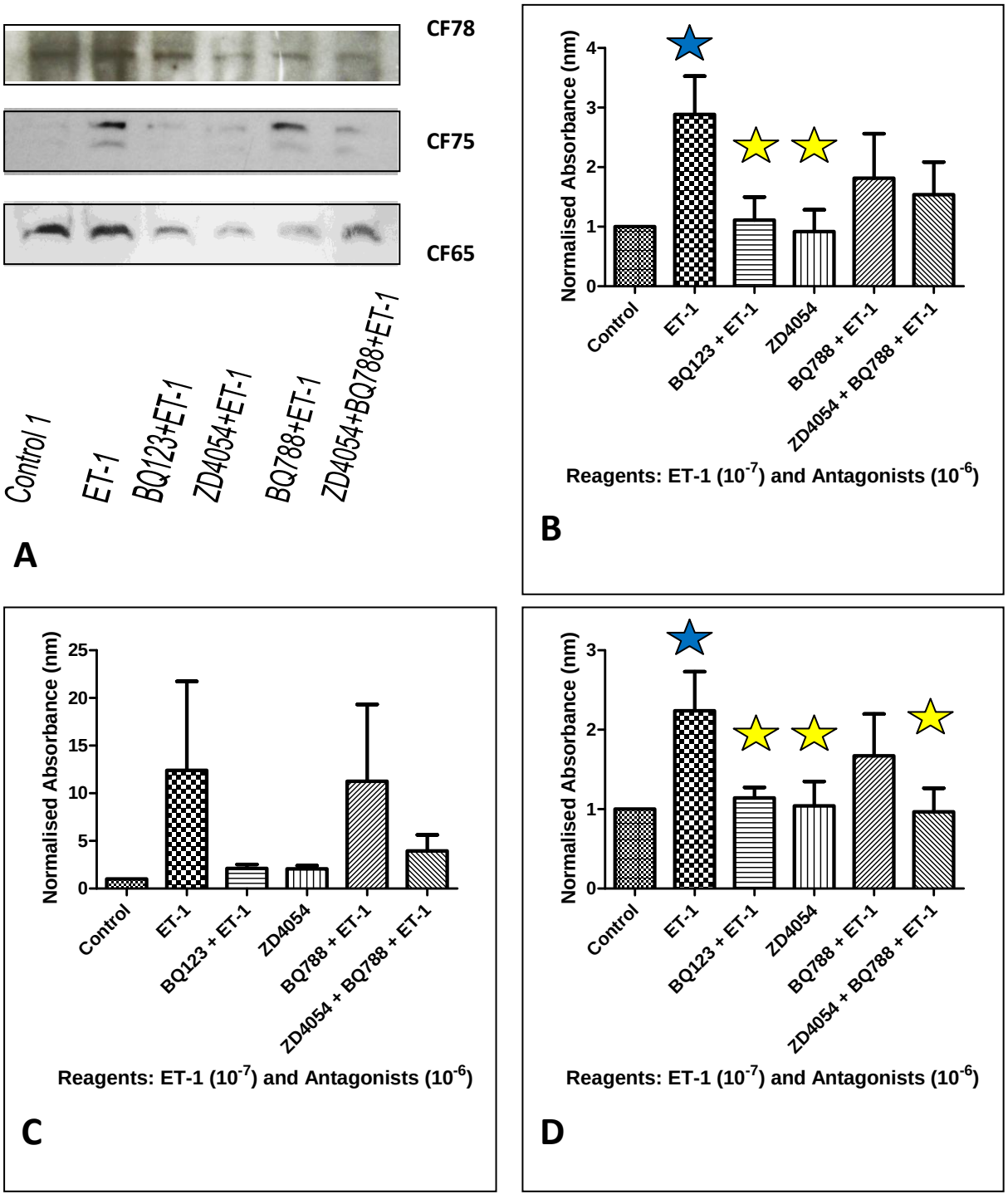
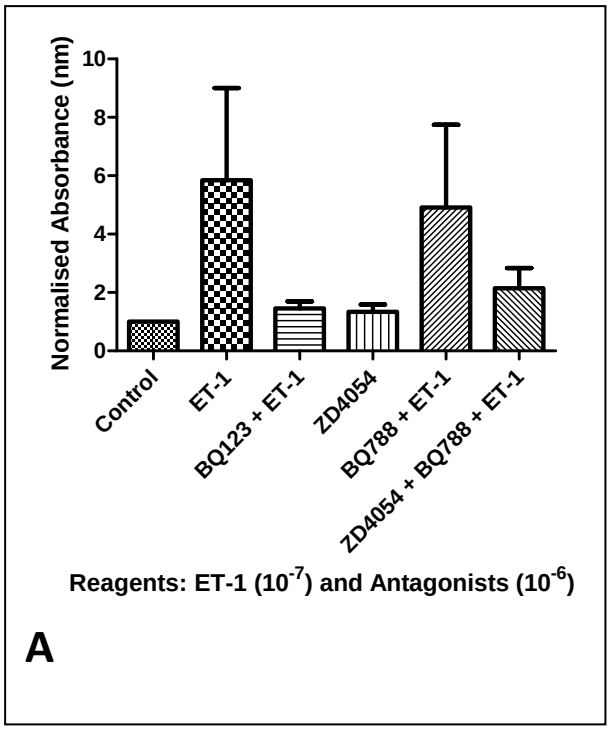


Figure 4.22. *AML-1 protein expression in* ***B:*** *CF65,* ***C:*** *CF75 and* ***D:*** *CF78 fibroblasts. Cells were incubated with ET-1 and/or $ET_{A/B}$ receptor antagonists. This graphical representation is produced by four independent repeats of these fibroblast strains using Western blotting with antibodies to AML-1. Values were normalised by using ratios to control for aiding comparison of strains. In CF65 and CF78 strains the ET_A receptor antagonist ZD4054 significantly reduced protein expression of AML-1.* ***A:*** *shows examples of Western blotting images for each of the fibroblast strains. The ET_A receptor demonstrated a significant inhibition of AML-1 protein expression with a much more variable ET_B receptor blockade response.*



Reagents	% Decrease
BQ123 + ET-1	75.1
ZD4054 + ET-1	77.1
BQ788 + ET-1	16.0
ZD4054 + BQ788 + ET-1	63.3

B

Figure 4.23. *Combined results of AML-1 protein expression for all colorectal fibroblasts.* ***A:*** *Graphical representation of protein expression for all 3 fibroblast strains combined, each with 4 independent repeats, following 24 hours of exposure to ET-1 (10^{-7}M) or ET-1 + receptor antagonists (BQ123, BQ788 and ZD4054, 10^{-6}M).* ***B:*** *Table showing percentage decrease in protein expression from ET-1 induced expression set at 100%. ET_A blockade with BQ123 and ZD4054 produced a 75.1% and 77.1% decrease in protein expression respectively (ZD4054 being the most efficacious at reducing AML-1 expression). ET_B blockade with BQ788 produced a 16% decrease.*

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4.5 RESULTS SECTION

SECTION C:

Expression of the Endothelin System in Colonic Fibroblasts & Cancer Cell Lines and Effect of SiRNA on the EGF Receptor

4.5.1 Expression of Epidermal Growth Factor Receptor and the Endothelin Axis in Colonic Fibroblasts and Cancer Cell Lines

Fibroblasts and cancer cells showed slight variations in responses to ET-1 and its receptor antagonists at the mRNA level. Therefore conventional RT-PCR was carried out to look at mRNA expression levels in continuously growing colonic fibroblasts and cancer cell lines (figure 4.24)

Cells were grown in 10cm dishes supplemented with 0.5% BSA for fibroblasts or 10% FCS for cancer cell lines in DMEM. Cells were grown to 60% confluence then mRNA extracted Conventional RT-PCR was carried out using primers designed to anneal at 58°C (appendix 11).

mRNA for ET-1 was only observed in the cancer cell lines and to a similarly weak extent. The ET_A receptor was equally expressed in the fibroblasts but variability was seen in the cancer cell lines. The highest levels of ET_A receptor mRNA were seen in the SW480 cells, followed by HT29 then LIM1215, with little expression seen in the SW620 cells. The ET_B receptor had varying levels of mRNA expression in fibroblasts with most seen in the CF65 and CF75 strains. Higher levels were seen in the cancer cell lines with the exception of SW620 which showed a low expression level. Epidermal Growth Factor Receptor (EGFR) mRNA levels were seen throughout the panel of cells with the lowest expression seen in the SW620 and LIM1215 cancer cell lines. GAPDH confirmed equal loading of all templates for the purpose of carrying out conventional PCR. Figure 4.24 shows mRNA expression levels as visualised on gels following conventional RT-PCR.

Two fibroblast and two cancer cell lines were also grown in the same conditions to evaluate protein expression, which may be either intracellular or on the cell surface (figure 4.25). The fibroblasts showed equal levels of both ET_A and ET_B receptors. The cancer cell lines showed greater ET_A receptor expression and little to moderate amounts of ET_B receptor expression. GAPDH confirmed equal loading of protein.

Expression at mRNA Level of ET-1, ET Receptors and EGF Receptor

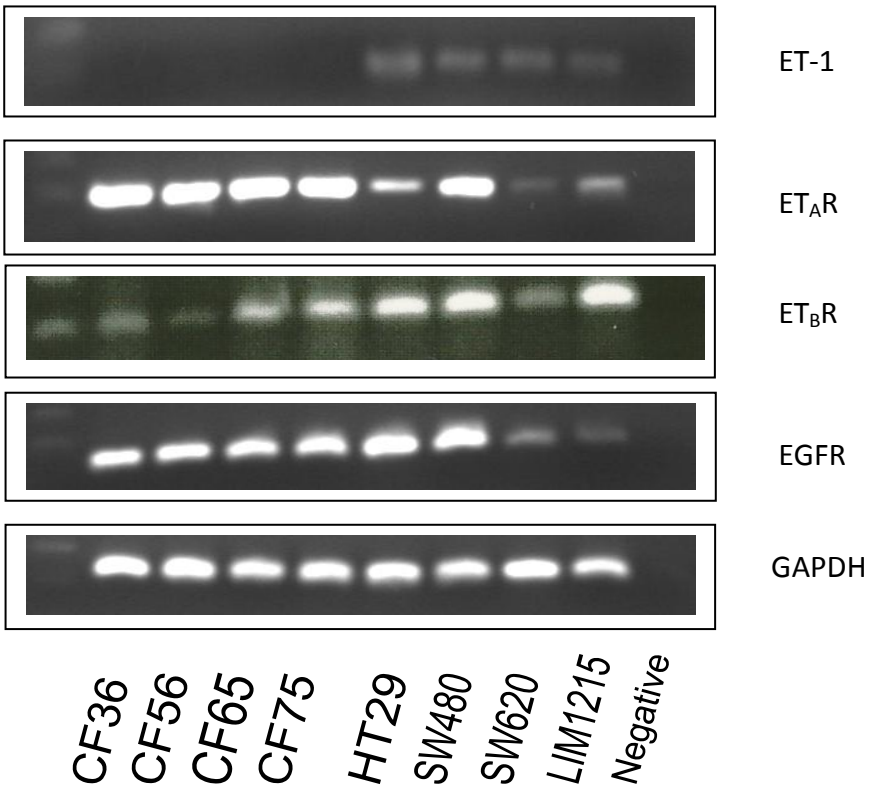


Figure 4.24. *Conventional RT-PCR showing expression levels of the EGR receptor and key genes within the Endothelin axis in both fibroblasts and colorectal cancer cell lines. Primers were designed to flank introns and with annealing temperatures of 58°C to be mRNA specific. Conventional RT-PCR was carried out as per standard conventional RT-PCR protocol using a Mastercycler Gradient thermal cycler (Eppendorf) up to 35 cycles. ET-1 was expressed in cancer cell lines and not in fibroblasts at the mRNA level. Varying degrees of ET_A, ET_B and EGF receptors were seen in both fibroblasts and cancer cell lines.*

Expression at protein level

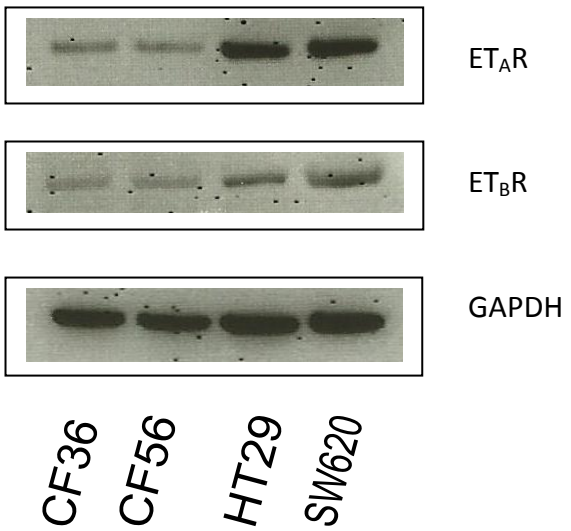
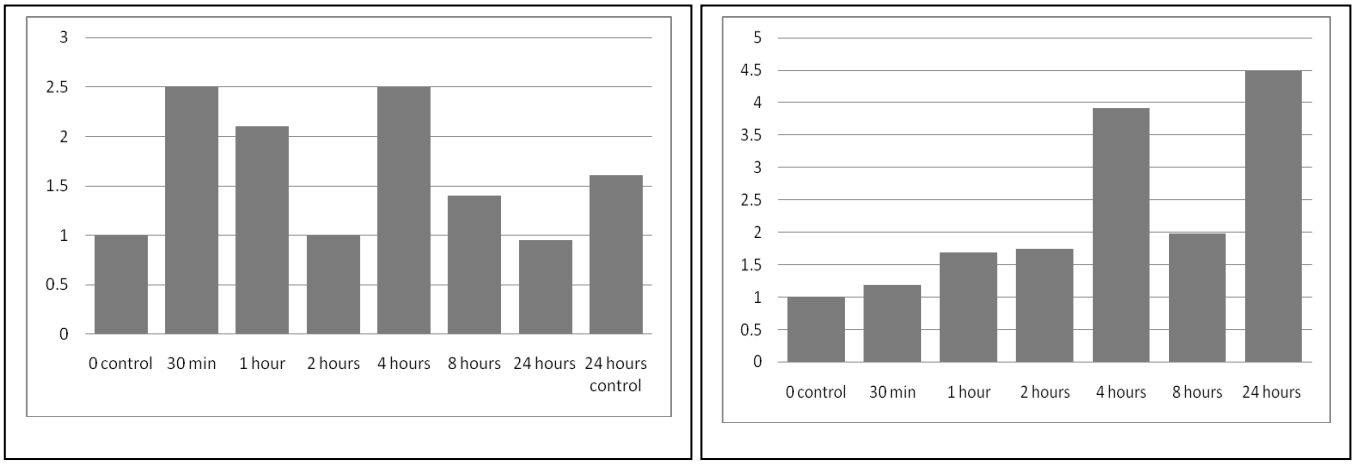


Figure 4.25. *Western blotting showing fibroblast (CF36 & CF56) and cancer cell line (HT29 & SW620) protein expression of ET_A and ET_B receptors. Cells were continuously grown in 3.5cm dishes containing 10% FCS in DMEM. Protein was extracted using RIPA Buffer. Fibroblasts showed similar expression levels of both receptor subtypes. Cancer cell lines showed more ET_A receptor protein expression with less ET_B receptors present.*

As the EGFR and ET_A receptors are associated with colorectal cancer progression, the response of these receptors to ET-1 was examined at the mRNA level. Fibroblasts were grown to 50-60% confluence, serum starved in 0.5% BSA, then exposed to ET-1 for 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours. At these time points, mRNA was extracted and qRT-PCR carried out for each receptor. Controls (at 0 and 24 hours) were exposed to 0.5% BSA alone (figure 4.26).

Quantitative real-time PCR showed a biphasic response to ET-1 in both receptors. The ET_A receptor demonstrated a 2.5 fold increase as early as 30 minutes, followed by a second peak of 2.5 fold at 4 hours. The EGF receptor showed a gradual increase in mRNA expression with a first peak seen at 4 hours (3.8 fold increase) followed by a 4.5 fold increase at 24 hours.

Induction of ET_A and EGF Receptors



ET_AR EGFR

Figure 4.26. *Real-time RT-PCR showing time point inductions of* ***A:*** *ET_A receptors and* ***B:*** *EGF receptors. Fibroblast strains were exposed to ET-1 (10^{-7}) for 0, 30 minutes, 1, 2, 4, 8 and 24 hours followed by RNA extraction using the Mini-RNeasy Kit. Real time RT-PCR was carried out using a QuantiTect SYBR Green RT-PCR Kit (Qiagen). Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated fibroblasts compared to untreated cells using the ΔCt method, as per manufacturer's instructions (Applied Biosystems). The ET_A receptor demonstrated a biphasic response to ET-1 exposure with a 2.5 fold increase as early as 30 minutes followed by a second 2.5 fold increase at 4 hours. The EGF receptor again showed a peak at 4 hours with a 3.8 fold increase and a 4.5 fold increase at 24 hours.*

SiRNA was used to knockdown the ET_A and/or ET_B receptor expression following optimization of this protocol: silencing of the ET_A receptor demonstrated 89% knockdown with a minor effect on the ET_B receptor (17% knockdown), whilst silencing of the ET_B receptor produced only 25.3% knockdown with a 10.5% knockdown of the ET_A receptor. Combined silencing produced 86.1% knockdown of the ET_A receptor and 36.8% knockdown of the ET_B receptor (figure 4.27A & 4.27B).

The effect on the EGF receptor was also evaluated, since this is known to be heavily involved in colorectal cancer progression. Interestingly the ET_A receptor silencing inhibited any ET-1 stimulated gene induction of the EGF receptor, maintaining it at control levels. Silencing the ET_B receptor had no effect on EGF receptor expression at the mRNA level (figure 4.27C).

Silencing of the ET_A and ET_B Receptors

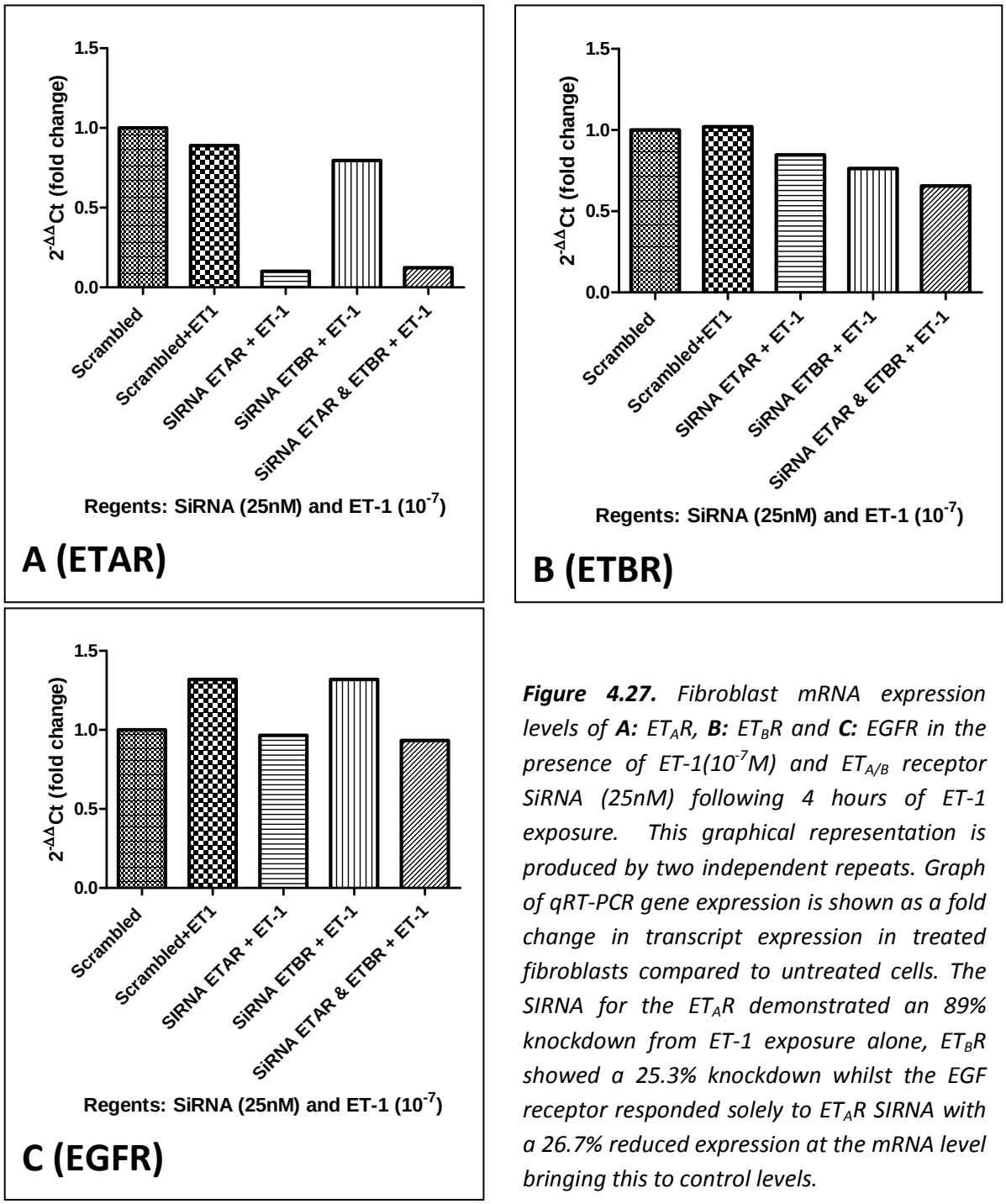


Figure 4.27. *Fibroblast mRNA expression levels of* ***A:*** *ET_AR,* ***B:*** *ET_BR and* ***C:*** *EGFR in the presence of ET-1(10^{-7}M) and $ET_{A/B}$ receptor SiRNA (25nM) following 4 hours of ET-1 exposure. This graphical representation is produced by two independent repeats. Graph of qRT-PCR gene expression is shown as a fold change in transcript expression in treated fibroblasts compared to untreated cells. The SIRNA for the ET_AR demonstrated an 89% knockdown from ET-1 exposure alone, ET_BR showed a 25.3% knockdown whilst the EGF receptor responded solely to ET_AR SIRNA with a 26.7% reduced expression at the mRNA level bringing this to control levels.*

4.6 DISCUSSION

4.6.1 Selected Genes Stimulated by ET-1 in Fibroblasts

4.6.1.1 CTGF and CYR61

The immediate early response genes connective tissue growth factor (CTGF; CCN2) and Cysteine-Rich 61 (CYR61; CCN1) were shown to be significantly up-regulated by ET-1. They are stimulated primarily by TGF-β, share 90% structure homology and belong to the CCN family of proteins. Structurally they all comprise 4 domains with differing functions: IGF binding (module 1), oligomerization (module 2), cell attachment (module 3) and dimerization (module 4). They are produced by and act on numerous cells including epithelial, endothelial, fibroblasts, myofibroblasts, smooth muscle and neural cells (Latinkic *et al.* 1991; Moussad *et al*. 2000). The CCN family collectively have numerous regulatory cellular functions involving cell cycle progression, division, differentiation, apoptosis, adhesion, chemotaxis, gene regulation and ion transport (Perbal 2001). They are therefore implicated in differentiation, embryological development, angiogenesis, tumour growth, wound healing and fibrosis. (Bornstein *et al.* 2002).

CTGF was up-regulated by ET-1 in all colonic fibroblast strains, as demonstrated by gene array analysis. This was confirmed by time-point induction studies with maximum expression reached between 1-2 hours. Further work showed that the ET_A receptor antagonist ZD4054 was most efficacious at inhibiting CTGF mRNA expression following ET-1 exposure, whereas a more variable non-significant response was seen with BQ123. A discussion of why this may be the case is found later in this chapter. The ET_B receptor antagonist BQ788 also significantly inhibited CTGF mRNA expression. Use of siRNA confirmed the dominant role of the ET_A receptor in CTGF regulation, as silencing almost completely inhibited its expression. Silencing the ET_B receptor also significantly inhibited CTGF expression, but to a much lesser extent. The greater inhibitory effect of the ET_B receptor antagonist BQ788 compared to ET_B receptor silencing can be explained by the specificity of the antagonist. As discussed previously (chapter 3) the affinity of BQ788 for ET_B receptors is only tenfold greater than its affinity for ET_A receptors. Therefore BQ788 will have some antagonistic action at the ET_A receptor which we have demonstrated significantly inhibits CTGF expression. In contrast, the ET_A receptor antagonist BQ123 has 1000-10,000 times greater affinity for the ET_A receptor than the ET_B receptor (Davenport, 2002). Inhibition of CTGF expression was also confirmed at the protein level, with ZD4054 having the greatest effect, followed by BQ788 and BQ123.

There are very few published reports on the role of CTGF in colorectal cancer development and progression. Knowles *et al*. (2011) is the only published study to date that mapped colonic fibroblast expression of CTGF and suggested a potential role in colorectal cancer development. Another recent study demonstrated increased overall cytoplasmic CTGF (and Cyr61) mRNA and protein expression in colorectal cancer specimens (Ladwa *et al*., 2011). Interestingly they found Duke's A and B tumours had higher mRNA expression levels then Duke's C tumours. Furthermore, higher CTGF levels were associated with better prognosis in colorectal cancers in two studies (Chang *et al*., 2006; Lin *et al*., 2005). However, this may correlate with findings of higher CTGF expression in early stage cancers with a decreased expression seen in more advanced tumours. Over-expression of CTGF was also observed in gliomas, oesophageal and pancreatic cancer (Koliopanos *et al*., 2002; Hartel *et al*., 2004; Xie *et al*., 2004). Conversely, in oesophageal and breast cancers, higher levels of CTGF were associated with more advanced disease (Koliopanos *et al*., 2002; Xie *et al*., 2001).
It has been suggested that CTGF plays an important role in angiogenesis. Human breast cancer (MDA-MB-231) and fibrosarcoma (HT-1080) cells showed high levels of CTGF expression. Xenographs in BALB/c nude mice showed high levels of neovascularisation, strongly correlating with CTGF levels. Furthermore, neutralising CTGF specific antibodies blocked tumour angiogenesis on chicken chorioallantoic membrane, producing a similar effect as anti-VEGF and anti-bFGF antibodies (Shimo *et al*., 2001). Breast cancer cells also increased levels of CTGF in response to hypoxia; it was suggested that tumour hypoxia promotes increase CTGF levels with subsequent MMP up-regulation and TIMP down-regulation (Chen *et al.* 2001). Increased MMP results in ECM degradation and facilitate endothelial migration and vascularity. Indeed HUVEC cells exposed to CTGF increased transcription of MMP1/2/3/7/9, MI1-MMP and decreased TIMP 1 and 2. In regards to related proteins, human fibroblasts exposed to CYR61 increased MMP1 and 3 expression, whilst CYR61 injected into RF-1 gastric adenocarcinomas and MCF-7 breast cancer cells produced larger and more vascular tumours (Xie *et al.* 2001).

CTGF and CYR61 may also promote angiogenesis indirectly. Ordinarily heparin sulphate proteoglycans (HSPGs) are bound to bFGF within the ECM. CTGF and CYR61 both bind to heparin sulphate proteoglycan (HSPGs) extracellularly via module 4 and therefore displace bFGF allowing it to bind to the FGF receptor, promoting an angiogenic response and mitogenic activity within fibroblasts (Koliopanos *et al.* 2002). Furthermore, adhesion of fibroblasts promoted by CYR61 or CTGF involve signalling events that include formation of filopodia and lamellipodia, formation of focal complexes containing integrin $\alpha_6\beta_1$, and activation of FAK, paxillin, Rac and MAPK (Chen *et al.* 2001). CYR61 and CTGF interact in a

dose-dependent manner with integrin $\alpha_v\beta_3$ to promote adhesion in endothelial cells and migration of cells. CYR61 also promotes chemotaxis, whilst CTGF promotes both chemotaxis and chemokinesis (Babic *et al.* 1998).

CTGF is associated with numerous tumourigenic actions and with such far reaching effects within the tumour environment, it seems hugely beneficial to be able to modulate its activity. ZD4054 significantly inhibited its expression in fibroblasts at both the mRNA and protein levels via the ET_A receptor. We would therefore expect this to decrease vascularity of tumours, inhibit its mitogenic effect on surrounding cells and reduce MMP production, thereby reducing metastatic ability of tumours. Its adhesion, migratory and proliferative effects on fibroblasts should also be curtailed. As ET_B receptor antagonists also had a small but significant effect, it is plausible that Bosentan, a dual ET_A and ET_B receptor antagonist may be of benefit.

4.6.1.2 Adrenomedullin (ADM/AM)

Adrenomedullin is a 52 amino acid peptide originally isolated from a human phaeochromocytoma and belongs to the calcitonin gene peptide superfamily (Poyner *et al.*, 2002; Nikitenko *et al.,* 2006). Similar to ET-1, it is a vasodilatory peptide with pleiotropic activities, such as: (1) a growth factor, (2) an angiogen both *in vitro* and *in vivo*, (3) an inhibitor of apoptosis in endothelium and isolated tumour cells, (4) a potent vasodilator, (5) regulator of endothelial permeability, as well as (6) contributing to adhesion and differentiation of bone marrow derived mononuclear cells into endothelial progenitor cells. (Hinson *et al*., 2000).

ET-1 was seen to up-regulate ADM on gene array analysis. This was confirmed on time point induction studies with maximum mRNA expression at 2-4 hours with up to a 2.6 fold increase.

Antagonistic studies showed that ZD4054 significantly inhibited ET-1 stimulated ADM induction ($p<0.05$). Both BQ123 and ZD4054 resulted in significant inhibition of expression in 2 of the 3 fibroblast experiments. However, on combining all repeats, significance was lost. ET_A receptor silencing demonstrated almost complete inhibition of ADM expression whereas the ET_B receptor seemed not to play any role.

A large body of literature has investigated the role of ADM in cell proliferation, angiogenesis, apoptosis and tumourigenesis. ADM is highly expressed in malignant tissues including lung, breast, colon, glioblastoma, pancreas and prostate with only a few reports on colorectal cancer. ADM has strong links with angiogenesis. Within leiomyomas, ADM expression correlated with increased vascular density and endothelial proliferation (Hague *et al.*, 2000). Kaafarani *et al*., (2009) carried out *in vivo* quantitative image analysis showing Matrigel plugs containing ADM demonstrating a 30-40% increase in endothelial and pericyte cells compared to VEGF treated plugs. In endometrial, breast and pancreatic xenograft tumours, vascular density or directed growth of blood vessels was increased in ADM over-expressing transfectants. In HCT116 colon cancer cell lines, hypoxia induced HIF-1α resulted in increased JMJD1A protein expression, which in turn demethylated a H3K9 hypoxic promoter thus inducing expression of ADM and ET-1 (Krieg *et al*., 2009).

ADM also stimulates growth and inhibits apoptosis. *In vitro* studies using anti-ADM receptor antibodies demonstrated a dose dependent inhibition of proliferation in HT29 colorectal cancer cells of 30%, 62% and 75% respectively with increased doses (10, 20 & 40μg/ml). Furthermore, in HT29 xenografts anti-ADM receptor antibodies inhibited growth with markedly diminished vascularity, compared to controls. Anti-ADM antibodies also significantly decreased growth *in vitro* and *in vivo* of ADM expressing U87 glioblastoma models (Oufik *et al*., 2002). In terms of apoptosis, ADM over-expressing endometrial tumour cells showed resistance to hypoxia-induced apoptosis via a bcl-2 mediated mechanism (Oehler *et al*., 2002). At the molecular level, ADM transfected tumour cells expressed higher levels of oncogenic proteins such as Ras, Raf, PKC and MAPKp49 and incorporated more bromodeoxiuridine, indicating increased proliferation (Martinez *et al*., 2002). There was also an up-regulation of anti-apoptotic factors and down-regulation of pro-apoptotic factors that contributed to tumour cell survival (Oehler *et al*., 2002, Martinez *et al*., 2002; Zudaire *et al*., 2003).

Treatment with ZD4054 showed significant reversal of the ET-1 induction of ADM. Therefore potentially its angiogenic, proliferative and anti-apoptotic effects would be of huge benefit in treatment of cancers in the clinical setting.

4.6.1.3 Stanniocalcin-1 (STC1)

STC1 is a glycoprotein that is expressed in numerous developmental and patho-physiological processes including intestinal calcium transport, organogenesis and carcinogenesis. Its chromosomal location falls within the metastatic susceptibility locus, 8p. Loss of this locus is associated with disease advancement and metastasis in colorectal cancers (Chang *et al*., 1998).

STC-1 was expressed in fibroblasts, with only a weak expression in SW620. Illumina data analysis showed up to a 1.9 fold down-regulation in STC-1 expression at 4 hours which was confirmed by time point induction experiments. However, a large up-regulation was observed as early as 30 minutes to 1 hour (3.8 fold increase): this indicates STC-1 is an early response gene.

Antagonistic studies were carried out at 4 hours and confirmed a down-regulation at this time point. All antagonists showed a trend to reverse the effects of ET-1. Two of the three experimental repeats carried out had shown significant return towards control levels for ZD4054 and BQ788, but when combined this significance was lost. When silencing the receptors, ET_A silencing returned STC-1 levels to control whilst the ET_B receptor SiRNA had no significant effect, indicating that ET_A receptor plays the main role in STC-1 expression.

STC1 has been linked to cell growth, apoptosis and angiogenesis. The first growth related properties were identified in fibroblasts on cDNA microarray analysis where STC-1 levels increased 6-8 fold in response to serum supplementation (Lyer *et al*., 1991). More recently knock-down of STC1 was shown to inhibit growth in breast cancer cells (Daniel and Lange, 2009). STC1 was also suggested to be a downstream target of VEGF/Wnt2 and involved in angiogenic responses (Klein *et al*., 2009).

Enhanced STC1 gene expression is seen in hepatocellular, colorectal, breast and medullary thyroid cancers (Fujiwara *et al*., 2000). Particularly for colorectal and hepatocellular cancers increased mRNA expression of STC1 was detected in all tissues compared to normal and 20 of the 21 cancer cell lines examined . In contrast, down-regulation of STC1 is seen in some breast and ovarian cancer cell lines (Yeung *et al*., 2010). Some evidence has linked STC1 expression to increased tumour vasculature and the use of its expression levels for the diagnosis of human breast, hepatocellular and colorectal cancers has been postulated (Fujiwara *et al*., 2000; Yeung *et al*., 2010). One of the few studies investigating STC1 in

fibroblasts was carried out in mouse embryo fibroblasts (MEF) by Nguyen *et al*., 2009. This study gave a mixed picture to the role STC1 has in tumour development. Their results indicated STC1 has negative effect on pro-survival signalling pathways (ERK1/2) and/or a positive effect on cell death signalling. Thus, data relating to STC-1 appears conflicting. Loss of its locus in colorectal cancer points to it acting as a tumour suppressor, however, a number of cancers including colorectal cancers demonstrate increased levels. Some cancer cells may lose expression of STC-1, as illustrated here by conventional RT-PCR work, thereby contributing to pro-survival of cancer cells and fibroblasts, as suggested above. Other supporting cells such as fibroblasts may increase their expression which would explain the higher amounts seen in a number of tumour tissues and support tumour growth through increased vasculature. Differences seen in individual cells maybe as a result of the early up-regulation then down-regulation, as observed by the time point induction studies. Further work is needed to delineate the exact contribution of STC-1 to cancer.

4.6.1.4 Collagen Type XI (COLXI)

Collagen Type XI was only investigated at the protein level as its differential expression following exposure to ET-1 was previously identified within the department (Knowles *et al*, 2011). Protein expression in fibroblasts was seen to be increased following 24 hours of ET-1 exposure. When combining 4 independent repeats for the 4 fibroblast strains examined, ZD4054 was the only antagonist that significantly inhibited collagen type XI expression (65.1%; $p<0.05$).

COLXI has been identified in the connective tissues of colorectal pathologies (Fischer *et al*., 2001a; Fischer *et al*., 2001b). This same group linked the expression of COLXI to stromal regions and co-localised to fibroblasts in colorectal cancers. A correlation between APC/β-catenin pathway and COLXI expression has been demonstrated in patients with both sporadic colorectal cancer and FAP. Our findings fit with the hypothesis that abnormal Wnt signalling induced ECM components such as WISP-1/CTGF that in-turn stimulate fibroblasts to secrete further WISP-1/CTGF and COLXI which aid tumourogensis. Furthermore, silencing COLXI affects the same extra-colonic tissues affected in FAP patients which have a dysregulated Wnt pathway. Knowledge that ET-1 acts via the Wnt pathway and these observations provide further evidence for the potential tumourogenic effect that ET-1 has on the morphology of fibroblasts, and the potential anti-tumourogenic effect that altering ET-1 may have in treating this condition.

4.6.1.5 Acute Myeloid Leaukaemia 1 (AML-1)

Most research involving AML-1 (also known as RUNX1: Runt-related transcription factors) has been carried out in leukaemic cells. However, increasing evidence is emerging that links it to numerous cancers and tumourogenic processes, either alone or when combined as a fusion protein with other transcription factors e.g. EVI-1 and ETS-1.

Expression was determined only at the protein level as up-regulation was previously reported in our department (Knowles *et al*, 2011). A significant up-regulation was seen at 24 hours following ET-1 exposure. Both the ET_A receptor antagonists (ZD4054 and BQ123) significantly inhibited protein expression in 2 fibroblast strains. Significance was not seen in the CF75 strain probably due to the huge variability of ET-1 induced expression.

At a molecular level, AML-1 combined with EVI-1, interacts with a number of signalling pathways. This fusion protein is linked to inhibition of c-Jun N-terminal kinase which is linked to apoptosis. Rat1 fibroblast cells that over-express EVI-1 show a down-regulation of JNK. Similar results are seen in human endometrial carcinoma cell lines HEC1B (Mitani, 2004). Proliferative effect of AML-1/EVI-1 has also been investigated in Rat1 fibroblasts by transfecting them with cDNA AML-1/EVI-1 (Kurokawa *et al*., 1995). Transfected cells formed macroscopic colonies whereas mock-transfected ones barely formed any. Within the TGFβ signalling pathway, over-expression of AML-1/EVI-1 binds to Smad3 resulting in inhibition of growth suppression (Mitani, 2004). A study looking at primary murine embryonic fibroblasts lacking functional p53 found that RUNX1 had pro-oncogenic effects on cell growth that included cytoskeletal re-organisation, reduced contact inhibition and accelerated tumour expansion *in vivo* (Wotton *et al*., 2004).

The only published study to date strongly linking AML-1/RUNX1 to colorectal cancer involved a population based study of 1555 patients (Slattery *et al*., 2011). They looked at genetic variations (tagSNPs) in RUNX1-3, MAPK1 and eIF4E transcription factors to determine association with colorectal cancers. There was statistically significant association of AML-1/RUNX1 with colon and rectal cancers and therefore important in the aetiology of both these cancers as well as abnormal TGFβ signalling.

With the role of AML-1 in TGFβ signalling, known to be dysregulated in colorectal cancer, the fact that ET receptor antagonism alters its expression is potentially exciting. By inhibiting AML-1 expression, Smad 3 would be able to propagate its signalling to inhibit cell growth.

There seems to be evidence that the apoptotic pathway may be enhanced and therefore use of ZD4054 in combination with other therapies may be of benefit too.

4.6.2 Selected Genes in Colorectal Cancer Cell Lines

4.6.2.1 Metallothioneins (MT1X)

Metallothioneins (MTs) are cysteine-rich low molecular weight protein localised to the Golgi apparatus membrane. They bind both physiological (zinc, copper, selenium) and xenobiotic (mercury, silver, cadmium) heavy metals, therefore providing protection from metal toxicity and oxidative stress, whilst playing a homeostatic role in the cell. MT genes can be induced by metal ions via metal responsive elements and controlled by glucocorticoids, various cytokines and growth factors. Elevated levels of MTs in rapidly proliferating cells have been attributed to increased Cu and Zn demand therefore potentially affecting genes associated with cell cycle regulation. Indeed Nagel *et al*., (1995) studied MT levels in HT29 cells using immunocytochemistry. Levels were higher in sub-confluent proliferating cells relative to growth inhibited confluent cells. They also found oscillating MT levels during the cell cycle reaching a maximum near G1/S transition, at the onset of DNA synthesis. Since cell growth and proliferation are closely related to Zn, they postulated the functions of Zn are mediated via MT for the use of cellular actions, including Zn requiring transcription factors. This correlates with observations that MTs localise to the nucleus around the G1/S phase of the cell cycle.

The Metallothioneins family of genes, most significantly MT1G and MT1X, were up-regulated following exposure to ET-1. MTX1 was chosen for further investigations due to the availability of primer sequences and antibodies. As early as 1 hour following ET-1 exposure, MT1X mRNA expression had increased by 12 fold; and to over 300 fold at 4 hours.

Both ET_A and ET_B receptor antagonists significantly inhibited expression of MT1X. The most efficacious of these was ZD4054 with 89.5% inhibition. Interestingly the response to receptor silencing was much more variable. ET_A receptor silencing actually increased MT1X expression whereas ET_B receptor silencing seemed to have no effect. One possibility for this unusual result may be due to sub-optimal experimental conditions as this was optimized for fibroblasts but not specifically for the cancer cell lines. It is possible that SiRNA concentration of 25nM was not high enough or the Oligofectamine concentration too low.

Even if silencing conditions were optimal, the timeline for the clearance of ET receptors from the cell surface membrane is unknown and therefore may confound the present results: mRNA expression for receptors may be inhibited, but the protein may still be present.

MT seems to relate to advanced and highly malignant tumours (Jasani *et al*., 1998). MT expression is associated with poorer prognosis in breast (Oyama *et al*., 1996), hepatic, pancreatic, gastric and oesophageal cancers (Hishikawa *et al*., 2001). It has been reported that MT over-expression can also occur in somatic mutations as a result of cis-activating mutations in the MT gene or trans-activating mutations in MT controlling genes (Jasani *et al*., 1998). Over-expression of MTs is reported to correlate with resistance to chemotherapeutic agents such as cisplatin and alkylating agents (Hishikawa *et al*., 2001). There is some limited information on the role of MTs in pathogenesis in colorectal cancer and drug resistance (Ofner *et al*., 1994; Giuffre *et al*., 1996; Sutoh *et al*., 2000). It has been suggested that MT expression neutralises many heavy metal chemotherapeutic drugs which aids in drug resistance. Another mechanism linking MTs to tumourogenesis involves p53. MTs induce a reversible conformation changes to the wild-type p53 protein, resulting in a mutant type unable to binding zinc needed for its stabilization. P53 also requires zinc for its DNA binding, and since MTs act as an intracellular scavenger for zinc, depletion of this would inactivate p53, leading to a proliferative advantage (Fan *et al*., 2002).

In colorectal tissue, Yoshitaka *et al*., (2001) used IHC to show MT positive expression in primary tumours in 7 out of 34 patients, with no expression in liver metastatic sites. MT positive expression was associated with higher degrees of lymph node involvement ($p=0.0122$). Survival rates were significantly better in MT negative tumours then MT positive ones ($p=0.0198$). Interestingly a similar down-regulation of MT expression in metastatic lymph nodes from breast cancers and metastatic adenocarcinomas was reported in other studies (Deng *et al*., 1998). This phenotypic change may be due to a change in the tumour environment. Bruewer *et al*., (2002) looked at patients with advanced ulcerative colitis and demonstrated an increased expression of MTs, suggesting abnormal expression may represent one of the first steps in the development of ulcerative colitis associated colorectal cancer.

The beneficial effect of using ZD4054 to reduce the expression of MTs seems overwhelming. With its range of activities affecting cell cycling, proliferation and p53 activity, it seems logical that reduced levels would be of benefit. Furthermore, increased evidence

suggesting its neutralising ability of chemotherapeutic drugs would support a role of ZD4054 as adjuvant treatment in colorectal cancer alongside 5-FU.

4.6.2.2 Matrix Metalloproteinase 7 (MMP7)

MMP7 is a zinc dependent enzyme with pro-metastatic functions relating to tumour development (Egeblad *et al*., 2002), metastatic potential (Kurokawa *et al*., 2005) and clinical outcome (Wang *et al*., 2006). It is also an independent prognostic factor for shorter survival in colorectal cancers (Maurel *et al*., 2007). It is not only implicated in ECM degradation and metastasis promotion, but also plays a role in Fas/FasL system regulation and in apoptosis responsiveness of tumour cells. MMP7 modulates Fas expression and activation, generates soluble FasL by cleaving its membrance form, and cleaving Fas receptors itself, blocking the induction of apoptosis (Almendro *et al*., 2009).

MMP7 expression was down-regulated in the HT29 cancer cell line and marginally up-regulated in SW480, SW620 and significantly in LIM1215 (even though this cell line was not further investigated) cell lines. Time point induction experiments showed a slight decreased expression at 30 minutes followed by a biphasic response to ET-1 in the HT29 cell line, with a 1.6 fold increase at 4 hours followed by a 5 fold increase at 24 hours. This indicates that it is a late responder gene, and rather than being down-regulated, is almost certainly up-regulated.

Both the ET_A and ET_B receptor antagonists significantly inhibited the expression of MMP7 ($p<0.05$). ZD4054 was the most effective at inhibiting its expression by 85%. Additionally, silencing of either ET receptor significantly reduced levels of MMP7 expression to below that of the control level. Receptor silencing may be having this effect by reducing the actions of endogenously produced ET-1, shown to be expressed by conventional RT-PCR (figure 4.24).

In HT29 cells and colon cancer tissue, MMP7 expression correlated with increased β-catenin levels and Wnt signalling via the epidermal growth factor (EGF) receptor (Ametler er al., 2011). Interestingly ET-1 is linked to both these pathways. Fang *et al*., (2009) also showed that oestrogen receptors (ER-β) in HT29 cells influenced apoptosis and MMP7 expression.

Both Tamoxifen and/or 5-FU down-regulated MMP7 and ER-β receptors, significantly inhibiting proliferation and migration whilst inducing apoptosis.

MMP7 is expressed in approximately 90% of human colonic adenocarcinomas and in the majority of human colon tumour cancer cell lines (Fang *et al*., 2009). In patient studies, Fang *et al*., (2010) demonstrated in 423 patient samples that high MMP7 compared to low expression was related to decreased overall survival (72% vs. 90%; p=0.008) and 5 year survival (86.6% vs. 88.8%; p=0.005). Kita *et al*., (2006) showed MMP7 was differentially expressed with higher levels on gene array, qRT-PCR and IHC in adenomas as compared to normal colon. Because a number of studies have shown its importance for transition from benign to malignant status, it is considered to be a biomarker of disease and prognosis (Nastase *et al*., 2011). MMP7 and its indication of prognosis may not only be related to increased malignancy, but also chemo-resistance. Almendro *et al*., (2009) compared HT29 and oxaliplatin resistant RHT29 cell lines. They found increased MMP7 expression in the RHT29 cell line. Furthermore, silencing of MMP7 with SiRNA restored drug sensitivity to oxaliplatin-induced apoptosis. Interestingly Fas receptor expression at the cell membrane was 3.8 fold lower in RHT29 cell lines than the HT29 cell line. This decrease was restored to HT29 levels once MMP7 was silenced in RHT29 cell lines, indicating MMP7 to be responsible for this.

There is overwhelming literature evidence that MMP7 is associated with colorectal cancers and similar pathways used by ET-1, namely EGF and Wnt signalling. Despite ZD4054 being the most efficacious at reducing its expression levels, both ET_A and ET_B receptor antagonist had a significant effect, suggesting drugs such as Bosentan may be of benefit in the clinical setting. Zibotentan (ZD4054) potentially could increase tumour sensitivity to drug induced apoptosis and possibly increase prognosis of patients.

4.6.2.3 PPP2R5D

Illumina gene array analysis demonstrated a 1.2 fold decrease in expression of this gene. Time induction studies showed a 1.2 fold decrease at 30 minutes followed by a 1.5 fold increase at 1 hour. Generally there was a non-significant fluctuation in expression levels. The use of antagonists demonstrated non-significant alterations in expression levels. This was partially due to the variable response to ET-1 in the triplicate repeat experiments.

Silencing the ET_A receptor marginally increased expression of PPP2R5D, whereas ET_B receptor silencing marginally decreased levels. However this was non-significant.

To date there is very limited information on the role of this protein. It is a 56kDa serine/threonine-protein phosphatase 2A regulatory subunit of the delta isoform. Phosphatase 2A (PP2A) consists of 3 subunits of which subunit A is a regulatory region and subunit C is an enzyme region. PPP2R5D is one of the regulatory subunit B families, which determines substrate specificity and sub-cellular localization (Ruediger *et al*., 2001). It is implicated in negative control of cell growth and division, therefore acting as a tumour suppressor (Sablina *et al*., 2010). All three regions together are termed a homoenzyme. Inactivation of a number of these subunit B phosphatase family members have been implicated in human cancers including colorectal (Wang *et al*., 1998) and suppression of these same PP2A components contribute to cell transformation. Deletion or alterations of other B subunit members (PPP2R1B) are observed in 30-50% of breast, lung, ovarian and cervical cancers (Calin *et al*., 2000). Within colon cancers, PP2A has not only been linked to the MAPK pathway, but also specifically the PPP2R5D subunit with PP2A has been linked with down-regulation of cellular β-catenin. Therefore decreased levels could be tumourigenic in the setting of colon, pancreatic, hepatic and skin cancers (Sparks *et al*., 1998; Seeling *et al*., 1999). Loss of Heterozygosity (LOH) of the B subunit region is seen in 29-34% of colon cancers (Takagi *et al*., 2000). As its function in the colorectal cells is linked to down regulation of the MAPK cascade, cell cycle check points and nuclear telomerase activity inhibition, this supports a role for this enzyme in tumourogenesis (Takagi *et al*., 2000).

Despite the links described above with the limited response to ET-1, it is difficult to assess the role that ET receptor antagonist would have in a tumour setting. Further experiments and optimizing conditions may be required before re-evaluating the ET receptor antagonists.

4.6.3 ET-1 and its ET_A and ET_B Receptor Expression

In continuously growing cells, there was a small endogenous expression of ET-1 seen within the colorectal cancer cell lines. Interestingly the colonic fibroblasts did not show any endogenous expression of ET-1. This differs from the finding of Knowles *et al*., (2011) where expression of ET-1 was demonstrated on cytospun colonic fibroblasts cells. One explanation for this difference is that the fibroblasts used in the present study were around passage 11-14, whereas fibroblasts used in the Knowles paper were all below passage 8. It has been

shown that fibroblasts change their phenotype with increasing passage number (Kernochen *et al*., 2002). The present fibroblasts may have lost expression of ET-1 in a similar manner to MC28 cell lines that lose ET-1 expression at around passage 32-36 (unpublished departmental data).

The mRNA expression of ET_A receptors in fibroblasts seemed to be expressed to similarly high levels when conventional RT-PCR was carried out to 35 cycles. More variability was seen in expression of the ET_B receptors, with lower levels in the CF36 and CF56, and higher levels in CF65 and CF75. Variability was also seen in mRNA expression of both ET_A and ET_B receptors in the colorectal cancer cell lines. Interestingly we see a lower than expected level of ET_A receptor mRNA expression considering this receptor subtype is expected to be up-regulated in colorectal cancer. One explanation of this may be that high protein expression of this receptor is present on the surface, and with no binding and internalisation, basal mRNA expression would remain low. In support of this, Western blotting shows high protein expression of the ET_A receptor in HT29 and SW620 cell lines, with a low expression of the ET_B receptors as would be expected. Protein expressions of both receptors in the colorectal fibroblasts were similar. The difference in responses to ET-1 and its antagonists in our previous experiments may have resulted from the variable expression levels of each receptor. However, increasing evidence points to a more complex ligand-receptor interaction that may account for the variability seen.

Recently studies on ET-1 have shown that it displays polyvalent binding to ET_A receptors, requiring the C-terminal, amino acids at the N-terminal and its disulphide bonds for its action (De Mey *et al*., 2011). Structural analysis also indicates that it has 2 functional units, one responsible for binding to ET_A receptor and the second for tighter binding and activation of a second orthosteric binding site (Lattig *et al*., 2009). This would explain why different ET_A receptor antagonists have different effects and why the same antagonist may have different effects in different tissues. By competing at either the first orthosteric binding site (pre-requisite for the second and tighter binding site), or the second orthosteric binding site (allowing partial signalling), this will alter competitive binding at the surface and signalling cascades downstream (neutral peptide or inverse agonist). This is supported by the observation that BQ123 is now known to promote receptor internalisation. Secondly, antagonist binding to an allosteric site separate from the orthosteric sites may modulate efficacy and affinity of binding. Some antagonists bind deeper in the ET_A receptor clefts, therefore only partially overlapping binding sites. This means our antagonists may actually

be acting as receptor allosteric modulators as opposed to just antagonists (De Mey *et al*., 2011).

Conditions of dynamic equilibrium also govern theories of molecular pharmacology and are relevant to ET-1. ET-1 is an effective paracrine agent as it associates with the ET_A receptor rapidly (like angiotensin II) but dissociates 100 times slower (0.0005/min). This is due to its tight binding which slows reversibility and dissociation. As a result, studies with agonists and antagonists become difficult as, for example, radiolabelled ET-1 and ET_A receptor complexes cannot be displaced by cold agonists and show a half life of between 7-77 hours (De Mey *et al*., 2011). This same group exposed arterial walls to BQ123 one hour prior to ET-1 exposure and found the concentration-response curve shifted to much higher concentrations. This pharmaco-dynamics along with potential different binding sites within the receptor itself may account for the variability in results observed.

4.6.4 Epidermal Growth Factor Receptor (EGFR)

Briefly, conventional RT-PCR has shown expression of the EGF receptor in all fibroblasts and cancer cell lines with extremely low levels seen in SW620 and LIM1215. Earlier, studies showed that SW620 cell lines do not express the EGF receptor (Coffey *et al*., 1987). The findings for LIM1215 are different from the expected high concentrations (Grant *et al*., 2007), and may be due to loss of expression with increased passages.

A biphasic increase in EGF receptor expression was demonstrated in colonic fibroblasts by 3.8 and 4.5 fold at 4 and 24 hours respectively. Furthermore, ET_A receptor silencing inhibited expression to that of the internal control (scrambled) levels. It is established that ET-1 transactivates the EGF receptor promotor in addition to using pathways such as PI3K and PKC (Grant *et al*., 2007). Additionally, phosphorylation of the EGF receptor in the presence of ET-1, was ameliorated by ET_A receptor blockade, suggesting ET-1 mitogenic signalling is propagated via the EGF receptor (Grant *et al*., 2007) similar to that of ovarian cancer (Vacca *et al*., 2000). Interestingly EGF receptor induced expression through the ET system has not been demonstrated before. This complexity of interactions suggests that the future of treating and managing cancers may lay in the use of multi-therapy targeted drugs.

Chapter 5:

ZD4054 ET_A Receptor Antagonism – Pharmacological Characteristics

Immunohistochemistry, Autoradiography & Receptor Binding

5.1 INTRODUCTION

In this chapter ET-1 binding is characterised in normal and tumour patient sections with preserved cellular architecture, within homogenates and cytospun cells. The ET-1 receptor binding characteristics were analysed along with IC_{50} for ET receptor antagonists.

5.2 MATERIALS AND METHODS

5.2.1 Tissues

Specimens were obtained from patients undergoing resection for colorectal cancer (2001-2004); normal and cancer samples were snap frozen and stored in liquid N_2 (n=6) 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 N_2 and homogenised (2 minutes; Mikro-Dismembranator U, Braun Biotech**,** Melsungen, Germany). The resulting powder homogenates were diluted in water (2ml). All were stored at -70^{o}C (Ethical approval, REC No 08/H0720/162, University College London Hospitals).

5.2.2 Cytospins

Fibroblast strains (CF35, CF56, CF65 & CF75) and cancer cell lines (HT29 & SW480; $2x10^6$ cells for gross autoradiography; $0.5x10^6$ cells for microautoradiography) were loaded onto polylysine-coated slides, centrifuged, air dried and stored at -70^{o}C (Shandon Cytospin3, WolfLabs Inc,Pocklington, York, UK). These were subsequently used for saturation and binding affinity analysis (K_d/B_{max}), autoradiography and inhibitory studies to determine IC_{50}.

5.2.3 Saturation Analysis: K_d/B_{max} Determination

Receptor binding studies were performed as described previously (Ali *et al*., 2000). Briefly, unfixed cytospins and tissue homogenates were allowed to equilibrate to room temperature (~21^{o}C, 20min). A preliminary step was performed for cytospins, where slides were preincubated at room temperature in 50mM tris-HCl, pH7.4, 20min, to reduce endogenous ET-1 levels. Cytospins and tissue homogenates were incubated with increasing ^{125}I-ET-1 concentrations (GE, Amersham, Bucks, UK, specific activity 2200 Ci/mmol: $3x10^{-12}$-10^{-9} M,

[total binding]) in 50mM tris HCl buffer, pH7.4, 5mM $MgCl_2$, 0.2% bovine serum albumin, 100 IU/ml aprotinin, 120min. This was followed by rinsing (2x10min) in 50mM tris-HCl, 4°C. Non-specific binding (NSB) was established by incubating in the presence of 1µM unlabelled ET-1. After incubation: (1) slides were briefly dipped into distilled water, 4°C, to remove salts in the buffer, and dried under a stream of warm air (~15min) followed by cool air (15min) and stored overnight. Slides were post-fixed under vacuum in paraformaldehyde vapour, 2hrs, at 80°C. (2) Homogenates were filtered and washed three times with buffer under vacuum through a cellulose GF/B filter attached to a 12-well manifold chambers (both Millipore, Watford, UK). Homogenate bound ^{125}I-ET-1 retained by the filter paper was measured to establish total and non-specific binding. At the end of each set of incubations, ^{125}I scales were prepared where 50µl aliquots of each of the serial dilutions of radioligand were spotted onto filter paper or cellulose filters and then attached to microscope slides that were co-exposed to radio-sensitive film along with the cytospins. (Appendix 14).

5.2.4 Inhibition Analysis

Relative affinities of receptor-selective antagonists were studied by comparing their ability to reduce ^{125}I-ET-1 binding to cytospins and frozen slide-mounted tissues. The same incubation conditions were used as described for saturation analyses, with cytospins/tissues incubated in a fixed ^{125}I-ET-1 concentration (150pM; ~K_d value determined initially in the saturation studies) in the presence of increasing concentrations ($3x10^{-9}$-$3x10^{-6}$M) of the ET_A receptor antagonists, BQ123, ZD4054, and the ET_B receptor antagonist BQ788. Two fixed concentrations (high=25µM; low=5µM) for each antagonist were used for autoradiographs that were produced as described below. (Appendix 15 and 16).

5.2.5 Autoradiography

(1) Low Resolution: Slide-mounted tissues and cytospins were placed in 24x30cm X-ray cassettes and apposed to Hyperfilm™MP (GE, Amersham) under dark-room conditions for 7-21 days exposure, 4°C. Films were subsequently processed following manufactuer's instructions (Appendix 15). Briefly, in a dark-room, they were immersed in undiluted D19 developer (Kodak), 5min, followed by a brief rinse in tap water and 5min immersion in Hypam™ fixative (Ilford, 1:3 in distilled water). After washing in running tap water (~20min), films were dried and subsequently autoradiographs used for densitometry; representative images were photographed. **(2) High Resolution:** Selected slides were used for microscopic localisation of radioligand binding. Tissues/cytospins were dipped in molten

(42^{o}C) K2 emulsion (Ilford; 1:1 in 2% glycerol/distilled water) and allowed to dry overnight (dark-room). Emulsion-coated slides were then placed in racks, stored in light-proof boxes containing silica gel dessicant (4^{o}C, 7-21 days). For microautoradiographs, slides were immersed in D19 developer, 5min, briefly dipped in rapid stop solution (Ilford, 1:10 in distilled water), fixed in Hypam™ fixative (1:3 in distilled water, 10min) and rinsed (3x10min) in distilled water. Tissues/cells were stained with haematoxylin & eosin (H&E), dehydrated by immersion in increasing ethanol concentrations (dewatered). Histo-clear™ (National Diagnostics, Hull, UK) was used as a clearing agent, then cover-slipped using DPX. Slides were viewed under an Olympus BX50 microscope (autoradiographs under dark-field illumination; staining under bright-field illumination), photographed using a Zeiss Axiocam™ digital camera and images stored on a KS400 imaging system (Imaging Associates, Bicester, UK).

In addition to densitometric analysis, radioligand binding was also assessed using a Wizard 1470 automatic gamma counter (Perkin Elmer, Cambridgeshire, UK). Once film images of cytopins had been generated for densitometry, cells were removed from the slides by digesting in 100µl 4M NaOH (~10 minutes), scraped off with filter paper then radioactivity was measured in the gamma counter (disintegrations/min).

5.2.6 Calculation of Binding Characteristics

Densitometric analysis of autoradiographic images 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 used. Maximum receptor binding (B_{max}) and affinity (K_d) were obtained using GraphPad Prism™ software (GraphPad, Santa Barbara, CA).The same approach was used for analysing data from cells removed from slides and measured in the gamma counter.

5.2.7 Immunohistochemistry

Standard immunohistochemistry was performed using the Vectastain alkaline phosphatase kit (Vector Labs, Peterborough, UK) as described previously (Ali *et al*., 2000; Hoosein *et al*., 2007). Slides were allowed to equilibrate to room temperature (20min) and fixed in acetone (-20^{o}C, 20min). Briefly, the protocol comprised the following steps (rinsing with phosphate buffered saline (PBS) in-between): Slides were blocked in 10% normal horse serum (NHS, in PBS, 10min), and incubated with primary antibody (1:200 in PBS, 30 min; AS02/Thy-1 for

fibroblasts, CD31 for vascular endothelial cells, anti-ET_A receptor and anti-ET_B receptor for ET receptors, ColXI for collagen Type XI), followed by universal secondary antibody (1:100 in NHS/PBS, 10min). Slides were then exposed to Vectastain ABC/AP reagent (1:100, 5min); followed by alkaline phosphatase (Vector red) substrate (1:50 in 200mM tris-HCL buffer, pH8) until colour developed (up to 30min). Slides were counterstained with haematoxylin, dehydrated in increasing alcohols, cleared in Histo-clear^{TM}, mounted, viewed and photographed as above. H&E staining was performed on selected sections.

5.2.8 ET_A Receptor localisation with Quantum Dots

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^oC), haematoxylin counterstained, and then observed under confocal microscopy.

5.3 RESULTS

5.3.1 Localisation and Distribution of ET-1 Binding within Human Colonic Tissues

In high resolution autoradiographs of both normal and tumour frozen tissue sections, ^{125}I-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 of underlying tissue (Figure 5.1).

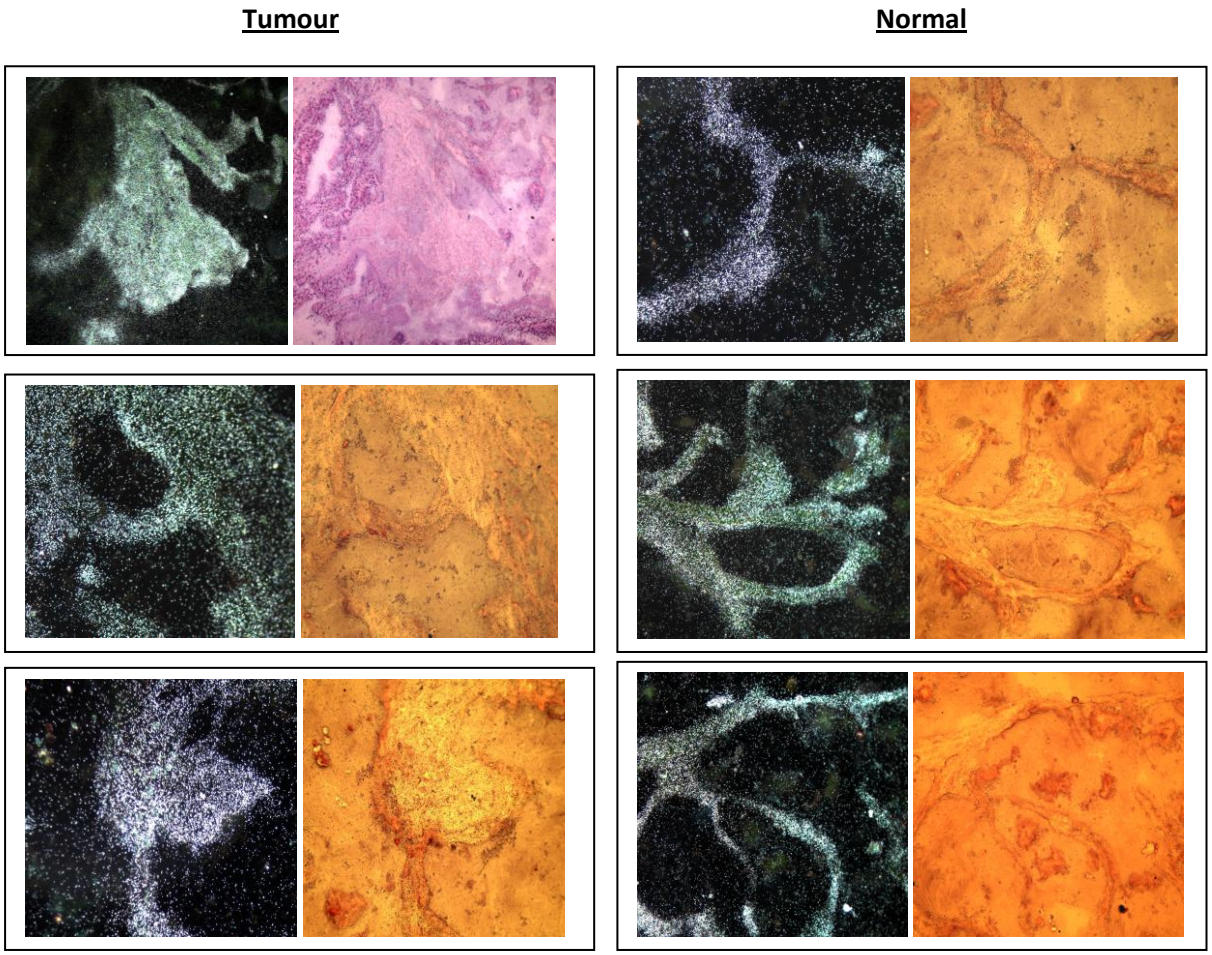


Figure 5.1 *ET-1 localisation on frozen sections in tumour (left panels) and normal tissue (right panels). Briefly, sections were fixed in paraformaldehyde vapour (80 ˚C; 2 hours), dipped into molton (42^{o}C) K2 emulsion, dried, stored at 4^{o}C for 7 – 21 days in light proof boxes containing silica gel dessicant, and then processed, fixed and washed. Underlying sections were H&E stained. Autoradiographs were visualised under dark-field illumination for radiolabelled ^{125}I-ET-1 binding (left) and H&E stained tissue under bright-field illumination (right). Highest density of binding is predominantly seen on connective tissues within both normal colon and tumour stroma.*

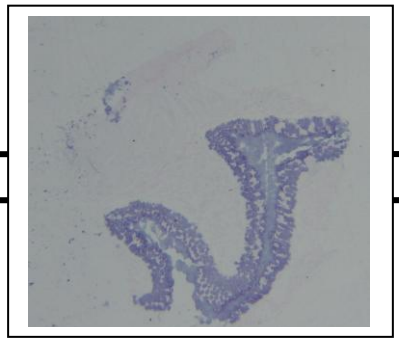
To identify associated structures, consecutive sections were stained immunohistochemically for: ET_A and ET_B receptors; stromal fibroblasts (Thy-1); endothelial cells (CD31) and collagen type XI (connective tissue) (Figure 5.2 & 5.3). Normal colon tissue had preserved structural architecture with a well defined epithelial mucosal layer whereas that of tumours was disorganised. In normal colon specimens, microautoradiography revealed ^{125}I-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}I-ET-1binding. ^{125}I-ET-1 bound strongly to regions that stained positively for endothelial cells and fibroblasts. In tumour tissue, both ET_A and ET_B receptors were present (ET_A>ET_B) and localised 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 tumour stroma, which was only present in tumour sections and not normal tissue, confirming previous reports of its association with colorectal pathology (Fischer *et al.*, 2001a; Fischer *et al*., 2001b).

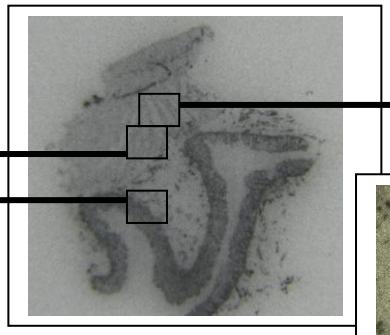
Figure 5.2 & 5.3 (following pages). *Immunohistochemistry to identify binding of ET-1 within normal and colonic tumour sections. Frozen sections were prepared then stained for ET_A receptor binding, ET_B receptor binding, vascular endothelial cells (CD31 antibody), fibroblasts (Thy-1 antibody) and collagen type XI (Col11A1 antibody). These sections were compared to the underlying regions of tissue that were exposed for autoradiography to identify binding regions.*

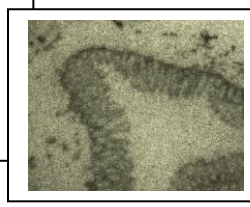
Normal Colonic Tissue – Staining for ET_A, ET_B, CD31, Thy-1 & Col XI

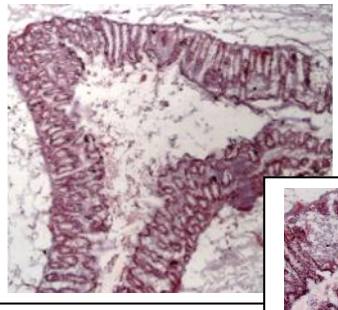
H&E Staining

^{125}I-ET-1 Total Binding

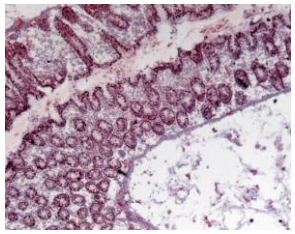








x2



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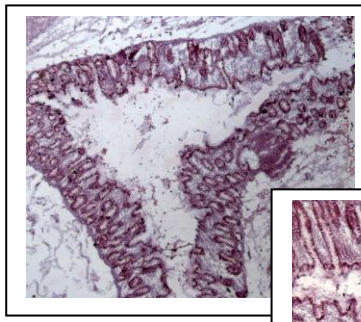
ET_A



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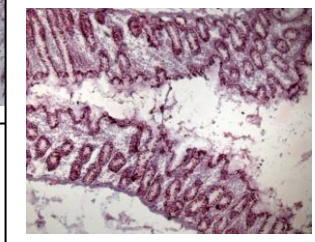
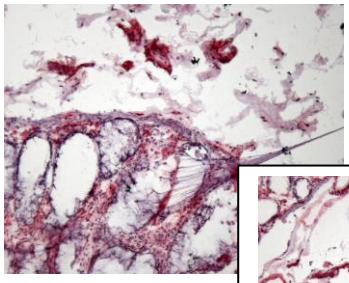


x4

ET_B



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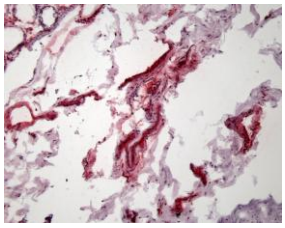

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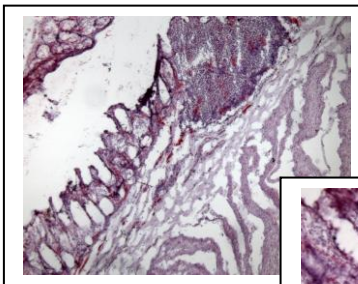
CD31



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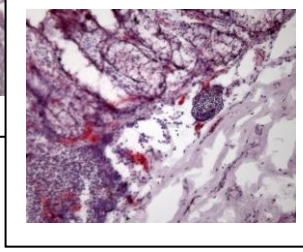

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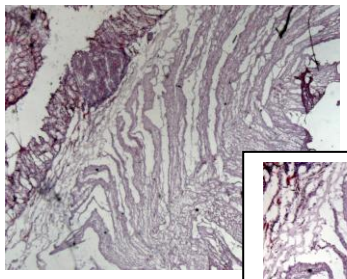
Thy-1 (Fibroblast)



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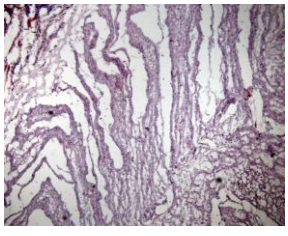

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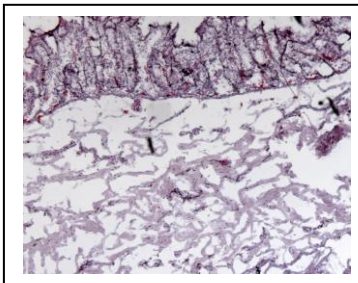
Col XI

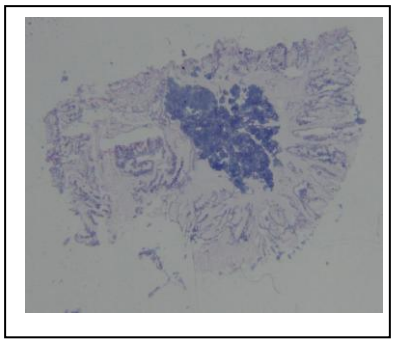


x2

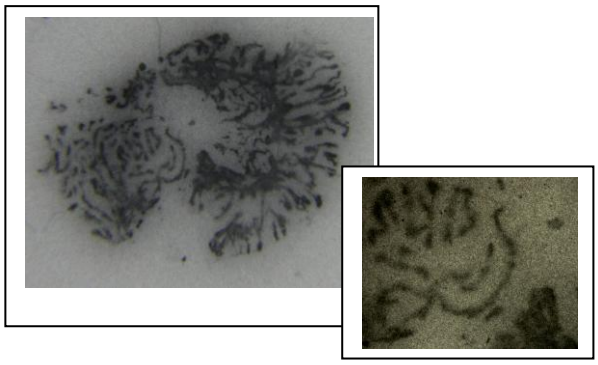
Control

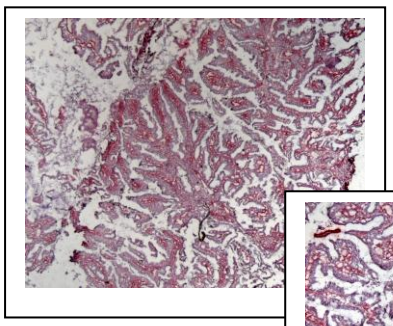
Colonic Tumour Tissue – Staining for ET_A, ET_B, CD31, Thy-1 & Col XI

H&E Staining



^{125}I-ET-1 Total Binding





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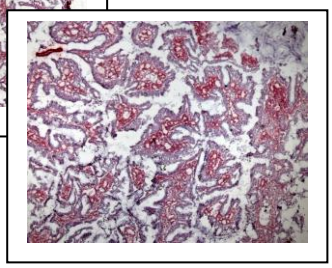

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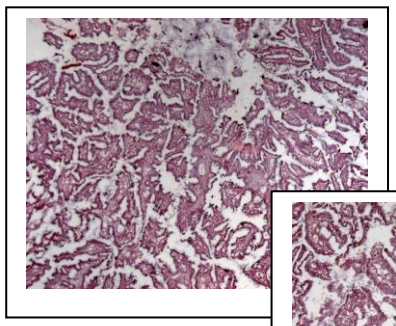
ET_A



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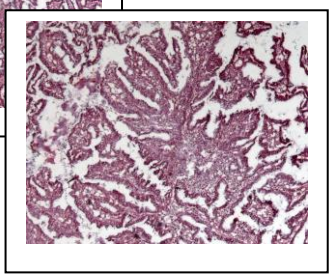
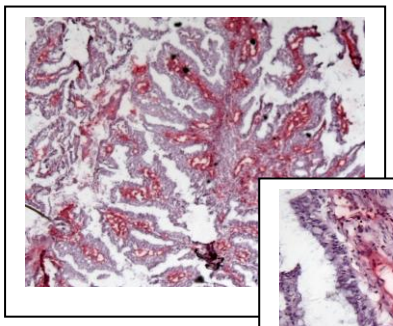


x4

ET_B



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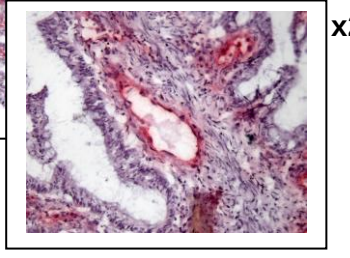
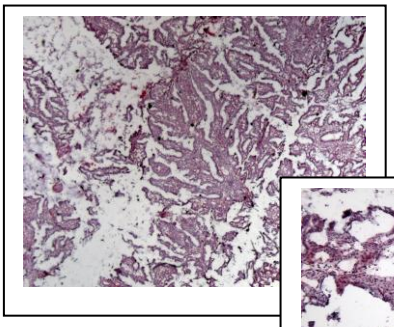


x20

CD31



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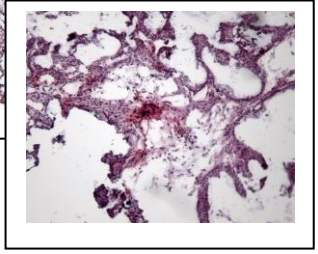

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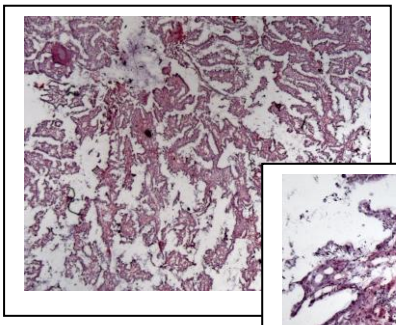
Thy-1 (Fibroblast)



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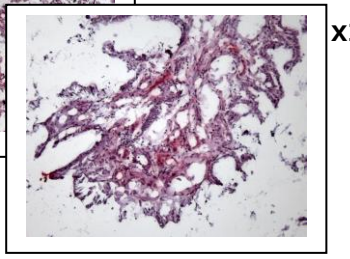
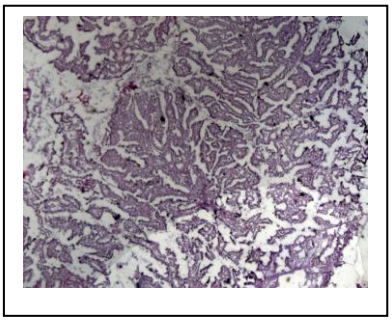


x10

Col XI



x2

Control

Normal colonic tissue had preserved structural architecture with a clear mucosal layer whereas tumour sections were more disorganised. Normal sections showed both ET_A and ET_B receptors to be distributed primarily within the mucosal and submucosal regions to a similar extent. There were small amounts distributed within the stromal connective tissue regions (Figure 5.2). The tumour sections showed both ET_A and ET_B receptor staining throughout the sections, however ET_A staining was more prominent. This correlates with our previous findings that ET_A receptors are up-regulated in colorectal carcinomas. All these binding regions closely matched the ^{125}I-ET-1 binding regions seen on autoradiography (Figure 5.3).

The distribution of blood vessel endothelial cells (CD31) and fibroblasts (Thy-1) were easier to correlate to ET-1 binding seen on autoradiographs within normal colonic sections (Figure 5.4). This was more difficult to correlate in tumour sections due to the architecture of the tissue. However, extent of blood vessel formation and neovascularisation within the tumour sections was much more extensive than that seen in normal sections (Figures 5.2 & 5.3).

Collagen type XI (Col XI) was only seen throughout tumour sections. Literature has shown that collagen type XI (as well as collagen type V) is specifically expressed in colonic pathologies and not within normal bowel, which correlates with the present findings.

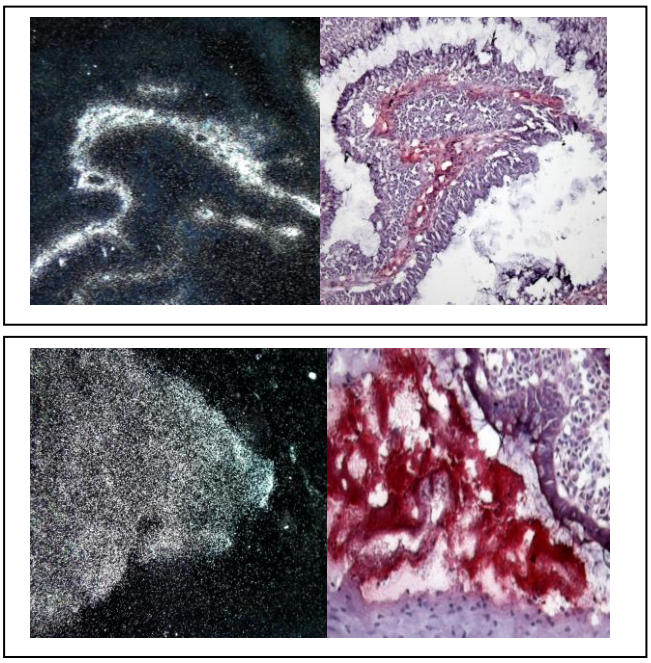


Figure 5.4 *Double panels showing dark field illumination of radiolabelled ^{125}I-ET-1 binding (left) with corresponding adjacent Haematoxylin stained frozen sections showing CD31 endothelial staining (red stain; top right) and Thy-1/ASO2 fibroblast staining (red stain; bottom right). Clear radiolabelled ^{125}I-ET-1 localization is seen to these regions.*

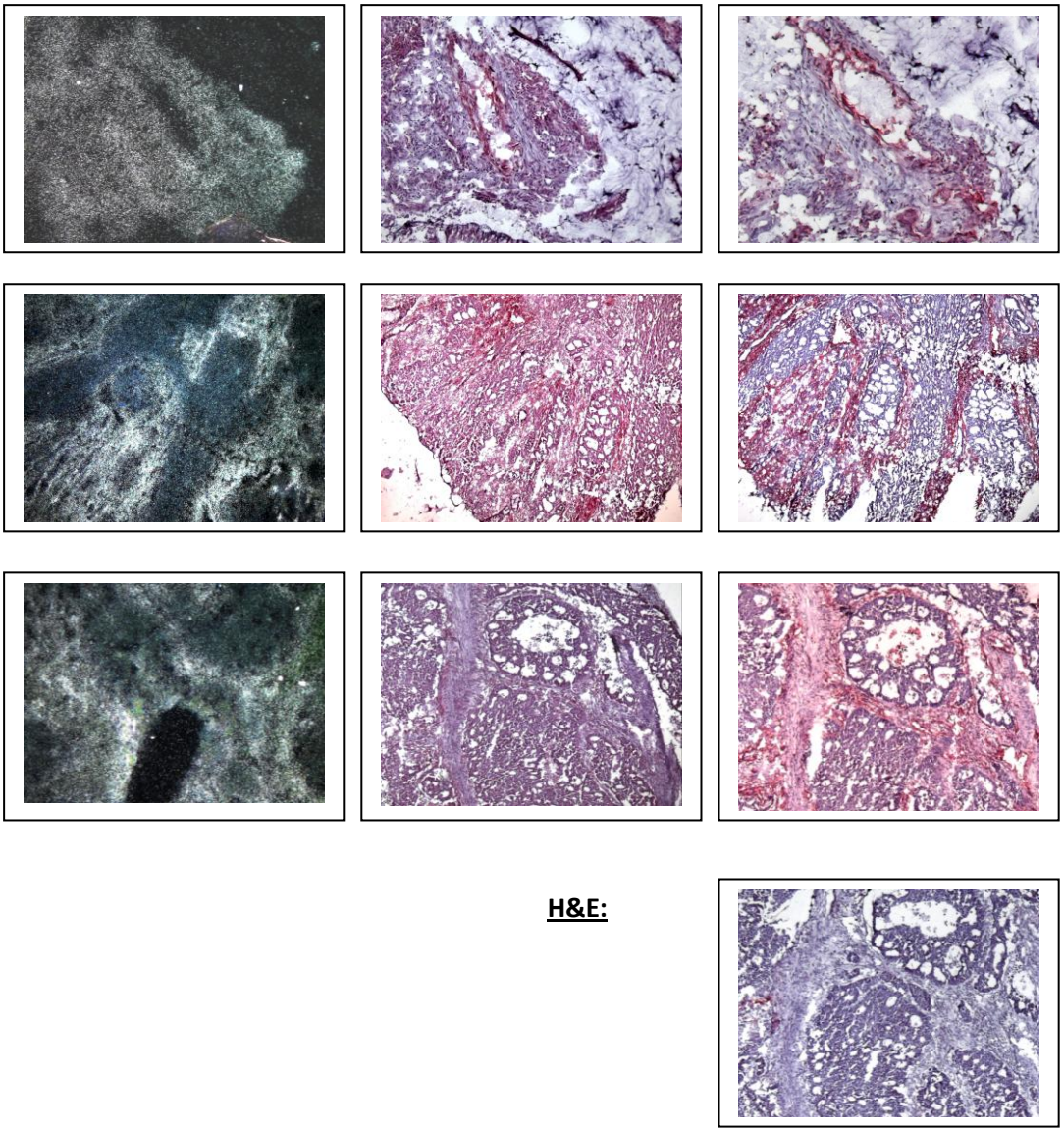


Figure 5.5 *Triple panels showing dark field illumination of radiolabelled ^{125}I-ET-1 binding (left) and cell type specific staining of serial sections for fibroblasts (middle) and endothelial cells (right) of the same cancer tissue. Lowest panel shows tissue histology of section using H&E staining alone. High binding density of ^{125}I-ET-1 coincides with endothelial cells and fibroblasts mostly within the tissue stroma as previously demonstrated.*

5.3.2 Receptor Subtype Distribution

To study ET_A and ET_B receptor distribution within normal and tumour sections, we inhibited ^{125}I-ET-1 binding using specific receptor antagonists, thereby demonstrating indirectly the presence of ET_A and ET_B receptors (Figure 5.6 & 5.7). The ET_A receptor antagonist, BQ123, in both normal and tumour sections, demonstrated a concentration dependent inhibitory effect with 25μM reducing ^{125}I-ET-1 binding to a greater degree than 5μM. Furthermore, the extent of inhibition was different between normal and tumour sections: more ^{125}I-ET-1 binding in normal tissue compared to tumour tissue with the use of BQ123 indicates more ET_B receptors in normal tissue. The ET_B receptor antagonist BQ788 also demonstrated a concentration dependent effect in both normal and tumour tissue with greater inhibition seen at 25μM compared to 5μM. More ^{125}I-ET-1 binding was inhibited in the tumour sections, consistent with a higher ET_A receptor density in tumours compared to normal tissue. Greater inhibition of ET_B receptor binding in normal colonic tissue suggests a higher ET_B receptors expression in healthy tissue than cancer specimens. 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 ZD4054 concentration dependent inhibition was not as great as either BQ123 or BQ788. On densitometry analysis there was more inhibition observed in tumours than normal colonic tissue specimens, again consistent with a higher concentration of ET_A receptors in cancer. ET_A receptor over-expression 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 5.8).

Patient Normal Colonic Tissue – ET_A and ET_B Receptor Inhibition

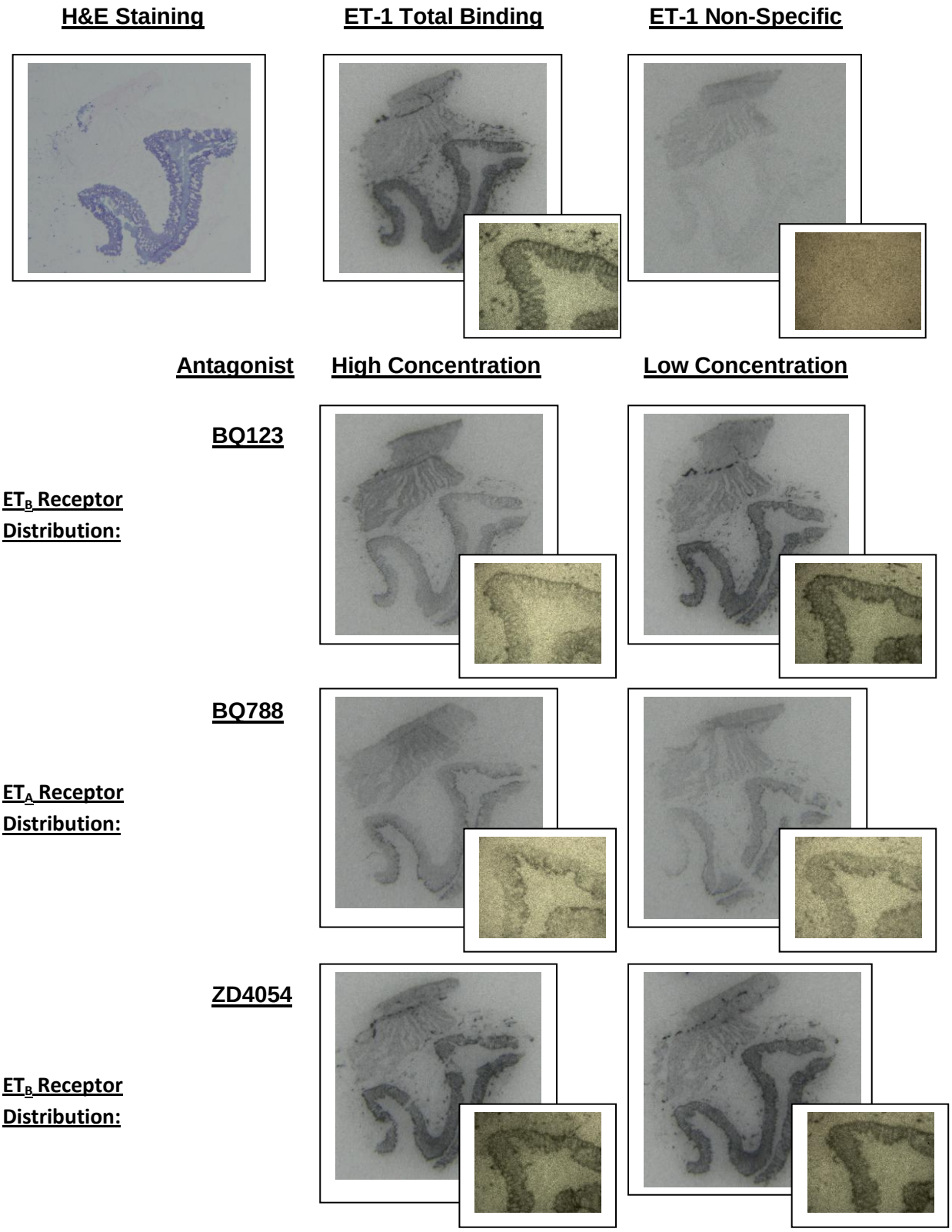


Figure 5.6 *Receptor selective antagonists ability to reduce ^{125}I-ET-1 binding to slide-mounted tissue. Briefly, slide mounted normal tissue sections were incubated in 150pM ^{125}I-ET-1 in the presence of BQ123, BQ788 or ZD4054 (high conc. 25µM; low conc. 5µM). ET-1 total binding had no antagonist present. The low resolution auto-radiographs were produced by opposing the slide mounted tissues to Hyperfilm™ within cassettes for up to 21 days. Films were processed with developer, fixative and then washed. H&E staining was done on underlying tissue sections.*

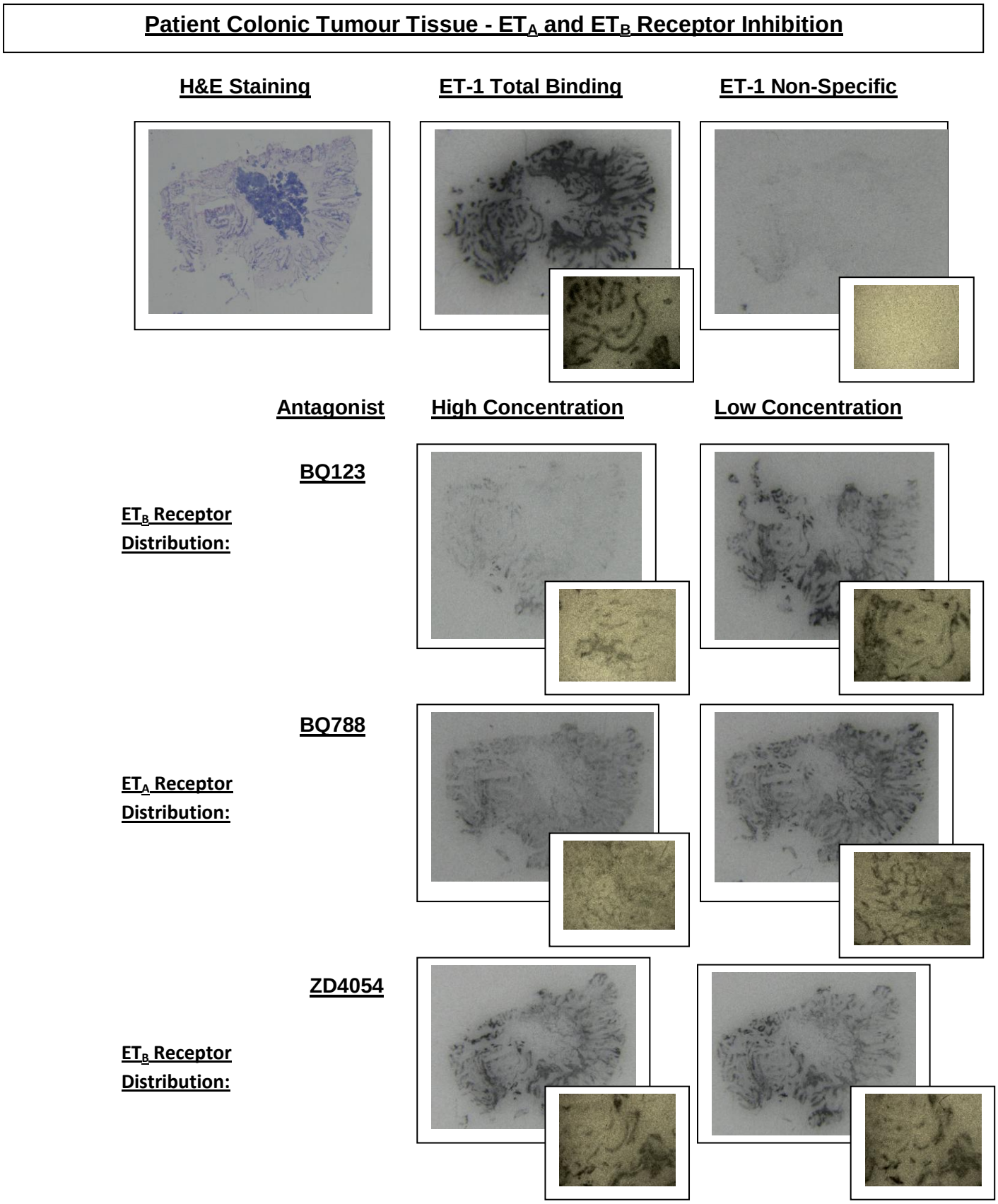


Figure 5.7 *Receptor selective antagonists ability to reduce ^{125}I-ET-1 binding to slide-mounted tissue. Briefly, slide mounted frozen tumour sections were incubated in 150pM ^{125}I-ET-1 in the presence of BQ123, BQ788 or ZD4054 (high conc. 25µM; low conc. 5µM). ET-1 total binding had no antagonist present. The low resolution auto-radiographs were produced by opposing the slide mounted tissues to Hyperfilm™ within cassettes for up to 21 days. Films were processed with developer, fixative and then washed. H&E staining was done on underlying tissue sections.*

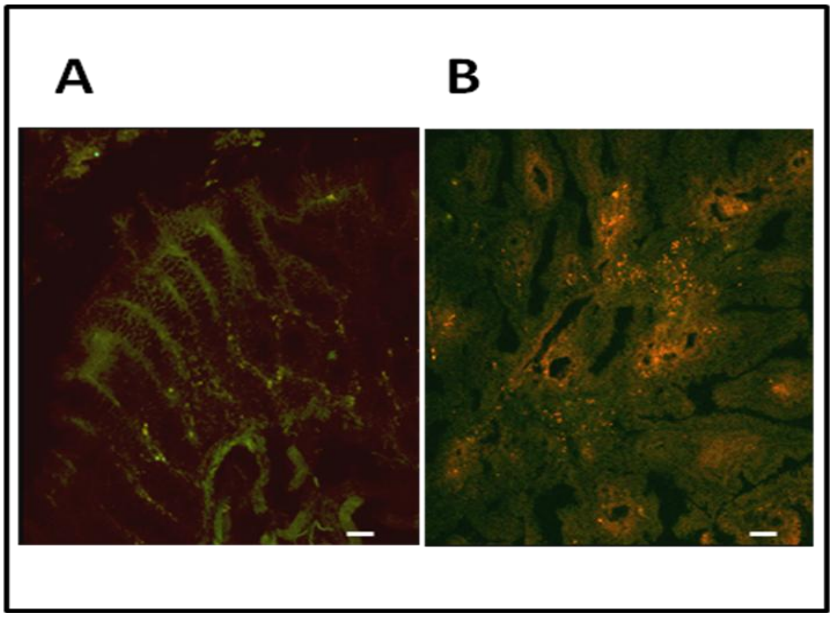
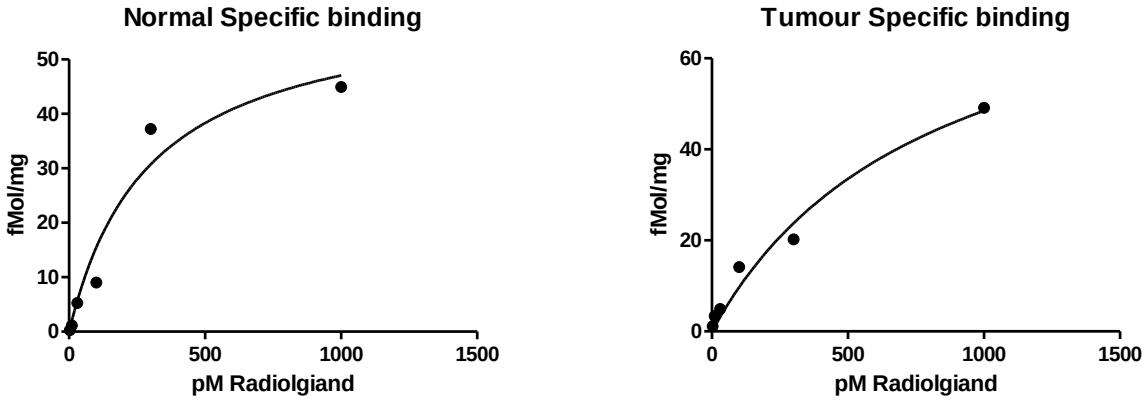


Figure 5.8 Localisation of Quantum Dot - BQ-123 conjugates in patient sections from normal (A) and tumour (B) specimens. Frozen sections were incubated with QD-BQ123 overnight (4^{o}C) and visualised by confocal microscopy. Punctate yellow-orange QD-BQ123 binding was more abundant in tumour versus normal sections. Green tissue autofluorescence is present. Bar=50 μm.

5.3.3 Binding Characteristics in Homogenate Tissue

To examine the general binding characteristics of ^{125}I-ET-1 in normal and tumour sections we used tissue homogenates (Figure 5.9). The K_d demonstrates that binding affinity of ^{125}I-ET-1 was similar in both the normal and tumour specimens (203pmol/L and 204pmol/L respectively). Maximum binding (B_{max}) was also similar in both normal and tumour samples (57.83fmol/mg and 58.31fmol/mg respectively; mg of tissue protein).



Specific Binding	Normal	Tumour
K_d (pmol/l)	203	204
B_{max} (fmol/mg)	57.83	58.31

Figure 5.9 *Calculation of K_d and B_{max} of radiolabelled ^{125}I-ET-1 binding in human tissue homogenate samples. Briefly, colonic normal and tumour tissue were snap frozen in liquid nitrogen, sonicated for 90 seconds, and then re-suspended in PBS. Homogenate was then incubated with increasing concentrations of ^{125}I-ET-1 at $3x10^{-12}$ to 10^{-9} M to determine total binding and 1µM unlabelled ET-1 for non-specific binding. Resultant homogenates were filtered and radioactivity measured. Specific ^{125}I-ET-1 binding was determined by subtracting non-specific binding from total binding. Maximum receptor binding (B_{max}) and affinity (K_d) were obtained using GraphPad Prism™ software. Binding affinities and maximum saturation binding for radiolabelled ^{125}I-ET-1 were similar for normal and cancer tissues.*

Since these homogenate studies only indicate ET-1 binding to mixed receptors (ET_AR or ET_BR), we cannot gain information as to what the distribution of each respective receptor is in each of the tissue specimens. Given that tissue homogenate contains a high degree of non-specific binding regions, subtle receptor density and affinity changes would be unlikely to be observed. However, previous studies within our department have shown a change in receptors on the cell surface with an up-regulation of ET_ARs and down-regulation in ET_BRs in cancer. If this was the case, the overall number of receptors within the specimens may remain approximately the same with just a change in the type of receptors present (ET_AR/ET_BR). In this case the K_d and B_{max} would remain similar. To determine if the type of receptors present have changed, we would need to use either individually labelled ET_AR or ET_BR antagonistic ligands, or inhibit ^{125}I- ET-1 binding with unlabelled ET_AR or ET_BR antagonists. The latter was carried out in patient sections.

5.3.4 Binding Characteristics in Colonic Fibroblasts and Colorectal Cancer Cell Lines

High resolution autoradiography showing overall ^{125}I-ET-1 binding to receptors on cytospun fibroblasts and cancer cell lines are shown in figures 5.10 & 5.11, with calculations of K_d and B_{max} in figures 5.12-5.17. The fibroblasts' combined K_d value of 213.6pM (0.213nM) is regarded as high affinity (near 1nM or less = high affinity; 1μM or more = low affinity). Fibroblasts also demonstrated a relatively high maximal binding of ^{125}I-ET-1 to its receptors (3.03 fmol/1x10^6 cells). The K_d and B_{max} values for all fibroblasts were similar with only one strain (CF56) demonstrating lower values. The CRC cell lines HT29 and SW480 had a combined B_{max} and K_d value of 2.43fmol/1x10^6 cells and 0.367nM respectively. Both cell lines had a similar B_{max} to the fibroblasts with an ^{125}I-ET-1 binding affinity which was marginally less (average HT29 & SW480: B_{max}: 2.435fmol/1x10^6 cells; K_d: 0.367nM; average fiboblasts: B_{max}: 3.03 fmol/1x10^6 cells; K_d: 0.213 nM).

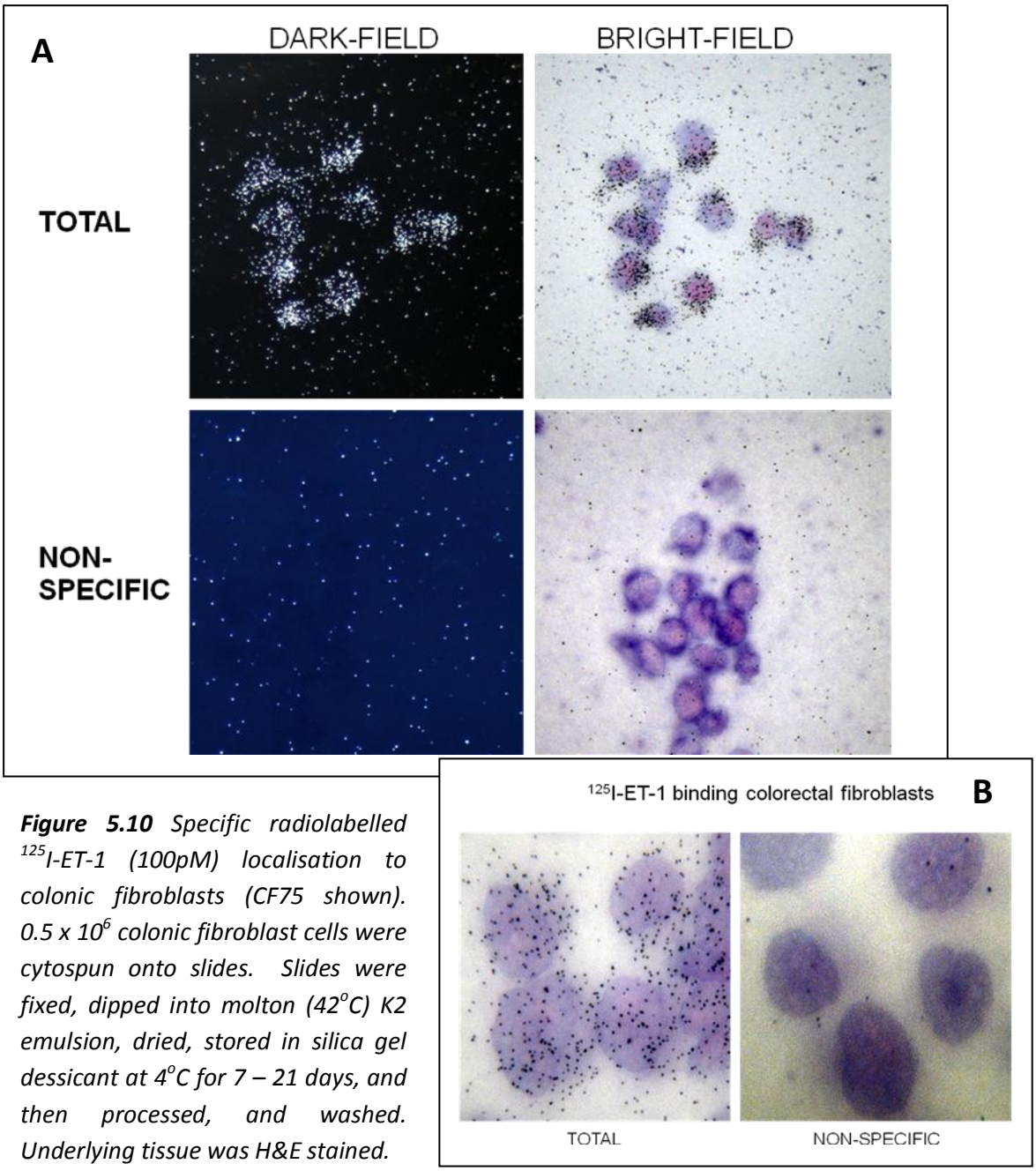


Figure 5.10 *Specific radiolabelled ^{125}I-ET-1 (100pM) localisation to colonic fibroblasts (CF75 shown). 0.5 x 10^6 colonic fibroblast cells were cytospun onto slides. Slides were fixed, dipped into molton (42^{o}C) K2 emulsion, dried, stored in silica gel dessicant at 4^{o}C for 7 – 21 days, and then processed, and washed. Underlying tissue was H&E stained.*

A. *Left panels show dark-field illumination and right show bright-field H&E stained cells. Both top panels demonstrate total ET-1 binding whilst non-specific is shown in the bottom panels.* ***B.*** *Magnification of ET-1 binding to colonic fibroblasts.*

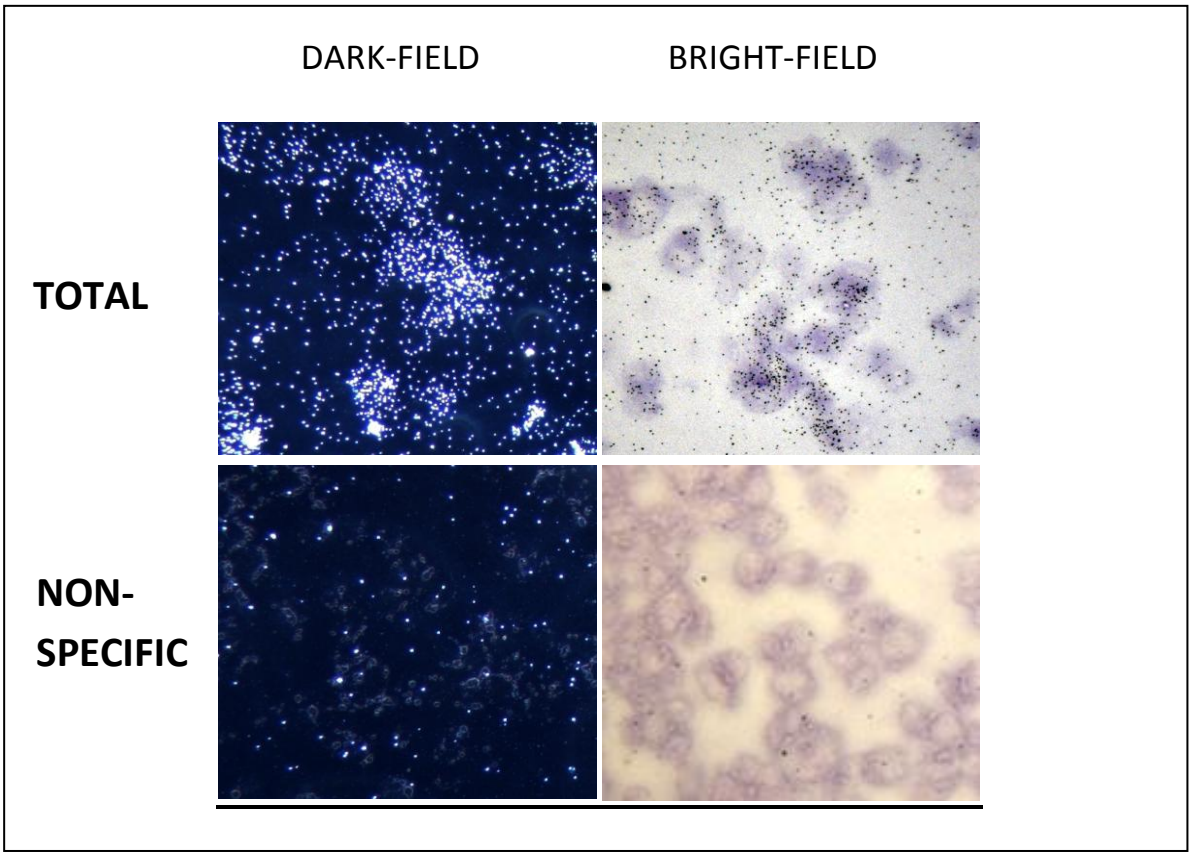


Figure 5.11 *Specific radiolabelled ^{125}I-ET-1 (100pM) localisation to colorectal cancer cell lines (SW480 shown). 0.5 x 10^6 colorectal cancer cells were cytospun onto slides. Slides were fixed, dipped into molton (42^{o}C) K2 emulsion, dried, stored in silica gel dessicant at 4^{o}C for 7 – 21 days, and then processed, and washed. Underlying tissue was H&E stained. Left panels show dark-field illumination and right show bright-field H&E stained cells. Both top panels demonstrate total ET-1 binding whilst non-specific is shown in the bottom panels.*

Specific binding of ^{125}I-ET-1 to either ET_A or ET_B receptors above (Figure 5.10 & 5.11) demonstrated that the ^{125}I- ET-1 used in this study does indeed bind to the cells of interest. The non-specific binding, which is produced by inhibiting ^{125}I- ET-1 by unlabelled ET-1, demonstrates that binding is specific to the receptors. The next step is to analyse binding characteristics in each cell line.

Calculation of K_d and B_{max} for Colonic Fibroblasts

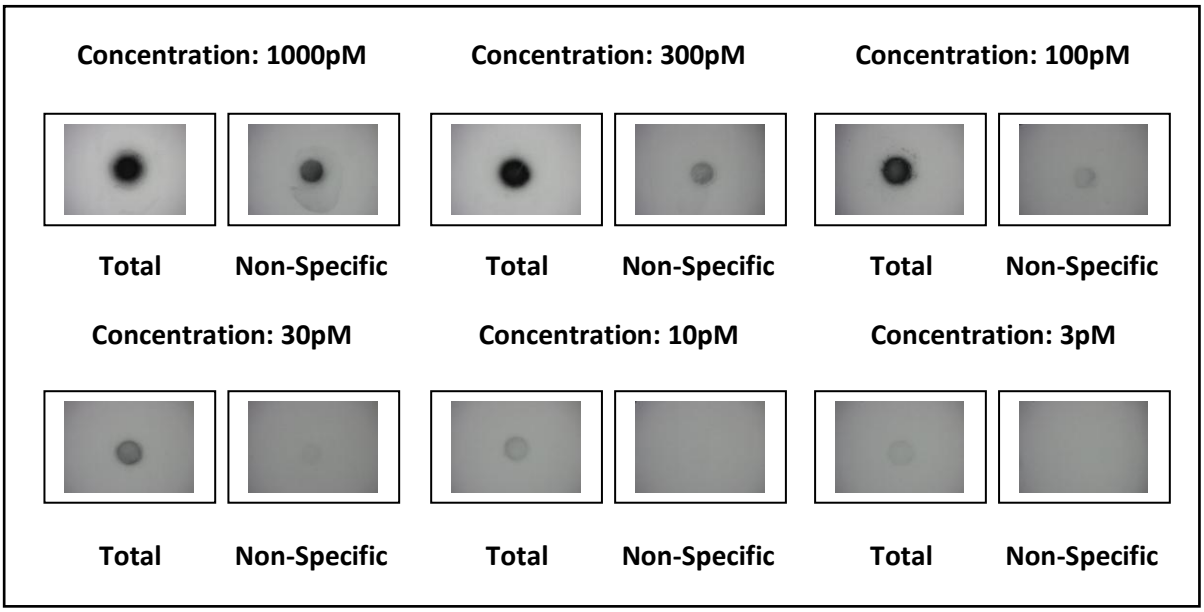


Figure 5.12 *Calculation of K_d and B_{max} of radiolabelled ^{125}I-ET-1 on fibroblast cytospins. Briefly, cells were cytospun onto slides at a density of $2x10^6$ cells then incubated with increasing concentrations of ^{125}I-ET-1 at $3x10^{-12}$(3pM) to 10^{-9} M (1000pM or 1nM) to determine total binding and 1µM of unlabelled ET-1 to establish non-specific binding. Radioactivity was measured with 2 methods: slides exposed to film with densitometry quantification (above) and cell removal with NaOH then radioactivity counted in a gamma counter. Specific ^{125}I-ET-1 binding was determined by subtracting non specific binding from total binding at each concentration used. Maximum receptor binding (B_{max}) and affinity (K_d) were obtained using GraphPad Prism™ software*

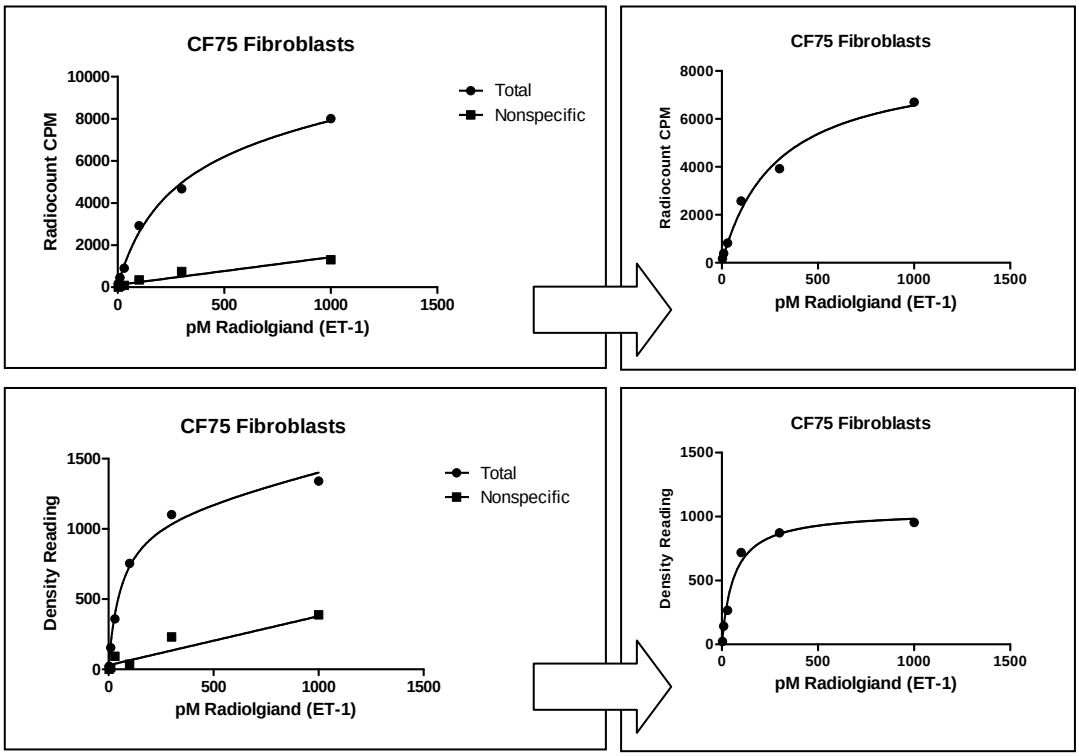
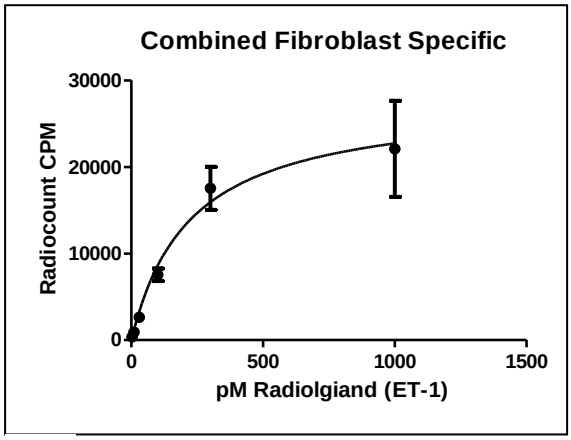
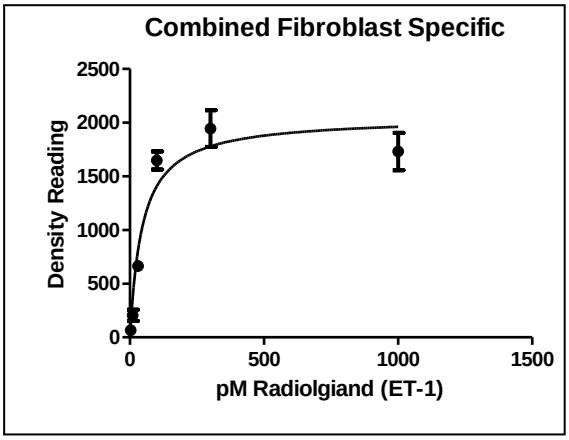


Figure 5.13 *Graphical representation of total and non-specific (left) and specific (right)* ^{125}*I-ET-1 binding using two quantification methods; gamma counts (top graphs) and film densitometry (bottom graphs). Prism software was used to determine the* K_d *(gradient of graph) and* B_{max} *(maximal binding).*





A

Fibroblast Strain	Radioisotope Count Readings		Densitometry Readings	
	B_{max} (fmol/1x10^6 cells)	K_d (pmol/L)	B_{max} (DPM/1x10^6 cells)	K_d
CF36	3.82	249.1	0.27	53.43
CF56	1.46	88.54	0.20	41.19
CF65	4.94	335.8	0.33	54.53
CF75	2.60	243.2	0.28	63.12
Combined	3.03	213.6	0.24	52.84

B

Figure 5.14 A: *Graphs showing combined ^{125}I-ET-1 specific binding to fibroblasts calculated as shown previously. Gamma radio-count in CPM shown on left and density readings shown on right.* ***B:*** *Table showing K_d and B_{max} values for individual and combined fibroblast readings using both methods.*

Calculation of K_d and B_{max} for Colorectal Cell Lines

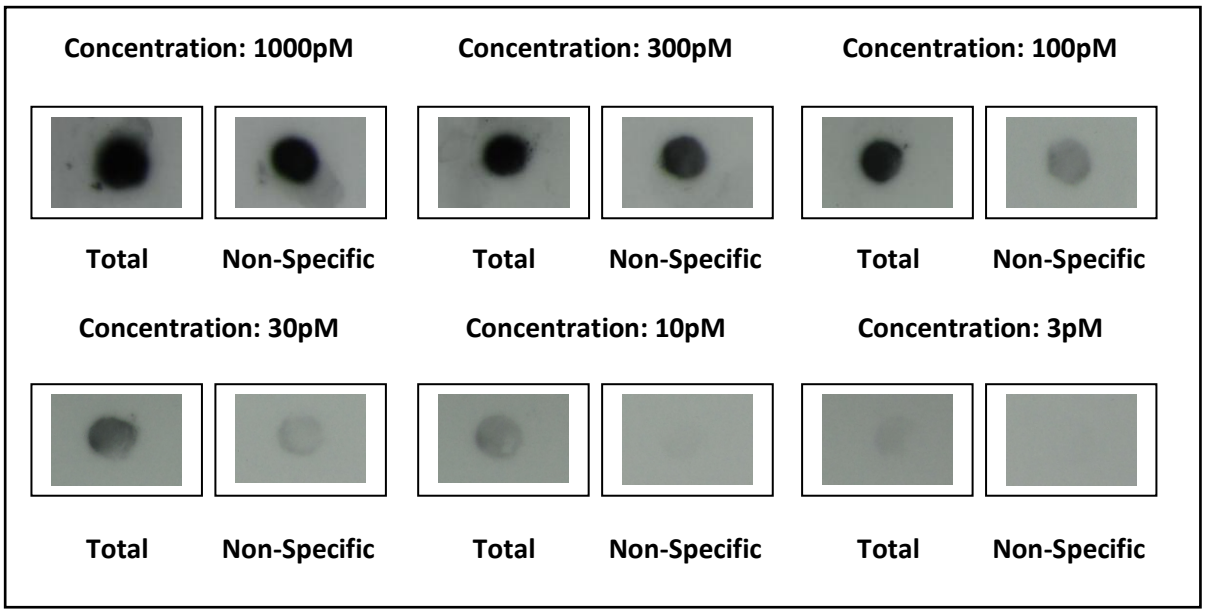


Figure 5.15 *Calculation of Kd and Bmax of radiolabelled ^{125}I-ET-1 on SW480 cancer cell line cytospins. Briefly, cells were cytospun onto slides at a density of 2x10^6 cells then incubated with increasing concentrations of ^{125}I-ET-1 at 3x10^{-12}(3pM) to 10^{-9} M(1000pM or 1nM) to determine total binding and in the presence of 1μM of unlabelled ET-1 to establish non-specific binding. Radiocount activity was measured with 2 methods: slides exposed to film with densitometry quantification (above) and cell removal with NaOH then gamma counted. Specific ^{125}I-ET-1 binding was determined by subtracting non specific binding from total binding at each concentration used. Maximum receptor binding (B_{max}) and affinity (K_d) were obtained using GraphPad Prism™ software.*

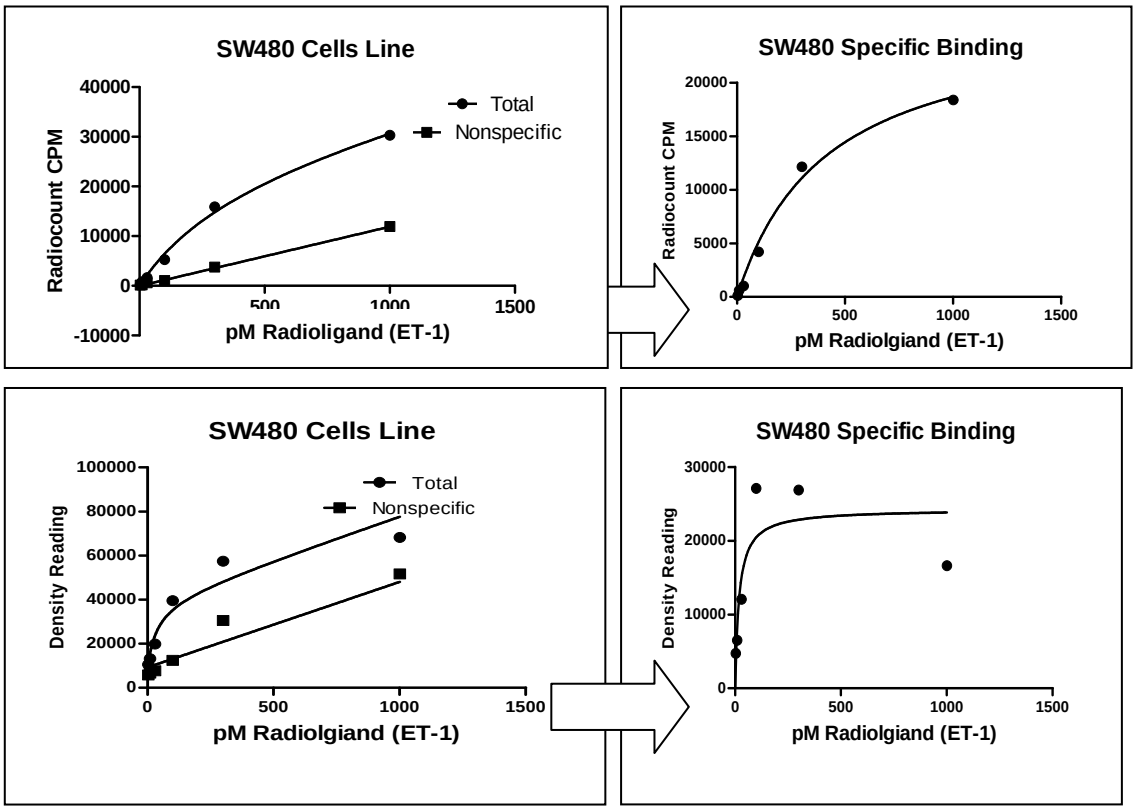
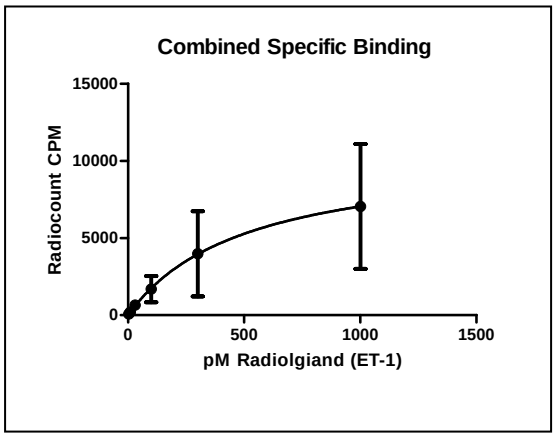
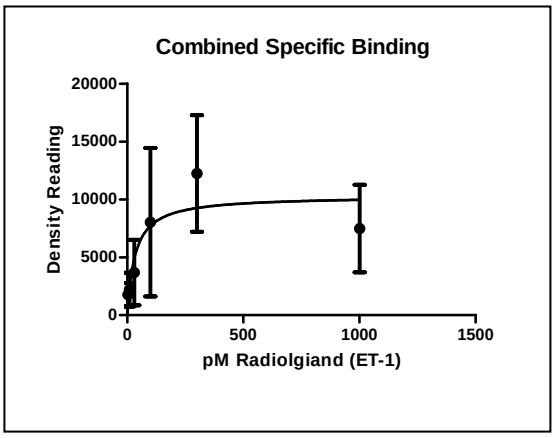


Figure 5.16 *Graphical representation of total and non-specific (left) and specific (right)* ^{125}*I-ET-1 binding using two quantification methods; gamma counts (top graphs) and film densitometry (bottom graphs). Prism software was used to determine the* K_d *(gradient of graph) and* B_{max} *(maximal binding).*





Cancer Cell Line	Radioisotope Count Readings		Densitometry Readings	
	B_{max} (fMol/1x10^6 cells)	K_d (pmol/L)	B_{max} (DPM/1x10^6 cells)	K_d
HT29	2.02	335.8	0.33	54.53
SW480	2.85	399.5	0.54	38.81
Combined	2.43	367.7	0.44	46.64

Figure 5.17 A: *Graphs showing combined ^{125}I-ET-1 specific binding to cancer cell lines calculated as shown previously. Gamma radio-count in CPM shown on left and density readings shown on right.* ***B:*** *Table showing K_d and B_{max} values for individual and combined fibroblast readings using both methods.*

5.3.5 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 radio-sensitive film and densitometric analysis, with readings categorised into high IC_{50} (<10μM), medium (10 – 100 μM) and low (>100 μM). Both methods gave similar IC_{50} results for colonic fibroblasts (BQ123: 0.1-2.2μM; zibotentan: 10-15.1 μM & BQ788: 0.96-1mM) (figure 5.19). The laboratory compound ET_A receptor antagonist BQ123 was ~10,000 fold more effective at inhibiting ^{125}I-ET-1 binding than the ET_B receptor antagonist BQ788. ZD4054 was more effective at inhibiting ^{125}I-ET-1 binding than BQ788 by ~1000 fold although ~1000 fold less effective than the ET_A receptor antagonist BQ123. The IC_{50} of CRC cell lines (figure 5.20) also indicated that ET_A receptor antagonists more effectively inhibited ^{125}I-ET-1 binding (BQ123: 4.43- 10μM; zibotentan: 0.1-1.01μM & BQ788: 0.013-1mM). The orally active ZD4054 was shown to be 1000 fold more effective than BQ123 and 10,000 fold more effective at inhibiting ^{125}I-ET-1 binding then BQ788.

Determining IC_{50} within Colonic Fibroblasts

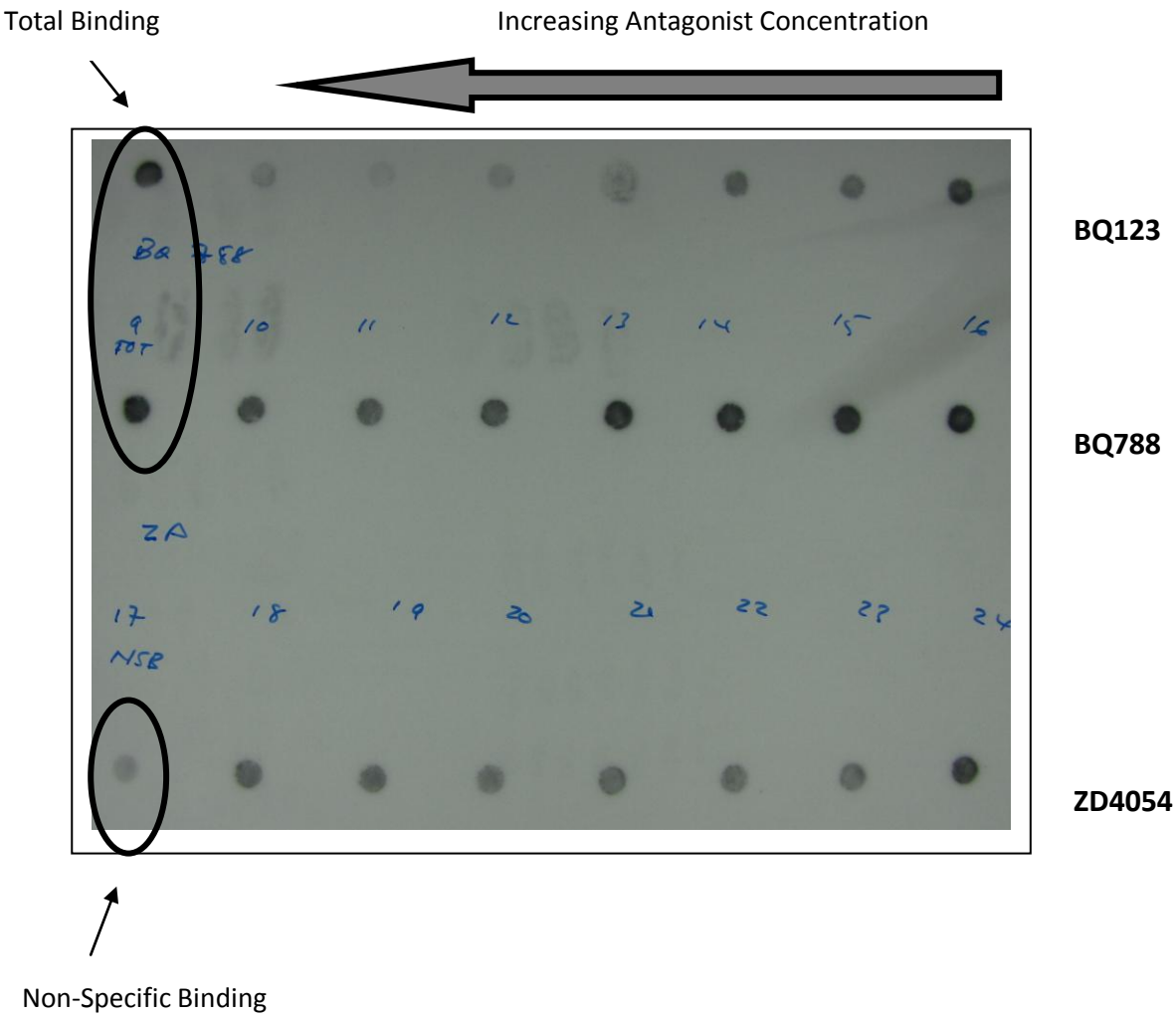
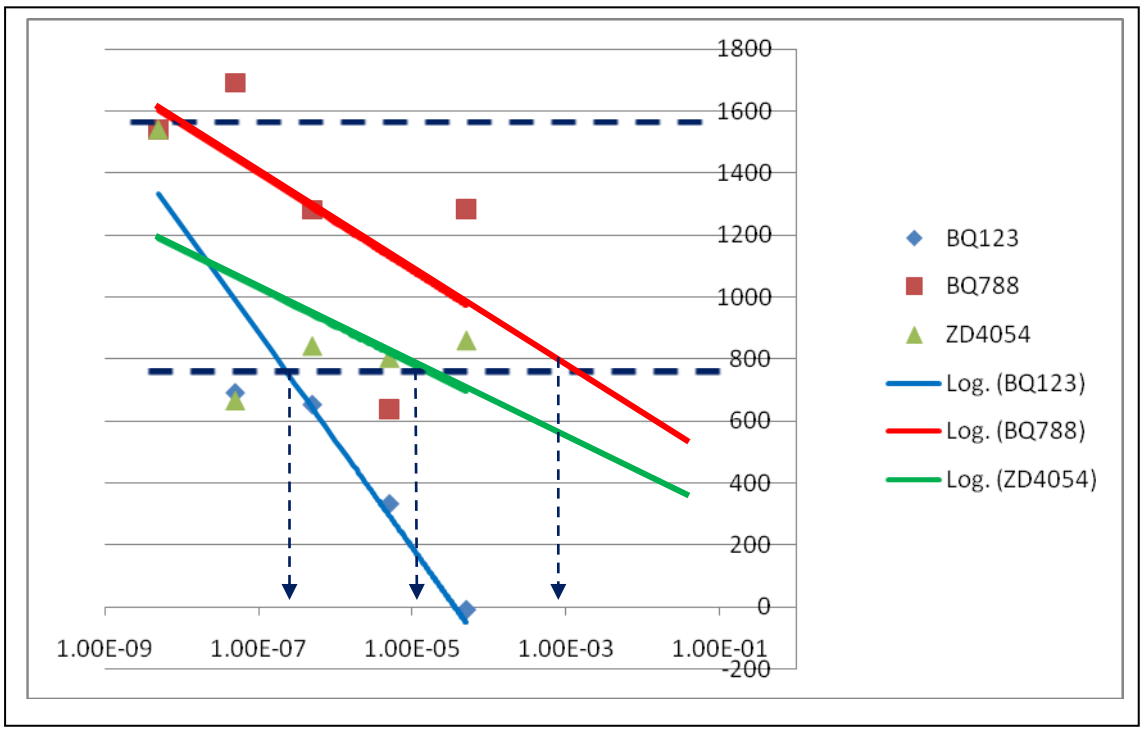
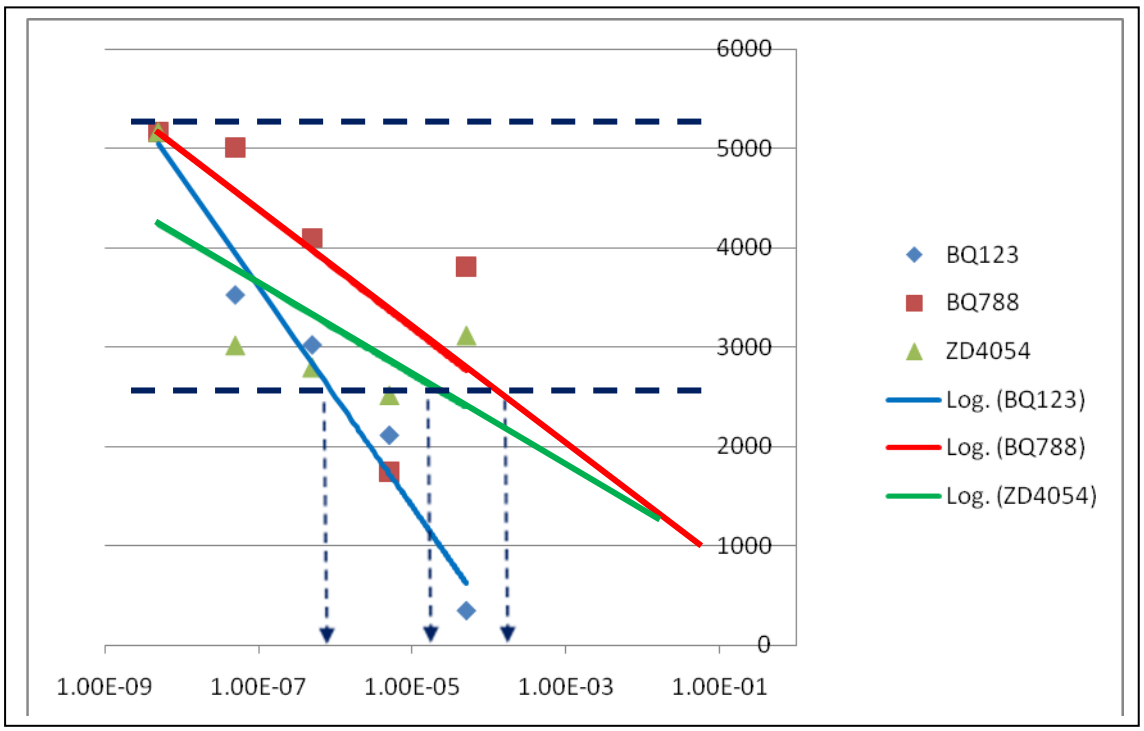


Figure 5.18 *Receptor selective antagonists' ability to reduce ^{125}I-ET-1 binding in slide-mounted tissue. Cytospun colonic fibroblasts were incubated in 100pM (approximately the Kd value determined initially by the saturation studies) ^{125}I-ET-1 in the presence of increasing concentrations of BQ123, BQ788 or ZD4054 ($3x10^{-9}$ to $3x10^{-6}$M). The above low resolution autoradiograph was then produced by opposing the slide mounted tissues to Hyperfilm™ within cassettes for up to 21 days. Films were processed with developer, fixative and then washed. They were photographed and quantified by densitometric analysis. Slide mounted tissues were then removed with NaOH and radioactivity measured with a gamma counter. Both quantification methods were used to determine the IC_{50}.*



(A) Radioisotope determined IC_{50} graph of combined fibroblasts



(B) Densitometry determined IC_{50} graph of combined fibroblasts

Figure 5.19 *Calculation of IC_{50} determined by* ***(A)*** *gamma counts and* ***(B)*** *densitometry analysis. Receptor selective antagonists ability to reduce ^{125}I-ET-1 binding to slide-mounted cells were determined by incubating slide mounted fibroblasts with 100pM ^{125}I-ET-1 in the presence of increasing concentrations of BQ123, BQ788 or ZD4054. Graph shows radioactivity/densitometry plotted against inhibiting concentrations of each antagonist (sample points shown for illustration purposes). Both methods gave similar IC_{50} results for fibroblasts (BQ123: 0.1-2.2µM; zibotentan: 10-15.1 µM & BQ788: 0.96-1mM).*

Determining IC_{50} within Colorectal Cancer Cell Lines

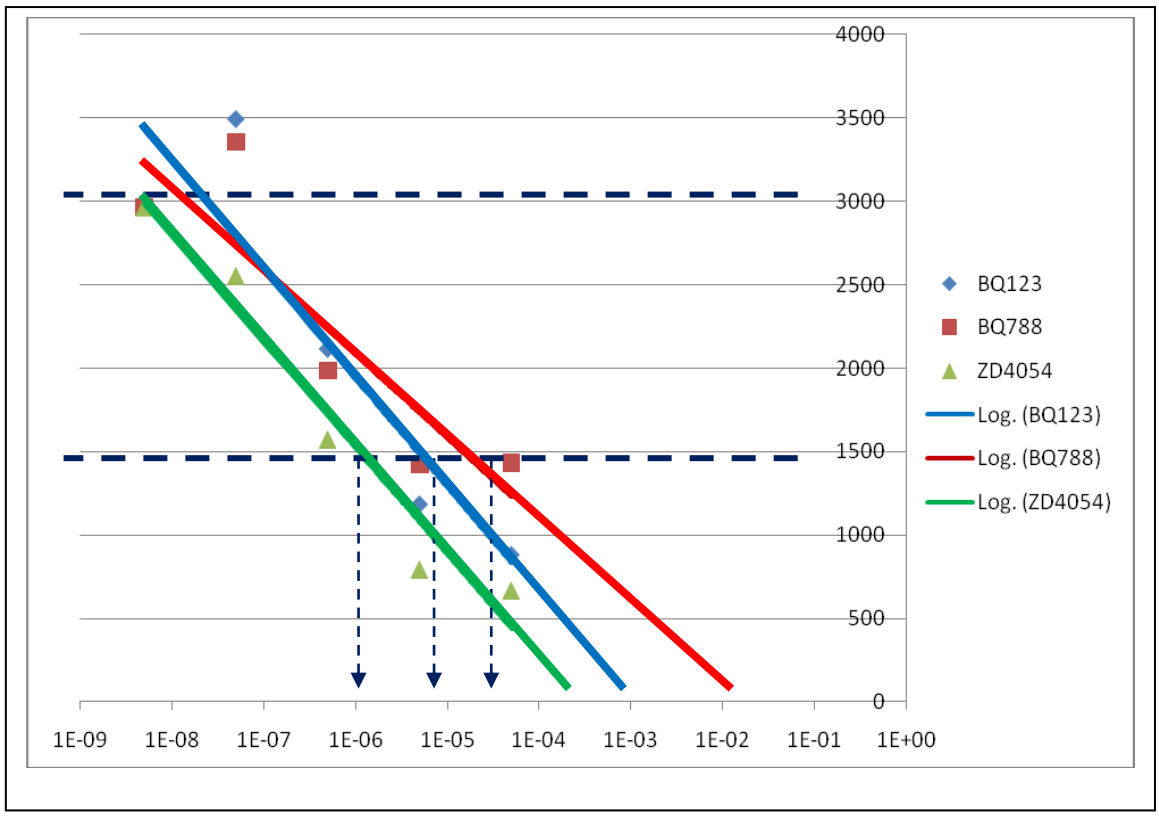


Figure 5.20 *Calculation of IC_{50} determined by densitometry analysis. Receptor selective antagonists ability to reduce ^{125}I-ET-1 binding to slide-mounted cells was determined by incubating slide mounted cancer cell lines with 100pM ^{125}I-ET-1 in the presence of increasing concentrations of BQ123, BQ788 or ZD4054. Graph shows densitometry plotted against inhibiting concentrations of each antagonist (sample points shown for illustration purposes). The IC_{50} of CRC lines also indicated that ET_A receptor antagonists more effectively inhibited ^{125}I-ET-1 binding (BQ123: 4.43- 10μM; zibotentan: 0.1-1.01μM & BQ788: 0.013-*

5.4 DISCUSSION

To investigate the efficacy of ZD4054 against ET-1 binding we carried out studies using specimens from patients with colorectal cancer.

5.4.1 ET-1 Binding within Patient Specimen Sections and Localisation by IHC

Autoradiography demonstrated ^{125}I-ET-1 binding to ET 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 staining for collagen XI confirmed the pathological state of the stroma associated with cancer lesions (Fisher *et al.*, 2001a). This correlates with our previous work on human colonic specimens which showed that radiolabelled ^{125}I-ET-1, ET_A receptor antagonist (^{125}I-PD151242) and ET_B receptor agonist (^{125}I-BQ3020) bound in a similar pattern within cancer and normal tissue (Hoosein *et al.*, 2007; Ali *et al.*, 2000). Other groups have also reported strong stromal binding of ^{125}I-ET-1 in intestinal tissues (Inagaki *et al.*, 1992; Egidy *et al.*, 2000).

To investigate ET-1 binding in patient samples, we carried out experiments in (1) whole tissue homogenates and (2) tissue sections.

5.4.2 ET-1 Binding Characteristics within Patient Tissue Homogenates

Tissue homogenates were used to determine ^{125}I-ET-1 binding characteristics. The binding affinity (K_d) and maximum saturable binding (B_{max}) of ^{125}I-ET-1 to its receptors were similar in both the normal and tumour specimens. These figures are closely matched to previously published data regarding the characteristics of ^{125}I-ET-1 binding in other tissues (Alexander *et al.*, 2009).

Since these homogenate studies only demonstrate ^{125}I-ET-1 binding to receptors ($ET_{A/B}$), we cannot gain information as to what the distribution of each respective receptor is in each of the tissue specimens. Given that tissue homogenates contain many non-specific binding regions, subtle receptor density and affinity changes would be unlikely to be revealed or observed. Previous studies within our department demonstrated a change in the ratio of receptor subtypes in colonic tissues, with up-regulation of ET_A receptors and down-

regulation of ET_B receptors in cancer compared to normal colon (Ali *et al.*, 2000; Hoossein *et al.*, 2007). Therefore, it is feasible that the overall number of ET_A and ET_B receptors within the specimens would remain constant with just a change in the proportions of receptors present (i.e. increase in ET_A and decrease in ET_B). In this case K_d and B_{max} would remain grossly similar and the main difference would result from individual receptor binding affinity differences for ET-1, which are known to exist (Ali *et al.*, 2000; Hoosein *et al.*, 2007). To determine changes in receptor subtypes in tissues, we inhibited ^{125}I- ET-1 with unlabelled ET_A or ET_B receptor antagonists.

5.4.3 ET-1 Binding Characteristics within Patient Tissue Sections

In tissues, the ET_A receptor antagonist BQ123, firstly demonstrated a concentration dependent inhibitory effect on ^{125}I-ET-1 binding in both normal and tumour specimens, (25μM>5μM). Secondly, most inhibition of ^{125}I-ET-1 binding with the use of BQ123 was observed in the tumour sections, consistent with a higher ET_A receptor expression in cancers compared to normal colon tissues. This is in keeping with previous findings: Specifically, Hoosein *et al.* (2007) used the selective radiolabelled ET_A receptor antagonist [^{125}I]PD-151242 to identify ET_A receptor binding sites and demonstrated that binding on cancer tissues was increased by 55.5% when compared to normal colon tissues.

The ET_B receptor antagonist BQ788 also inhibited ^{125}I- ET-1 in a concentration dependent manner (25μM>5μM). There was generally greater inhibition of ^{125}I- ET-1 binding with the use of BQ788 in normal colon tissues than within tumour sections, consistent with higher ET_B receptor expression in normal colon and a down-regulation observed in colorectal cancer (Ali *et al.*, 2000; Hoosein *et al.*, 2007). As BQ788 inhibits ET_B receptor binding, the increased ^{125}I-ET-1 binding in tumour tissue compared to normal tissue also indicates a higher ET_A receptor expression in tumour. Hoosein *et al.,* (2007) by using a specific ET_B receptor agonist, [^{125}I]BQ3020, demonstrated a 45% decrease in ET_B receptor expression within cancer sections compared to normal tissues. However, this is contrary to findings by Egidy and colleagues (Egidy *et al.*, 2000) who demonstrated a quantitative increase in mRNA expression of both ET_A and ET_B receptors in colorectal cancer specimens. However, increases at the mRNA level are not necessarily followed by translation at the protein level.

The ET_A receptor antagonist ZD4054 did not display a concentration dependent inhibition to the extent observed with BQ123 and BQ788. There was overall more extensive inhibition observed in tumour specimens than normal tissues, in keeping with the over-expression of

ET_A receptor in colorectal cancer. The extent of inhibition of ^{125}I- ET-1 binding may not have been as expected as ZD4054 is a specific ET_A receptor antagonist whilst BQ123 and BQ788 at higher concentrations are known to act on both receptor subtypes (loss of selectivity; Alexander *et al*., 2009). Therefore use of these laboratory compounds may not be truly subtype-selective when used at high concentrations.

5.4.4 Identifying ET-1 Localisation within Tissues using Immunohistochemistry

^{125}I-ET-1 binding to specific tissue structures is further clarified by the use of immunohistochemistry to localise ET_A and ET_B receptors, in addition to fibroblast, endothelial cell and collagen type XI mapping. ET_A and ET_B immunostaining closely correlated to ^{125}I-ET-1 binding in underlying tissue sections, localising to mucosa, dense stromal regions and surrounding blood vessels. CRC specimens demonstrated increased ET_A receptor staining localised to epithelial cells and tumour stroma, and reduced ET_B receptor staining on the epithelial cell surface compared to normal tissue specimens. This altered receptor expression in CRC is consistent with our autoradiographic work, other CRC studies (Hoosein *et al.,* 2000; Ali *et al.*, 2000; Asham *et al.*, 2001; Shankar *et al.*, 1998) and studies in prostate and ovarian cancer (Nelson *et al.*, 1996; Bagnato *et al.*, 1999). 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. This is the first time that BQ123 has ever been conjugated to Quantum dots and have been used to visualise ET_A receptor distribution in normal and tumour specimens.

^{125}I-ET-1 binding co-localised to CD31 immuno-positive vascular endothelial regions that were predominantly associated with the ET_B receptor subtype, determined by both autoradiography and immunohistochemistry (Figure 5.2 & 5.3). ET_A binding was prominent on vascular smooth muscle cells as found in other studies (Sullivan *et al.*, 2000; Hansen-Schwartz *et al.*, 2002). This receptor has been shown to be involved in cell proliferation, survival (Pedram *et al.*, 1997; Shichiri *et al.*, 2000) and down-stream signalling (VEGF, PKC; Alanen *et al.*, 2000). This work confirms vascular in-growth and neovascularisation which is crucial for tumour development (Knowles *et al.*, 2000) and highlights ET-1 angiogenic actions.

Collagen Type XI was only seen throughout stromal regions of tumour sections and co-localised to fibroblasts where ^{125}I-ET-1 binding was predominantly to the ET_A receptors.

These results are in agreement with previous studies linking its expression to CRC (Fisher *et al.*, 2001a) and confirmed the nature of our specimens to be cancer. Interestingly ET-1 affects APC/β-catenin and Wnt signalling which is the pathway also involved in regulating COLXI and CTFG expression that are known to have potential tumourogenic effects on fibroblasts.

Interestingly within normal mucosa, ET_A receptors appeared closer to the luminal surface whereas ET_B receptors were towards the muscularis mucosa and lamina propria (determined from Figure 5.6), a distribution pattern not previously reported. One possible reason for increased ET_B receptors may be due to endothelial cell capillary networks that supply nutrients to the mucosa, although this is not fully supported due to lack of observed CD31 staining. This leads us to consider possible trophic signalling roles for ET receptors. It is known that basal to apical cell movement in intestinal epithelium is accompanied by a change in cell signalling and receptors. Stem cells give rise to progenitor cells which undergo growth arrest at the crypt-villus junction then turn into mature cells where most signalling to directed towards differentiation. This movement is accompanied by an increase in multiple receptors including retinoid x receptor/retinoic acid receptors, vitamin D receptors, oestrogen receptor β (ERβ) and a decrease in others such as ERα found predominantly in basal regions. Therefore it is possible that the ET_B receptor is associated with growth arrest signalling whilst ET_A receptor expression towards the apical regions are associated with differentiation signalling, an aspect where little research has been done.

5.4.5 High Resolution Autoradiography of Colonic Fibroblasts and CRC Cell Lines

High resolution autoradiography demonstrated that ET-1 binds to both fibroblast cell strains and colorectal cancer cell lines. The importance of studying this by autoradiography is that we are actually visualising the binding of ET-1 to its receptors. This is unlike Western Blotting that only shows that the receptor proteins are present but not indicating if on the surface or within the cytosol of the cells. IHC would show us the presence of ET_A and ET_B receptors on the surface but would not necessarily indicate if ET-1 has the ability to bind to them since structural receptor variations may exist.

5.4.6 Characterisation of Colonic Fibroblasts and CRC Cell Lines (K_d and B_{max})

The combined colonic fibroblasts' K_d value of 213.6pM (0.213nM) is regarded as high affinity (≤1nM) with these cells demonstrating a relatively high maximal ^{125}I-ET-1 binding (3.03 fmol/1x10^6 cells). The CRC cell lines exhibited a similar B_{max} (2.43fmol/1x10^6 cells) and affinity that was marginally less than the fibroblasts (K_d: 0.367nM), suggesting that fibroblasts have more ET surface receptors than cancer cells and fibroblasts also had a higher binding affinity than cancer cells.

The K_d and B_{max} values for all fibroblasts were similar except within the CF56 strain which were lower. A lower B_{max} may have been caused by high non-specific binding (NSB), which would have lowered the calculated specific binding. A similar result would occur if total binding was underestimated which may have resulted from inadequate removal of cells from slides before gamma counting. These would also lead to a falsely low K_d value. Since K_d is defined as the concentration of ligand that occupies 50% of receptors, if there were less receptors calculated, a lower concentration would be needed to occupy these, and therefore result in a falsely low K_d value (falsely high affinity) as we observed.

Looking at the two methods employed to determine the binding characteristics, both radioisotope and densitometry readings seemed to display similar trends in results within the population of cancer cell lines or fibroblasts investigated. There was more variability seen within the densitometry reading data. This can be explained by the huge number of variables encountered when using this technique. These include time of exposure to radioactive sensitive film, differences between films, apposition variability between slide-film, cell density and surface area variability on slides, and difference in densitometry reading by software on different days. The studies measuring radioactivity of ^{125}I-ET-1 binding (in a gamma counter) was the more accurate method of determining binding characteristics.

Overall these results suggest that colonic fibroblasts have more ET surface receptors than CRC cell lines and this is supported by the high resolution autoradiographs (Figure 5.10). Fibroblasts also had a higher binding affinity than CRC cell lines with similar figures to previous studies using human colonic mucosa and human skin fibroblasts with K_d values of 0.41nM and 0.4nM respectively (Inagaki *et al.*, 1992; Inagaki *et al.*, 1991; Kusuhara *et al.*, 1990). This is the first time that individual cellular components of colonic tissue have been evaluated for ET-1 binding characteristics with the overall results suggesting a higher binding affinity and maximal binding within fibroblasts.

5.4.7 Determining Inhibitory Concentration of 50 (IC_{50})

Competition studies were performed using both radioactivity measurements and film densitometry. Both methods gave similar IC_{50} results for fibroblasts (BQ123: high; ZD4054: 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, ZD4054, 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 (ZD4054: 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, ZD4054 was the most effective at inhibiting ^{125}I-ET-1 binding in cancer cells.

The only other published study to investigate selective binding of ZD4054 was by Morris *et al.,* in 2005. In this paper, human recombinant ET_A or ET_B receptors were expressed in mouse erythroleukaemic cells and competitive binding assessed using radioligand ^{125}I-ET-1 and ZD4054 (concentrations between 100pM and 100μM). Experiments revealed ZD4054 had high affinity for the ET_A receptor (Ki=13nM) with a pIC_{50} of 8.27nM. There was minimal interaction at the ET_B receptor (Ki=1.2nM) and none seen below 10μM. To assess specificity, the ability of ZD4054 to inhibit ET_A receptor induced forearm vasoconstriction (using venous occlusion plethysmography) was used. ZD4054 significantly inhibited vasoconstriction following oral administration of 10mg and 30mg by 18.8% and 23.7% respectively. ET_B receptor interaction was assessed by measuring plasma ET-1 levels in healthy individuals (as ET_B is linked to ET-1 clearance pathway). Following administration of between 2.5mg – 240mg, there was no significant difference in plasma ET-1 levels compared to placebo. This paper once again demonstrates high ET_A receptor specificity of ZD4054. The difference in the IC_{50} between our study and the Morris study could be due to the fact that the authors used recombinant ET expression and different cell lines.

Chapter 6

Overall Discussion, Future Directions and Clinical Implications

6. OVERALL DISCUSSION AND FUTURE DIRECTIONS

As described in chapter 3, ET-1 stimulated proliferation of both colonic fibroblasts and colorectal cancer cell lines. There have been a limited number of studies investigating the effect of ET-1 on colonic fibroblasts, one study showing significance in growth of only one of six fibroblast strains (Knowles *et al.,* 2011) while a second study on embryonic fibroblasts failed to demonstrate significance (Kernochan *et al.,* 2002). The current study showed significant proliferation in all fibroblast strains used. Surveying the literature addressing fibroblasts from other organs, there have been only two studies on fibroblasts associated with tumours, one in ovarian cancer and the other in primary oral cancer, both of which reported a proliferative response to ET-1 (Moraitis, 1999; Hinsley *et al.,* 2012). Fibroblasts from non-tumour sites such as human dermal and rat cardiac tissues also demonstrated ET-1 stimulated growth (Piacentini *et al.,* 2000; Xu *et al.,* 1998). The proliferative effects of ET-1 in colorectal cancer cell lines reported in this study were in line with previous findings and consistent with the extensively described stimulatory effects of ET-1 in epithelial cancers.
This is the first time that ET_A receptor antagonism using ZD4054 was investigated in colorectal cancer models. ET_A receptor antagonism significantly inhibited proliferation in both fibroblasts and cancer cell lines, with ZD4054 being the most efficacious of all receptor antagonists tested. Due to the variation in response to ET-1, I propose future studies to correlate the ET-1 proliferative effect with levels of endogenous ET-1 and the number and affinity of cell surface receptors. This would generate a complete map of the above and determine the efficacy of anti-ET_A receptor treatment.

However, it is widely accepted that monotherapies are not appropriate for eradication of cancer in the clinical setting. As described in ovarian cancer models, and in line with our knowledge of how non-efficacious cancer chemotherapy can be, future work should also include combining chemotherapeutic agents with ZD4054 (Rosano *et al.,* 2007a, 2007b). In regards to combination therapy a number of trials in other cancers have taken place. The availability of orally active bio-available endothelin receptor antagonists offers potential opportunities in adjuvant cancer treatment. One example is Atrasentan with a 1000-fold greater affinity for the ET_A receptor than the ET_B receptor. A phase II randomized, placebo-controlled trial (288 patients with asymptomatic hormone-resistant metastatic prostate cancer) evaluated three groups: placebo, 2.5mg, or 10mg Atrasentan. Delayed time to progression (TTP) was observed in the group that received 10mg and stabilization of biochemical markers (prostate specific antigen and lactate dehydrogenase) was compared to controls. In two phase III clinical trials (809 prostate cancer patients) 10mg of Atrasentan daily was shown to delay TTP when compared to placebo metastatic hormone resistant

prostate cancer, albeit statistically non-significant (Carducci *et al.*, 2007). However, secondary endpoint analysis demonstrated significant delayed progression of bone acid phosphatase levels and preserved prostate cancer-specific quality of life, particularly in terms of pain-related symptoms. It was suggested that the future use of Atrasentan may lie in combining it with other drugs. This was demonstrated by a phase I-II trial in patients with resistant prostate cancer, where results from this drug combined with docetaxel were comparable to results produced by docetaxel and prednisolone (Armstrong *et al.*, 2008). More recently the novel specific ET_A receptor antagonist Zibotentan (ZD4054) -which is the drug under investigation in this body of work - has also been investigated within the field of prostate cancer. A phase II double blinded clinical trial allocated a total of 312 patients with pain free or mildly symptomatic hormone resistant prostate cancer patients with bony metastases to either receive daily doses of ZD4054 (10mg-15mg) or placebo. There was no significant difference seen for progression-free survival although there was a difference in the overall survival of these patients compared to the placebo group. Importantly this drug had an acceptable safety and tolerability profile (James *et al*., 2008 and 2009). A recent Phase III clinical trial examined ZD4054 in non-metastatic hormone resistant prostate cancer, but was terminated early (in 2011) as it was unlikely that the trial would meet its primary efficacy end point of progression free survival and overall survival benefits. Due to the disappointment of monotherapy, interest has grown in its use as adjuvant therapy; for example the latest on-going phase II clinical trial (FOLFERA) which combines ZD4054 with chemotherapy agents Irinotecan, Fluorouracil and Folinic acid (FOLFIRI) in patients with advanced colorectal cancer. Hopefully results will be available in the near future. The endothelin axis is altered in cancer, aiding both tumour growth and progression and accumulating evidence supports selective ET_A antagonism as an effective method to inhibit endothelin action in tumourigenesis. The indicated anti-tumour activity, with generally acceptable side effects, warrant further clinical evaluation of these agents to determine therapeutic potential in an adjuvant setting.

Further work in chapter 3 investigated migration of fibroblasts under the influence of ET-1 which was detected in all fibroblasts strains tested. Migration was inhibited by both ET_A and ET_B receptor antagonists. Most inhibition appeared to be blocked via the ET_B receptor, whilst similar inhibitory effects were seen by both ZD4054 and BQ123. The ET_B receptor being predominantly involved in migration is in keeping with previous work looking at colonic fibroblast migration (Kernochan *et al*., 2002). The mixed effects of blocking different combinations of receptors leads us to hypothesize a more complex interaction of ET-1 with its receptors that involve second messengers and other regulatory peptides e.g. PDGF. This follows from other work that looked at fibroblast responses to regulatory peptides. Jiang *et*

al., (2008) and Kinnman *et al*., (2000) demonstrated that PDGF stimulated migration in dermal fibroblasts (through activation of Rac causing protrusion of fibroblast dendritic extensions) and stellate cells (3 fold increase). The work presented here demonstrated using gene arrays that PDGF was also stimulated in our fibroblasts in response to ET-1 which could have been via either receptor; this could account for some of the migratory response observed. Adding to the complexity of second messenger signalling, Jiang *et al*., (2008) reported that ET-1 was a Rho activator which resulted in retraction of dendritic extensions and contraction. Furthermore Shlyonsky *et al*., (2011) showed that ET-1 increased α-smooth muscle actin and cell migration in lung fibroblasts. They also found that BMP-2, which inhibits PKC in a dose dependent manner, inhibited ET-1 ability to increase α-smooth muscle actin and migration.

The colorectal cancer cell lines did not demonstrate any migration in response to ET-1. Despite no published work demonstrating ET-1 induced migration in cancer cell lines, it has been shown that HT29 cell lines do have the ability to migrate (along with other CRC cell lines). Rubie *et al*., (2011) examined the response of HT29, SW480 and CaCo2 cell lines in response to increased exogenous CXCL12 (ligand for the G-protein receptor CXCR4 or stromal derived factor-1; SDF-1). They found that migration was increased in all cell lines examined however to a much lesser extent in HT-29 cells, as CXCR4 expression was less. HT29 cells also express MMP7 and LN5 (Laminin-5/Laminin-332), the latter causes firm adhesion and hemi-desmosome formation. Remy *et al*., (2006) found that increasing MMP7 concentrations cleaved LN5 and therefore increased cell motility. The gene array data in chapter 4 showed significant MMP-7 up-regulation but only after 24 hours which was beyond the time scale used in this experiment. I therefore postulate that increasing the experimental time scale to 72 hours could reveal an observed migration response enhanced by MMP7 expression. Furthermore changes in experimental conditions may be of benefit, as other studies had used 1% BSA rather than 10% FCS, and used Cycloheximide rather than Mitomycin C.

Finally on contraction studies in chapter 3, ET-1 stimulated contraction of fibroblasts with both ET_A and ET_B receptor antagonists inhibiting its effects. Contraction seemed to be stimulated via both receptors and hence for this reason the highly specific ET_A receptor antagonist ZD4054, although significantly inhibiting contraction, was least effective out of all three antagonists as it has little or no effect on the ET_B receptor. Published work suggests that ET-1 acts synergistically with other factors such as PDGF, bFGF and insulin like growth factors for producing contractile effects (Takuwa *et al*., 1989). I have previously summarised

a large volume of literature that had studied the complexities and interactions of pathways for cell contraction (see chapter 3).

In chapter 4 gene arrays were carried out to identify novel genes that were up or down regulated by ET-1. Significantly regulated genes identified in fibroblasts included CTGF, ADM and STC-1. Time point inductions confirmed ET-1 stimulation of the former two genes at between 1-2 hours and 2-4 hours respectively. Interestingly, time point inductions actually demonstrated an up-regulation of STC-1 at 30 minutes (not seen in the arrays which used 4 hours as the snapshot time of investigation). Antagonistic work revealed the ET_A receptor antagonist ZD4054 was the most efficacious at inhibiting induction of all these genes. This was confirmed as driven via the ET_A receptor through experimental work I designed with silencing SiRNA. Furthermore the effects of silencing the ET_A receptor on targets were confirmed at the protein level (on Western blotting of CTGF); similar results were found when using the receptor antagonist ZD4054. Studies have linked both CTGF and ADM to a number of cancers including colorectal (Ladwa *et al.*, 2011; Koliopanos *et al.,* 2002; Nikitenko *et al.*, 2006), with only one other study mapping CTGF expression to colonic fibroblasts (Knowles *et al.*, 2011). However, the majority of fibroblasts from various other sites do produce CTGF (Shi-Wen *et al*., 2004, 2006, 2007). With both CTGF and ADM linked to cell proliferation, angiogenesis, survival (anti-apoptosis) and tumourogenesis, the fact that ET-1 alters their expression is exciting and provides more specific information on the molecular pathways utilised to propagate the signal. Furthermore, STC-1 is linked to a number of cancers as well as tumourogenic processes (Fujiwara *et al.*, 2000; Yeung *et al.*, 2010). However, there are conflicting roles of STC-1 (discussed in detail, chapter 4) ranging from the molecule acting as a tumour suppressor that is lost in colorectal cancer at locus 8p (Chang *et al.*, 1998) to increasing tumour support through increased vascular formation. This is the first time that ZD4054 has been investigated in this setting and has been demonstrated to be effective in altering the expression of these key genes. Of interest would be carrying out further work in co-culture or 3D models to investigate whether ET-1 from tumour cells alter the expression of these genes in fibroblasts as a means to support tumour growth.

Collagen type XI and AML-1 were previously shown within our department to be up – regulated in response to ET-1 (Knowles *et al.*, 2011). Here we confirmed this at the protein level using Western blotting. Furthermore, ZD4054 via the ET_A receptor had significantly inhibited the protein expression of both genes. COLXI has only been shown to be expressed in colorectal pathologies within stromal regions and correlating with the APC/β-catenin pathway that is altered in colorectal cancers (Fischer *et al.*, 2001a/b). On the other hand only

one population based study had linked AML-1 to colorectal cancer (Slattery *et al.*, 2011); the majority of studies link this gene to leukaemia. This makes the current findings to be all the more exciting for both these proteins, especially as AML-1 seems to be linked to abnormal signalling of various molecular pathways including TGF-β signalling - which is deregulated within colorectal cancers. Future work in this field would therefore be novel and possibly open up new avenues to target cancers. With some common signalling pathways shared with leukaemia and lymphomas, drugs used in these fields may also warrant investigating in the field of colorectal cancer, either individually or in combination therapy. The more we explore the cross-reactivity and interconnectivity of molecular events and pathways, the smarter the design of multi-treatment pathways will become.

While investigating colorectal cancer cell lines, gene array data revealed an up-regulation of MT1X and MMP7 following exposure to ET-1. Time point induction experiments showed a 300 fold increase at 4 hours and 5 fold at 24 hours for both genes respectively. ET_A and ET_B receptor antagonists inhibited the expression of both of these genes, with ZD4054 being the most efficacious for both genes. Silencing of both ET receptors individually caused a reduction in MMP7 expression, confirming our antagonistic findings. Importantly MT1X was shown to be associated with poor prognosis in a number of cancers (Hishikawa *et al.*, 2001), resistance to chemotherapeutic agents and inactivation of p53 via a number of mechanisms (Fan *et al.*, 2002). MMP7 is also linked to a number of tumourogenic signalling pathways (β catenin, EGF and Wnt), decreased survival and chemo-resistance (Ametler *et al.*, 2011; Wang *et al.*, 2006; Maurel *et al.*, 2007). These, along with links to advanced colorectal cancer for both MT1X and MMP7, point to potential benefits in using ZD4054 as adjuvant or combined therapy to improve the effects of chemotherapeutic regimens.

Expression of endogenous ET-1 was demonstrated in colorectal cancer cell lines but was absent in fibroblasts most likely due to the high passage number. Cancer cell lines demonstrated great variability in mRNA expression of both ET_A and ET_B receptors. At the protein level the high ET_A and low ET_B expression levels are consistent with previous studies including our chapter 5 results. Discrepancies between mRNA and protein levels were discussed and may be related to receptor internalisation and cycling. Fibroblasts had shown high protein expression levels of ET_A and ET_B receptors. I had discussed theories of allosteric and orthosteric binding sites and the way this affects binding and activation of various pathways (De Mey *et al.*, 2011). The faster association and slow dissociation of ET-1 to the ET_A receptor will also affect binding and effects of different antagonists.

Of major interest is the relationship of ET-1 with the EGF receptor. A previous study in our department had shown ET-1 mitogenic signalling propagates via the EGF receptor similar to ovarian cancer models (Grant *et al.*, 2007; Vacca *et al.*, 2000). The work carried out here reports for the first time that ET-1 induces a biphasic EGF receptor expression. Furthermore ET_A receptor silencing had returned EGF expression to scrambled levels. Currently Cetuximab, an EGFR monoclonal antibody is used clinically in colorectal cancers. We also know that ZD4054, Zibotentan, has been used in clinical trials in prostate cancer with a good safety profile. Therefore there seems to be good clinical rationale for combining both treatments in a phase 1 clinical trial which should be easier to gain ethical approval for. This is an area of great interest to pursue. Relating this complex interaction between different pathways highlights the possible use of multi-therapy treatments for cancer.

Chapter 5 characterises ET-1 binding in normal and tumour patient sections with preserved cellular architecture, homogenates and cytospun cells. Maximal binding was observed in stromal regions around vascular endothelial cells and fibroblasts. Immunohistochemistry confirmed this with staining for fibroblasts, CD31 endothelial cell and COLXI, the latter only seen in stroma of tumour sections. These observations fit with the increasingly important role of fibroblasts in cancer, the vascular role demonstrated in previous studies and specific COLXI expression seen in colorectal cancers (Hoosein *et al.*, 2007; Fischer *et al.*, 2001). Higher ET_A expression was seen in colorectal cancer sections as demonstrated in previous studies compared with normal sections (55.5% increase). This was mostly around cancer epithelial, stroma and blood vessels. Higher ET_B expression was seen in normal tissue with decreased levels in tumour sections, again consistent with expected observations for epithelial regions (45% decrease). For the first time we have demonstrated normal mucosa expressing higher ET_A levels at the luminal surface and more ET_B towards the muscularis mucosa. Understanding of the physiology and changes within the mucosa, we hypothesise a possible trophic signalling role of ET receptors with ET_B linked to growth arrest and ET_A involved with differentiation. This would be interesting to investigate in the setting of regenerative medicine and colonic reconstruction. Autoradiography demonstrated that ET-1 actually bound to receptors on the surface of cancer cell lines and fibroblasts. Fibroblasts demonstrated a higher maximal binding and affinity for ET-1 than cancer cell lines. This is the first time that individual colonic components have been evaluated and findings emphasise the importance of fibroblasts in colorectal cancer development and progression.

Lastly it could be argued that the IC_{50} value and lack of concentration dependent inhibition of ZD4054 points to its specificity to the ET_A receptor. ZD4054 acting via the ET_A receptor has been shown to significantly inhibit proliferation, migration and contraction at the cellular level,

therefore inhibiting tumour progression. Its Inhibition of key genes (CTGF, ADM, STC-1, MT1X and MMP7) at the molecular level seem to inhibit progression of tumours by inhibiting proliferation, migration, invasion, stroma formation, neovascularisation and induce sensitivity to chemotherapeutic agents. Autoradiography has also demonstrated high expression levels of the ET_A receptor in tumour sections, therefore being an abundant and probable key target. With ZD4054 (Zibotentan) being shown to be clinically safe in clinical trials and interacting with the EGFR pathway, currently used in colorectal cancer therapy, it is a promising agent to investigate in the clinical setting. This may well be in an adjuvant chemotherapeutic setting.

In summary, within this body of work, Zibotentan reduces tumourogenic effects in both epithelial and stromal environments. It has been shown at mRNA and protein levels in fibroblasts to block CTGF (heavily associated with angiogenesis, adhesion, migration, proliferation and anti-apoptosis), ADM (associated with proliferation, angiogenesis and vasodilatation) and to reverse effects of STC-1 (loss within MSL 8p in advanced CRC). Within CRC cells, MT1X is inhibited by Zibotentan. MT1X is heavily linked to cell proliferation and chemo-resistance via p53 inactivation and heavy metal chemotherapy scavenging. MMP7 which is highly specific for CRC cells (90% expression) is also inhibited, therefore reducing metastatic capabilities via ECM degradation and cleavage of Laminin 5 – the latter reducing cell adhesion, aiding cell migration. As MMP7 cleaves Fas receptors thereby inhibiting caspase apoptotic mechanisms, Zibotentan will also reduce chemoresistance and induce apoptosis in the presence of chemotherapeutic drugs. The tumour suppressor PPP2R5D is lost in CRC and effects reversed by Zibotentan. This leads to reduced MAPK signalling, increased cell cycle checkpoint activity and nuclear telomerase activity, all leading to decreased proliferation and potentially increasing apoptosis in hostile environments. It has also been shown in this thesis that Zibotentan is highly specific for the ET_AR and binds on both CRC cells and fibroblasts, affecting both epithelial and stromal compartments of the tumour.

As the safety profile has already been proven for Zibotentan via a number of clinical trials, there is no reason why it cannot be used in CRC patients. The work described here has demonstrated a considerable number of mechanisms by which Zibotentan acts to reduce CRC advancement, therefore it seems a hugely promising drug to use in the clinical setting. Furthermore, with its added anti-metastatic and anti-chemoresistant mechanisms, it would make sense for use at an earlier stage of treatment in CRC and as adjuvant therapy along with current treatment, whether chemotherapeutic or biological. It should be endeavoured to use this drug in phase 1-2 clinical trials as adjuvant to either FOLFOX/FOLFIRI or

Cetuximab. Further combination with nano-particles such as quantum dots may be an interesting step forward with potential for targeted combined therapy and imaging, but requires further research and evaluation.

Chapter 7

Prizes, Presented and Published Work Arising from this Thesis

PRIZES

BJS Prize:

Winner of British Journal of Surgery prize at the British Association of Surgical Oncology (BASO) & Association of Cancer Surgeons (ACS). Two oral presentations selected for the BJS Prize Session,

Royal College of Surgeons of England, UK; 2011

Young Investigators Award at ET-12:

Twelfth International Conference on Endothelin.

Cambridge University, UK; 2011

PUBLICATIONS

Haque S*, Dashwood M*, Heetun M, Shiwen X, Farooqui N, Welch H, Savage FJ, Ogunbiyi O, Abraham DJ, Loizidou M. **Efficacy of the specific endothelin A receptor antagonist Zibotentan (ZD4054) in colorectal cancer: a preclinical study.** (*Joint first authors). *Molecular Cancer Therapeutics*. *Published OnlineFirst, doi: 10,1158/1535-7163. MCT-12-0975; May 30, 2013*

Haque S, Welch H & Loizidou M. **The Endothelin Peptide.** Book chapter for *The Handbook of Biologically Active Peptides. 2nd Edition; Chapter 70. ISBN: 978-0-12-385095-9, 2013*

Haque S. Endothelin-1 and future colorectal cancer research. *Invited article summarising ET-1 and colorectal cancer research. BASO Yearbook 2012.*

Haque S, Morton D, Welch H, Loizidou M. **Biologics against cancer-specific receptors - challenges to personalised medicine from early trial results.** *Review article, Invited review. Current Opinions in Pharmacology; 12 (4): 392-397, 2012*

Knowles JP, Shiwen X, **Haque S**, Bhalla A, Dashwood MR, Yang S, Taylor I, Winslet MC, Abraham DJ, Loizidou M. **Endothelin-1 stimulates colon cancer adjacent fibroblasts.** *Int. J. Cancer: 130; p1264-p1272, 2011*

Heetun M, **Haque S**, Loizidou M. **p53 at a Glance: The Story so Far.** *OncologyNew Volume 4 Issue 2 May/June; 49-51, 2009*

Haque S, Heetun M, Ogunbiyi G. **Surgical Management for Inflammatory Bowel Disease: Implications for Bowel Cancer** *OncologyNews Volume 4 Issue 1: March/April; 20-22, 2009*

Bhalla A, **Haque S**, Taylor I, Winslet M, Loizidou M, **Endothelin Receptor Antagonism and Cancer.** *Eur J Clin Invest; 39 (S2): 74-77, 2009*

Haque S, Bhalla A, Winslet M, **Clinical Management of Familial Adenomatous Polyposis (FAP) and Lynch Syndrome (Hereditary Non-Polyposis Colon Cancer – HNPCC).** *Oncology News Volume 3 Issue 3: October/November, 2008*

ABSTRACT PUBLICATIONS

Haque S, Loizidou M, Dashwood M, Shiwen X, Abraham D, Welch H. **Novel Molecular Pathways by which ET_A Receptor Mediates Tumourogenic Signals in Colorectal Cancer: Support for ET_A Receptor Antagonism as Adjuvant Treatment.**
Accepted to ET-13 Tokyo, 2013

Haque S, Ramesh B, Welch H, Abraham D, Ogunbiyi O, Loizidou M, Dashwood M. **The Localisation and Distribution of Endothelin Receptors in Normal and Cancer Colon Tissue: Confirmation by Autoradiography, Immunohistochemistry and Quantum Dot Targeting.** *Accepted to ET-13 Tokyo, 2013*

Haque S, Loizidou M, Shiwen X, Abraham D, Winslet M, Welch H. **Identification of Endothelin-1 stimulated genes in colon cancer cells and fibroblasts and the effect of Endothelin receptor antagonism on their regulation.** *EJSO; 37 (11): p1012, 2011*

Haque S, Dashwood M, Welch H, Ogunbiyi O, Loizidou M. **Characterizing colorectal cancer binding sites for Endothelin-1 and antagonistic action by the Endothelin A receptor antagonist Zibotentan (ZD4054).** *EJSO; 37 (11): p1011, 2011*

Goodyear G, **Haque S**, Bartnik A, Loizidou M, Welch H. **The E_2, ERα, EFP and 14-3-3δ pathway mediates breast cancer cell proliferation.** *EJSO; 37 (11): p1006, 2011*

Haque S, Welch H, Dashwood M. Shiwen X, Abraham D, Heetun M, Farooqui N, Taylor I, Winslet M, Loizidou M, **Endothelin-1 stimulated gene induction within colonic cancer**

cells and fibroblasts and the effect of Endothelin receptor antagonism on key genes. *ET-12 conference & published in the BPS, 2011*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Winslet M, Ogunbiyi O, Dashwood M. **Characterisation of Endothelin-1 binding sites in colorectal cancer and antagonistic action of the Endothelin A receptor antagonist Zibotentan (ZD4054).** *ET-12 conference & published in the BPS, 2011*

Haque S, Welch H, Dashwood M. Shiwen X, Abraham D, Heetun M, Farooqui N, Taylor I, Winslet M, Loizidou M, **Endothelin-1 stimulated gene induction within colorectal fibroblasts and the effect of endothelin receptor antagonism on key genes.** *Brit J of Surg;98(S2):6-39 pp38, 2011*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Winslet M, Ogunbiyi O, Growcott J, Dashwood M. **Characterisation of endothelin-1 binding sites in colorectal cancer and antagonistic action of the endothelin A receptor antagonist Zibotentan (ZD4054).** *Brit J of Surg;98(S2):6-39 pp36, 2011*

Haque S, Winslet M, Loizidou M, Taylor I. **Endothelin receptors as an emerging target in colorectal cancers.** *Therapeutic Strategies-Oncology, (Review article – currently as ebook*: http://viewer.zmags.com/publication/8da7a9fc#/8da7a9fc/86*), Pg86-91, 2011*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Ogunbiyi O, Growcott J, Dashwood M. **Localisation and characterization of ET-1 binding to human colorectal cancers and evaluation of the orally active ETA receptor antagonist Zibotentan (ZD4054).** *AACR Conference, Berlin, Germany, 2010. Abstract Number 161. http://www.ecco-org.eu/Conferences-and-Events/Past-events/2010-Past-Events/EORTC-NCI-AACR-2010/Abstracts-online/page.aspx/2528*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Ogunbiyi O, Growcott J, Dashwood M. **Localisation and characterization of ET-1 binding to human colorectal cancers and evaluation of the orally active ETA receptor antagonist Zibotentan (ZD4054).** *NCRI conference 2010; 7-10 Nov; Liverpool, UK. NCRI 2010 Abstract LB89: http://www.ncri.org.uk/ncriconference/2010abstracts/abstracts/LB89.htm*

Heetun M, **Haque S**, Farooqui N, Dashwood M, Winslet M, Shiwen X, Abraham D, Loizidou M. **Efficacy of the specific ETA receptor antagonist zibotentan (ZD4054) in cancer cells and fibroblasts from colorectal cancer.** *Brit J of Surg; 97(S6):7-38 pp16, 2010*

Pimenta D, **Haque S**, Heetun M, Dashwood M, Shiwen X, Farooqui N, Abraham D, Loizidou M. **The Endothelin Axis in Drug Sensitive and Multidrug Resistant Bladder Cancer Cells.** *Brit J of Surg; 97(S6):7-38 pp27, 2010*

Haque S, Heetun M, Farooqui N, Loizidou M, Dashwood M, Shiwen X, Abraham D. **Efficacy of the Specific ETA Receptor antagonist Zibotentan (ZD4054) in Cancer Cells and Fibroblasts from Colorectal Cancer.** *Mol. Cancer Ther; 8(12 Suppl):B190, 2009*

Haque S, Heetun M, Taylor I, Shiwen X, Winslet M, Loizidou M. **Efficacy of the Specific ETA Receptor Antagonist Zibotentan in Colorectal Cancer Cells and Colorectal Fibroblasts.** *EJSO; 35 (11): 1206, 2009*

PRESENTATIONS

Haque S, Loizidou M, Dashwood M, Shiwen X, Abraham D, Welch H. **Novel Molecular Pathways by which ET_A Receptor Mediates Tumourogenic Signals in Colorectal Cancer: Support for ET_A Receptor Antagonism as Adjuvant Treatment.** *Accepted to ET-13 Tokyo, 2013*

Haque S, Ramesh B, Welch H, Abraham D, Ogunbiyi O, Loizidou M, Dashwood M. **The Localisation and Distribution of Endothelin Receptors in Normal and Cancer Colon Tissue: Confirmation by Autoradiography, Immunohistochemistry and Quantum Dot Targeting.** *Accepted to ET-13 Tokyo, 2013*

Haque S, Loizidou M, Shiwen X, Abraham D, Winslet M, Welch H. **Identification of Endothelin-1 stimulated genes in colon cancer cells and fibroblasts and the effect of Endothelin receptor antagonism on their regulation.** *BJS Prize session oral presentation, BASO 2011.*

Haque S, Dashwood M, Welch H, Ogunbiyi O, Loizidou M. **Characterizing colorectal cancer binding sites for Endothelin-1 and antagonistic action by the Endothelin A**

receptor antagonist Zibotentan (ZD4054). *BJS Prize session oral presentation, BASO 2011.*

Goodyear G, **Haque S**, Bartnik A, Loizidou M, Welch H. **The E_2, ERα, EFP and 14-3-3δ pathway mediates breast cancer cell proliferation.** *Poster Presentation, BASO 2011.*

Haque S, Welch H, Dashwood M. Shiwen X, Abraham D, Heetun M, Farooqui N, Taylor I, Winslet M, Loizidou M, **Endothelin-1 stimulated gene induction within colonic cancer cells and fibroblasts and the effect of Endothelin receptor antagonism on key genes.** *ET-12 international conference oral presentation, Cambridge 2011*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Winslet M, Ogunbiyi O, Dashwood M. **Characterisation of Endothelin-1 binding sites in colorectal cancer and antagonistic action of the Endothelin A receptor antagonist Zibotentan (ZD4054).** *ET-12 international conference poster presentation, Cambridge 2011*

Haque S, Welch H, Dashwood M. Shiwen X, Abraham D, Heetun M, Farooqui N, Taylor I, Winslet M, Loizidou M, **Endothelin-1 stimulated gene induction within colorectal fibroblasts and the effect of endothelin receptor antagonism on key genes.** *Oral Presentation, SARS 2011*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Winslet M, Ogunbiyi O, Growcott J, Dashwood M. **Characterisation of endothelin-1 binding sites in colorectal cancer and antagonistic action of the endothelin A receptor antagonist Zibotentan (ZD4054).** *Oral Presentation, SARS 2011.*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Ogunbiyi O, Growcott J, Dashwood M. **Localisation and characterization of ET-1 binding to human colorectal cancers and evaluation of the orally active ETA receptor antagonist Zibotentan (ZD4054).** *Poster presentation for NCRI, 2010.*

Haque S, Loizidou M, Shiwen X, Abraham D, Farooqui N, Welch H, Ogunbiyi O, Growcott J, Dashwood M. **Localisation and characterization of ET-1 binding to human colorectal cancers and evaluation of the orally active ETA receptor antagonist Zibotentan (ZD4054).** *Poster presentation for AACR, 2010.*

Heetun M, **Haque S**, Farooqui N, Dashwood M, Winslet M, Shiwen X, Abraham D, Loizidou M

Efficacy of the specific ETA receptor antagonist zibotentan (ZD4054) in cancer cells and fibroblasts from colorectal cancer. *Oral Presentation, SARS 2010*

Pimenta D, **Haque S**, Heetun M, Dashwood M, Shiwen X, Farooqui N, Abraham D, Loizidou M. **The Endothelin Axis in Drug Sensitive and Multidrug Resistant Bladder Cancer Cells.**

Oral Presentation, SARS 2010

Haque S, Heetun M, Farooqui N, Loizidou M, Dashwood M, Shiwen X, Abraham D. **Efficacy of the Specific ETA Receptor antagonist Zibotentan (ZD4054) in Cancer Cells and Fibroblasts from Colorectal Cancer.** *Poster Presentation at AACR International Conference, Boston. 2009*

Heetun M, **Haque S**, Farooqui N, Dashwood M, Winslet M, Shiwen X, Abraham D, Loizidou M. **Efficacy of the specific ETA receptor antagonist zibotentan (ZD4054) in cancer cells and fibroblasts from colorectal cancer.** *Oral presentation at ET-11 Conference, Canada. 2009*

Pimenta D, **Haque S**, Heetun M, Dashwood M, Shiwen X, Farooqui N, Abraham D, Loizidou M. **The Endothelin Axis in Drug Sensitive and Multidrug Resistant Bladder Cancer Cells.** *Poster presentation at ET-11 Conference, Canada. 2009*

Haque S, Heetun M, Taylor I, Shiwen X, Winslet M, Loizidou M. **Efficacy of the Specific ETA Receptor Antagonist Zibotentan in Colorectal Cancer Cells and Colorectal Fibroblasts.** *Oral presentation, BASO, 2009*

Chapter 8

Appendices

APPENDIX 1:

Alamar Blue Proliferation Colorimetric Assay

Purpose: Pilot experiments were carried out to determine optimal conditions (seeding cell concentration; time of incubation with the dye) for the Alamar Blue colorimetric assay for cell growth.

Method: Cells at different concentrations made in serum containing medium were seeded in 24 well plates and left overnight at 37°C, 5%CO_2. Medium was replaced with serum free medium and growth assessed at 48 hours as described in the methods. Alamar Blue was incubated at either 1 hour or 4 hours to determine optimal incubation time.

Results: Incubation at either 1 hour or 4 hours gave near identical results (figure A.1) and therefore 1 hour incubation was chosen for subsequent experiments, for convenience. Cell seeding concentrations from 0.5 – 2.0 x 10^5 resulted in a linear growth curve which plateaued thereafter. Since the purpose of this assay is to determine further growth advantages or decreases stimulated by ET-1 and various antagonists, the dose of 1 x 10^5 cells per well, which lies approximately half way up the linear growth curve, was deemed to be the most appropriate for further experimentation.

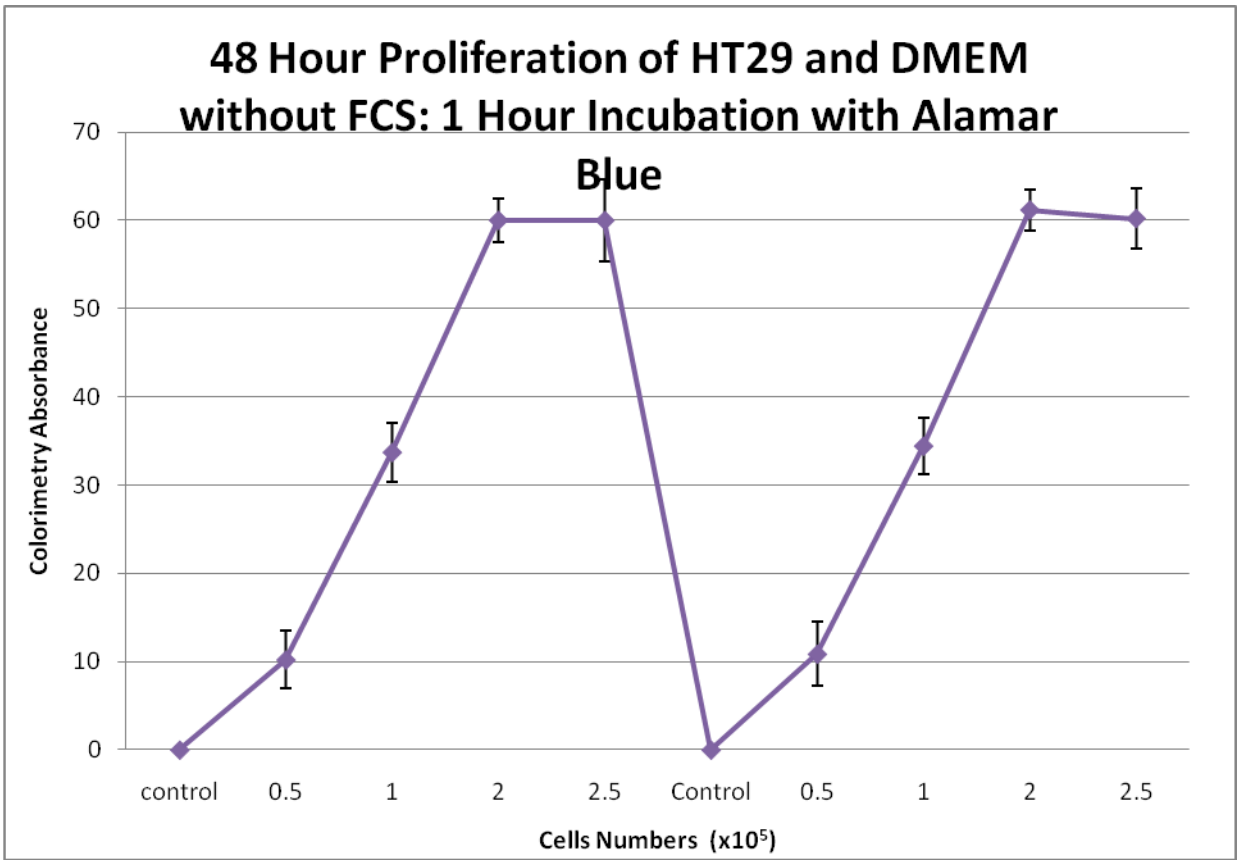


***Figure A.1**: Proliferation of HT29 colorectal cancer cells measured by Alamar Blue colorimetric assay. Cell growth at 48 hours for various cell concentrations seeded (X-axis) was determined by Alamar Blue and measured at 650nm absorbance (Y-axis). The dye was incubated for 1 hour. Similar results were produced at 4 hours of incubation and therefore not shown here. The experiment was carried out twice using quadruplicate wells for each time point.*

APPENDIX 2

Methylene Blue Assay for Proliferation Assays

Reagents

1. Normal Saline: 8.5g NaCl in 1L of distilled water, Formal Saline: 10% formaldehyde in normal saline
2. Methylene Blue: 1% methylene blue; 1g in 100mls of borate buffer
3. Borate Buffer (0.01 M): 38.14g of disodium tetraborate in 5L of distilled water; pH adjusted with 1M HCL to a pH of 8.5
4. Elutant: 0.1M HCL : Ethanol (1:1 ratio)

Protocol:

1. 96 well plates were thoroughly washed with normal saline and patted dry on tissue paper
2. Cells were fixed with 100µl/well of 10% formal saline for a minimum of 30 minutes
3. Formal saline was discarded and the plates patted dry on tissue paper.
4. 100µl of filtered methylene blue was added to each well and left for at least 30 minutes.
5. Six baths were filled with borate buffer and the plates were washed in each bath in turn.
6. Excess buffer was shaken off the plates and patted dry on tissue paper.
7. To each well, 100µl of elutant was added and the plates placed on a plate shaker for 1 hour
8. The absorbance was read at 650nm on a plate reader. To determine background absorbance, one well was left with elutant only. The absorbance level from this well was subtracted from the remaining plate readings.

APPENDIX 3

Floating Collagen Gels

Reagents

1. 2% Bovine albumin (Sterile): 1g 98% bovine albumin (Sigma-Aldrich) in 50mls
2. Type 1 collagen: Rat tail collagen, type 1 (First Link (UK) Ltd)
3. HEPES buffer: 0.2m 4-(2hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer, adjusted to pH 8 with sodium hydroxide

Protocol

1. Using 24 well plates, 2mls of 2% bovine serum albumin were added to each well.
2. The plates were incubated in a humidified incubator at 37°C for 24 hours.
3. The albumin solution was discarded from the plates and the plates were washed three times with sterile PBS
4. Collagen suspension was made using 40% collagen, 10% HEPES buffer and 50% serum free medium.
5. 100,000 fibroblasts/ml were added to the solution.
6. Anatagonists were added to collagen/cell suspension as necessary for pre-incubation.
7. 1ml of collagen solution was added to each well.
8. The plates were incubated for 1 hour at 37°C or longer until the gel had solidified.
9. The gels were floated by the addition of 1ml of ET-1

APPENDIX 4

Protocol for Antagonistic work

1. Grow cells in 6 well plates. Cancer cell line seeding concentrations vary from 150,000 to 300,000 cells per well.

2. Cells grown until 50-60% confluent

3. Serum starvation for 12-18 hours in serum free medium for cancer cell lines and 0.5% BSA for fibroblasts.

4. Cells are then pre-incubated with antagonists alone at the appropriate concentration for 1 hour in serum free medium

5. Media is then removed and medium with antagonists and/or ET-1 are added to relevant wells. Serum free media or 0.5% BSA medium is added to the control well.

6. RNA extraction: Follow protocol for RNA extraction at 4 hours.

7. Protein extraction: Follow protein extraction protocol at 24 hours.

Protocol for Silencing (SiRNA)

1. Grow cells in 6 well plates. Cancer cell line seeding concentrations vary from 150,000 to 300,000 cells per well. Fibroblasts are grown at 20,000-50,000 cells per well. All media is antibiotic free.

2. Cells grown until 40% confluent.

3. Change to serum free media containing 4µ oligofectanime and a final concentration of 25nM SiRNA is added to wells.

4. Following 24 hours incubation, this is changed to 10% FCS containing medium for a further 24 hours.

5. Cell are then serum starved for 12-18 hours

6. ET-1 at the appropriate concentration for cancer cell lines or fibroblasts is then added to stimulate cells.

8. RNA extraction: Follow protocol for RNA extraction at 4 hours.

9. Protein extraction: Follow protein extraction protocol at 24 hours.

APPENDIX 5

Total Protein Extraction

Protocol:

Once cells have grown within 6 well plates with their respective media, they are ready for trypsinization.

1. Each well is washed with 5ml PBS then suctioned out from the corner of the well. They are then washed with 5mls PBS EDTA for 2 minutes followed again by suction.
2. 500µl of Trypsin is added and incubated for 3 minutes at 37C. This is then neutralised with 5mls DMEM containing 10% FCS. Each well is transferred to a separate Universal Tube. Each well is further washed with 5ml of media and transferred to their respective Universal Tube.
3. Spin each tube at 1500rpm, brake 2 for 5 min. The media is tipped out, cells re-suspended 10mls PBS then spun down again, discarding the PBS after this.
4. Cells are transferred to an eppendorf with 2x 500µl of PBS then spun down at 4C for 5 minutes. Pipette the PBS leaving the cells at the bottom.
5. Add 200µl RIPA Buffer to each eppendorf.
6. Vortex for 15 minutes with intermittent breaks.
7. Centrifuge down for 15 minutes at 4C.
8. Pipette 50µl of supernatant into each eppendorf (total of 4 for each sample) leaving pellet at bottom and freeze down for protein analysis at -20C.

Protocol (Modified 1):

Once cells have grown within 6 well plates with their respective media, they are ready for trypsinization.

1. Each well is washed twice with 5ml PBS then suctioned out from the corner of the well.
2. 300µl of RIPA buffer is added to each well and cells scrapped off using a cell scrapper. This is pipette into an eppendorf for each well.
3. Vortex for 15 minutes with intermittent breaks.
4. Centrifuge down for 15 minutes at 4C.
5. Pipette the supernatant into eppendorfs and store ready for Western blot analysis.

Protocol (Modified 2):

Once cells have grown within 6 well plates with their respective media, they are ready for trypsinization.

1. Each well is washed twice with 5ml PBS then suctioned out from the corner of the well.
2. To each well is added between 100µl - 200µl (depending on confluence of cells) of solution containing: 60µl RIPA Buffer, 20µl Lamella Buffer (8x) and 20µl Urea (8M).
3. Cells were scrapped off using a cell scrapper and pipette into an eppendorf for each well.
4. Vortex for 15 minutes with intermittent breaks.
5. Store at -20C to be used for Western blotting. Samples will need spinning prior to running on gels.

APPENDIX 6

Determining Protein Concentration

Material/Reagents:

1. Modified Lowry Protein Assay Kit
2. PBS (1 tablet PBS (Sigma-P4417) in 200ml Distilled H2O
3. Prepare 1X Folin-Ciocalteu reagent by mixing 1ml of the supplied 2X concentration in a 1:1 ratio with 1ml of ultra pure water.

Protocol:

1. Prepare the following eppendorfs:
    a. Label 10 eppendorfs A-J which will contain Standard Albumin Concentrations.
    b. Labelled eppendorfs A2-J2.
    c. Labelled eppendorfs for each protein sample 'S1x10, S2x10, S3x10....etc'
    d. Labelled eppendorfs for each protein sample 'S1x100, S2x100, S3x100...etc'
2. To the labelled eppendorfs A-J make up and add the Standard Albumin Concentrations (as below).
3. To the S1x10, S2x10 etc. Tubes add: 20µl Protein sample for determination and 180µl PBS.
4. To the S1x100, S2x100 etc. Tubes add: 10µl Protein sample for determination and 990µl of PBS.
5. To the A2-J2 eppendorfs add: 1ml of the Modified Lowry Protein Assay Reagent and 200µl of the corresponding A-J tube (containing Standards) to each A2-J2 eppendorf.
6. Now add 200µl of the Modified Lowry Protein Reagent into each of the eppendorf tubes labelled S1x10 etc and S2x100 etc.
7. Vortex all tubes for a few seconds and leave to incubate at room temperature for 10 minutes.
8. Add 100µl of 1X Folin-Ciocalteu reagent to each eppendorf A2-J2, all samples S1x10 etc and all samples S2x100 etc.
9. Vortex and incubate at room temperature for 30 minutes.
10. Samples were then read using the Spectrophotometer (read at 750nm) to determine the protein concentrations.

APPENDIX 7

Polyacrylamide Gel for SDS-PAGE for Western Blotting

Materials/Reagents:

1. Distilled Water (dH2O)
2. 30% Acrylamide Mix: Solution from ProtoFLOWGel (Flowgen, Findel House, Leicestershire)
3. 1.5M Tris (pH 8.8): ProtoGel Resolving Buffer (National Diagnostics, 305 Patton Drive, Atlanta, 30336 USA)
4. 10% SDS and 10% AP: 10g Sodium Dodecyl Sulphate in 100ml water with 10% Ammonium Persulphate
5. TEMED: N,N,N,N-Tetramethylethylenediamine

Protocol:

Make up 30ml of 10% solution and 20ml of 6% solution for upper cassette.

1. To make 30ml of 10% mix: 11.9ml dH2O, 10ml of 30% Acrylamide, 7.5ml Tris, 0.3ml SDS with AP and 0.012ml of TEMED just before pouring so gel not hardens before in cassette.

2. Use manual pipette to pour into gel cassettes (Invitrogen Life Tech) up to ¾ level.

3. Add 2ml Butanol with manual pipette to remove bubbles.

4. Allow to stand for 1 hour to set.

5. Tip out Butanol and wash upper cassette well with distilled water, tip out and dry with filter paper.

6. To make 20ml of 6% mix: 10.6ml dH2O, 4ml of 30% Acrylamide, 5ml of Tris, 0.2ml of SDS and AP, and 0.016ml of TEMED (added just before pouring into cassettes.

7. Fill to the upper limit then insert well comb at top and ensure no bubbles by adding additional mix with manual pipette.

8. Then transfer to fridge 4 degrees Celsius overnight.

APPENDIX 8

Western Blotting : Electrophoresis of Protein in Polyacrylamide Gel (Samples used from modified protein extraction method 3)

Materials/Reagents:

1. Running Buffer (100mls TrisGlycine Gel (10X TGF) and 900mls dH2O)
2. 2-Metacaptoethanol in a fume cupboard.

Protocol:

1. Take 200µl protein sample in eppendorfs and add 10µl of 2-Metacaptoethanol. Then leave in water bath at 95 C for 5 min.
2. Spin down samples for 5 minutes to remove cell debris so supernatant can be aspirated to load gel columns.
3. Make up Running Buffer.
4. Slightly remove well comb and remove white strip from cassette. Assemble electrophoresis apparatus and insert cassette, finally removing the comb completely.
5. Pour Running Buffer into middle compartment around cassette until filled to top and inside wells. Pour distilled water to lower marker in outside compartments of apparatus.
6. Add 10µl of ladder and 15µl of protein sample to wells.
7. Run gels for 90 minutes at 125V, topping up buffer if needed.

Western Blotting: Protein Transfer

Materials/Reagents:

1. Transfer Buffer (40ml TrisGlycine Transfer Buffer 25X (Novex, Invitrogen), 120ml Methanol (BDH) and dH2O up to 1 litre volume

Protocol:

1. Cut 1 nitrocellulose membrane and 2 filter papers per gel.
2. Soak 4 sponges per gel in transfer buffer removing any bubbles from the sponges.

3. Remove the gel from the cassettes by incising the gel where the white tape was and opening the cassette around the edges.
4. Stack from bottom to top as follows: Sponge, filter, nitrocellulose membrane, gel, filter paper then sponge. Two gels per stack can be used in the same order as above.
5. Pack into blotting module with membrane facing anteriorly.
6. Cover with transfer buffer centrally.
7. Surround central well with cold dH2O.
8. Run at 30V for 90 minutes.

Western Blot: Antibody Labelling Membranes

Materials/Reagents:

1. PBS-Tween: 20 tablets PBS in 2 Litres distilled water and 1ml Tween 20
2. 5% Milk blocker: 20g dried milk powder (Marvel) and 400ml PBS-Tween (above).

Protocol:

1. Place nitrocellulose paper into a weighing boat and add 25ml milk blocking solution and rock for 1 hour.
2. Wash 3x with PBS-Tween for 10 minutes each wash.
3. Make Primary Antibody solution with 15µl antibody with 15ml milk solution (1:1000).
4. Remove PBS-Tween and replace with antibody solutions. Leave overnight in fridge 4 C.
5. Wash off primary antibody with PBS-Tween 3x over a total of 45-60 minutes.
6. Make secondary biotinylated AB solution with 15µl antibody in 15ml milk solution. Rock at room temperature for 1 hour.
7. Wash 3x with PBS-Tween for 45-60 minutes.

8. Add 2mls reagent A and 2mls Reagent B into universal tube and mix. Drop mixture onto nitrocellulose membrane and leave to soak for 5 minutes at room temperature on rocker.

Western Blotting: Development of Image

Protocol 1:

1. Place the nitrocellulose membrane in cling-film onto the Hyperfilm cassette hard surface and tape down ensuring no air pockets.
2. In dark room open cassette and place film with one side folded into cassette and expose for 20 seconds to 10 minutes.
3. Develop film with exposure to developing medium, fixing medium and water washes. Then leave to dry.

Protocol 2:

1. Make up active Super-Signal (1ml bottle 1 and 1ml bottle 2) reagent.
2. Apply to Western blotting membrane
3. Visualise using Chemi-doc software

APPENDIX 9

Total RNA Extraction

Materials/Reagents:

1. RLT Buffer (1ml RLT + 10µl β-Mercaptoethanol)
2. RPE Buffer
3. RW1 Buffer
4. 2ml and 1.5ml collection tubes and RNAeasy columns
5. RNAase-free water

Protocol:

Once cells have grown within 6 well plates with their respective media, they are ready for trypsinization.

1. Each well is washed with 5ml PBS then suctioned out from the corner of the well. They are then washed with 5mls PBS EDTA for 2 minutes followed again by suction.
2. 500µl of Trypsin is added and incubated for 3 minutes at 37C. This is then neutralised with 5mls DMEM containing 10% FCS. Each well is transferred to a separate Universal Tube. Each well is further washed with 5ml of media and transferred to their respective Universal Tube.
3. Spin each tube at 1500rpm, brake 2 for 5 min. The media is tipped out, cells re-suspended 10mls PBS then spun down again, discarding the PBS after this.
4. Cells are transferred to an eppendorf with 2x 500µl of PBS then spun down at 4C for 5 minutes. Pipette the PBS leaving the cells at the bottom. (At this stage the pellet can be frozen down at -20C if needed prior to completing extraction).
5. Into the pellet, add RTL buffer according to the cell number (350µl if $<5x10^6$ cells or 600µl if $0.5\text{-}1x10^7$ cells). Vortex the tubes then further homogenize with a 20 gauge needle in the same eppendorf.
6. Add the same volume (as RTL buffer) of 70% Ethanol and vortex
7. Pipette 700µl of the sample to an RNAeasy mini column placed in a 2ml collection tube and vortex for 15 seconds at 10,000rpm. The collection tube overflow is discarded and the if any sample remains, this process is repeated discarding the overflow once again.
8. Add 700µl RW1 buffer to the RNAeasy column and vortexed for 15 seconds at 10,000 rpm. The overflow and collection tubes are discarded.
9. The RNAeasy column is placed into a new 2ml collection tube. Pipette 500µl RPE buffer into the column and discard the overflow. Add another 500µl RPE buffer to the column and centrifuge for 2 minute at 10,000 rpm.

10. Transfer the RNAeasy column to a new 1.5ml collection tube; pipette 50µl RNAase-free water onto the column silica-gel membrane. Centrifuge for 1 minute at 10,000rpm. Keeping the overflow, add 30µl of RNAase free water to the membrane and centrifuge for 5 minutes.
11. Dispose of the RNAeasy column keeping the collection tube of the RNA. This is now ready for analysis.

The was analysed using the Gene Spec 1 Program .

Protocol (Modified 1):

Once cells have grown within 6 well plates with their respective media, they are ready for trypsinization.

1. Each well is washed trice with 5ml PBS then suctioned out from the corner of the well.
2. Directly into each well is added RTL buffer according to the cell number (350µl if $<5x10^6$ cells or 600µl if $0.5\text{-}1x10^7$ cells).
3. A cell scrapper is used to scrap off wells which are pipette into individual eppendorfs for each well.
4. At this stage the samples can be stored at -80C before proceeding with total RNA extraction outlined below.
5. Vortex the tubes then further homogenize with a 20 gauge needle in the same eppendorf.
6. Add the same volume (as RTL buffer) of 70% Ethanol and vortex
7. Pipette 700µl of the sample to an RNAeasy mini column placed in a 2ml collection tube and vortex for 15 seconds at 10,000rpm. The collection tube overflow is discarded and the if any sample remains, this process is repeated discarding the overflow once again.
8. Add 700µl RW1 buffer to the RNAeasy column and vortexed for 15 seconds at 10,000 rpm. The overflow and collection tubes are discarded.
9. The RNAeasy column is placed into a new 2ml collection tube. Pipette 500µl RPE buffer into the column and discard the overflow. Add another 500µl RPE buffer to the column and centrifuge for 2 minute at 10,000 rpm.
10. Transfer the RNAeasy column to a new 1.5ml collection tube; pipette 50µl RNAase-free water onto the column silica-gel membrane. Centrifuge for 1 minute at 10,000rpm. Keeping the overflow, add 30µl of RNAase free water to the membrane and centrifuge for 5 minutes.

11. Dispose of the RNAeasy column keeping the collection tube of the RNA. This is now ready for analysis.

APPENDIX 10

Making Conventional PCR Gels

Apparatus:

1. Agarose Gel powder (A9539-500G)
2. Ethidium Bromide in 1xTBE Buffer
3. Cassette and comb to run samples
4. 250ml Conical Flask and spoon
5. Power supply and holder for Cassette

Method:

1. Make up running gel (1g Agarose powder; 70ml Ethidium Bromide and Buffer 1xTBE) in a 250ml conical flask.
2. Place on a hot plate (temp. setting 8 and stir rate 8) until solution clear and bubble free.
3. Securely apply tape to cassette ends and pour solution into the cassette. Place a comb for the sample wells at one end.
4. Cool for 30 minutes at room temp. then place in fridge for 30 minutes.
5. Take RNA samples and thaw at room temperature
6. Add colour dye to this in a ratio of just over 1/10 (i.e. 10µl sample add 1.2µl dye).
7. Place cassette into electrophoryses unit and top up with Ethidium Bromide to cover surface.
8. Remove comb and add 11-15µl of samples into wells along with a marker ladder.
9. Run gels at 50V. (Initially running at 100V to see bubbles in solution ensures circuit is running).

APPENDIX 11

Conventional RT-PCR

One step RT-PCR Qiagen kit (210212)

Make up the Reactants and Primers in separate reaction tubes. Total volume per reaction is 15µl.

Components	Volume per Reaction	Final Concentration
Tube 1 (Reactants)		
tRNA Template (e.g. HT29/CF56)	5µl	0.066µg/µl
40u/µl RNasin	0.375µl	1u/µl
5x Qiagen Buffer	3µl	x1
10mM dNTPs	0.6µl	400µM
RT-PCR	0.6µl	
RNA Free Water	0.425µl	**Total Tube 1: 10µl**
Tube 2 (Primers)		
10µM Sense Primer	0.9µl	0.6µM
10µM Anti-Sense Primer	0.9µl	0.6µM
RNA Free Water	3.2µl	**Total Tube 2: 5µl**

If 6 reactions required then make up 7 times the above quantities so enough reactant mixture for all reactions.

Protocol:

1. In an eppendorf for each reaction, pipette 3.2µl into each.
2. Pipette 0.9µl of Sense and 0.9µl of Anti-Sense Primers into each eppendorf.
3. Now make up the Template tRNA and place onto ice.
4. Make up the Core reagents (tube 1; except tRNA made in step 3 and the RNA Free Water).
5. Add Template tRNA to the Core reagents.

6. Add Primers (Tube 2) to the Core reagents.
7. Now add RT-PCR enzyme to the Reactant mixture.
8. The total Reactant mixture is placed into the PCR cycler.

One completed then the Reactants and product can be run on a gel to analyse.

APPENDIX 12

Real Time PCR Protocol

Quantitect Real Time PCR Qiagen kit

Make up the Reactants and Primers in separate reaction tubes. Total volume per reaction is 15µl.

Components	Volume per Reaction
Tube 1 (Reactants and Primers)	
PCR Mastermix	10µl
10µM Sense Primer	2µl
10µM Anti-Sense Primer	2µl
Quantitect Enzyme (added last)	0.2µl

Tube 2 (Template and Water)

Require 100ng of template (e.g. HT29) made up to 5.8µl for each reactant.

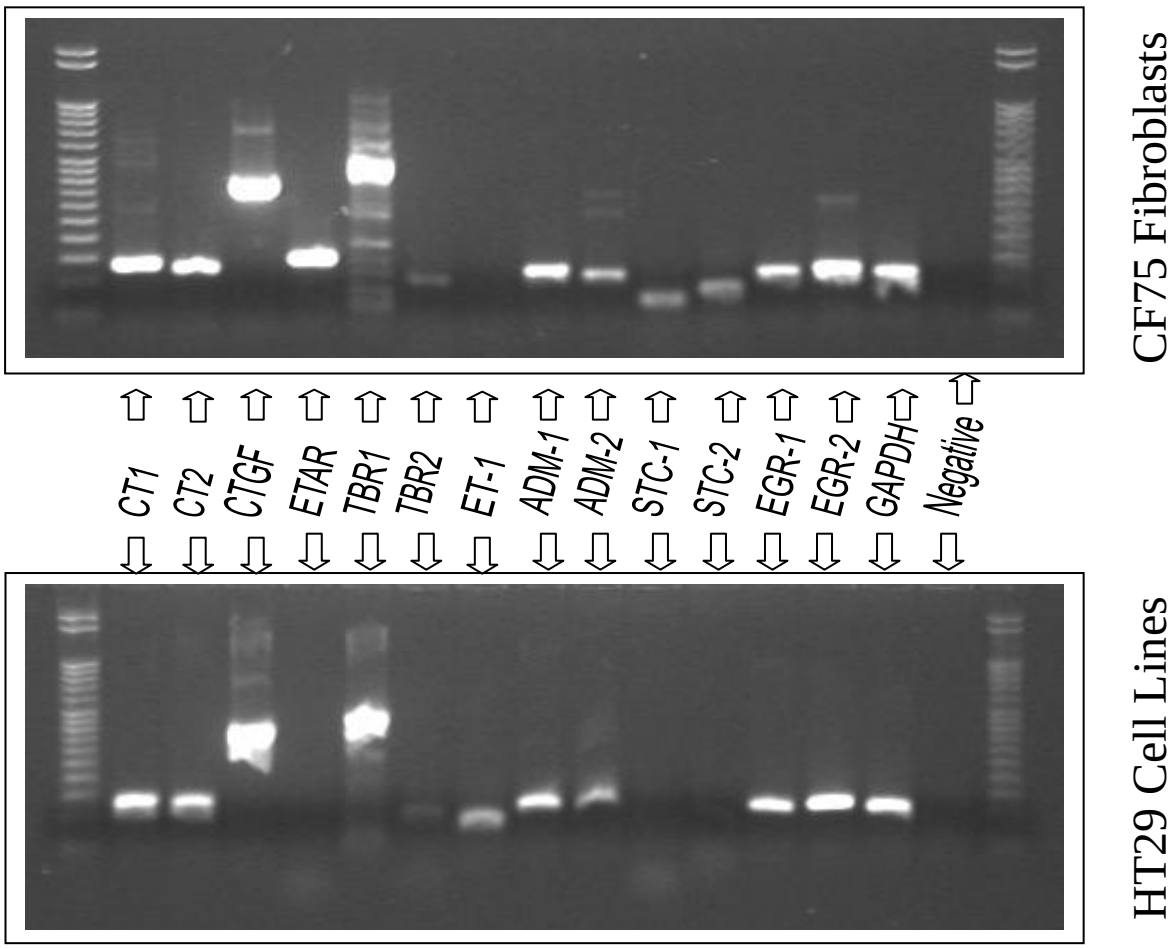
When calculating quantities, 10% excess should be calculating for each reactant.

Protocol:

1. Templates are made first in a reactant tube with water then stored on ice.
2. All reactants in tube 1 can be made up with the exception of the Quantitect enzyme, then stored on ice.
3. Reactants can be added to template tubes.
4. BEFORE PROCEDING: set up the Light cycler ready to start.
5. Quantitect enzyme can now be added to tubes when sitting on ice.
6. All contents can be added to individual reaction capillaries to go into the Light cycler.
7. All capillaries are added to the Light cycler carousel and the program started.

APPENDIX 13

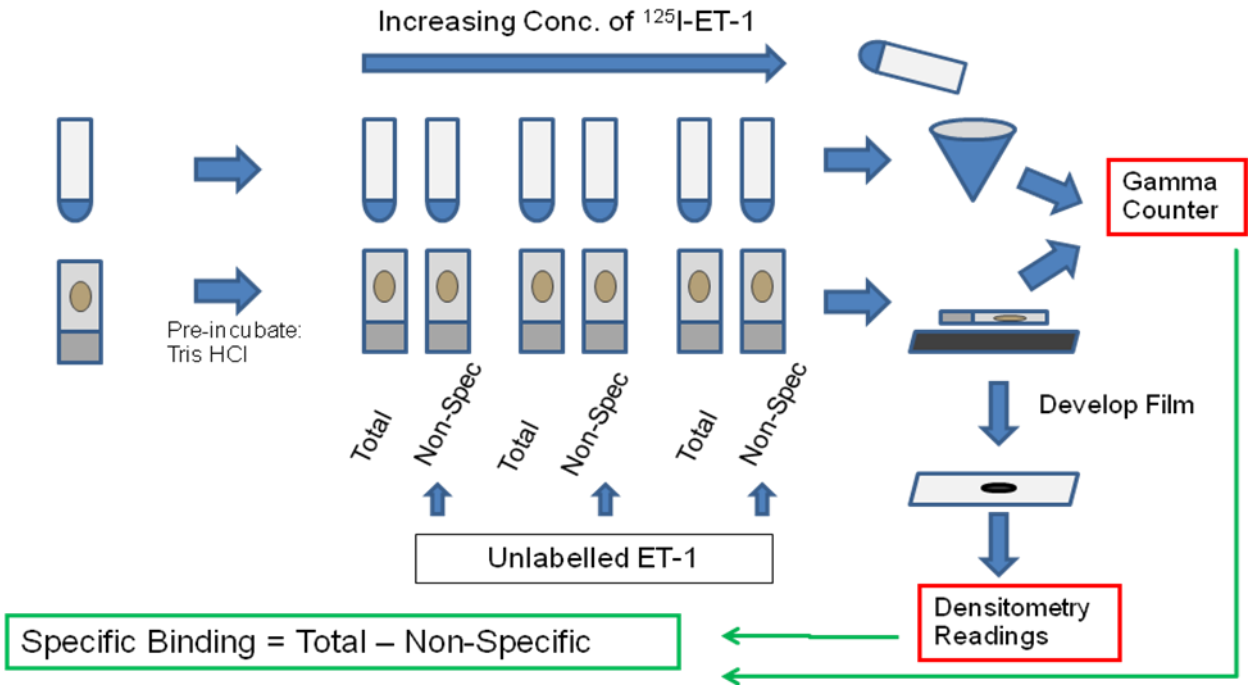
Initial Testing of All Primers



Appendix A.2. Conventional RT-PCR showing expression levels of all key genes within the Endothelin axis that were chosen to be examined in both fibroblasts and colorectal cancer cell lines. Primers were designed with annealing temperatures of 58°C and flanking introns to be RNA specific. Conventional RT-PCR was carried out as per standard conventional RT-PCR protocol using a Mastercycler Gradient thermal cycler (Eppendorf) up to 35 cycles. This confirmed the primers were working and enabled decision making as to which primers to use if more than one had been designed.

APPENDIX 14

Saturation Analysis: K_d/B_{max} Determination



Appendix A.3. Schematic diagram showing the method used to calculate specific binding and determining K_d and B_{max}.

Procedure

1. Unfixed cytospins and tissue homogenates were equilibrated to room temperature (~21^{o}C, 20min). A preliminary step was performed for cytospins, where slides were preincubated at room temperature in 50mM tris-HCl, pH7.4, 20 min, to reduce endogenous ET-1 levels.

2. Both were incubated with increasing ^{125}I-ET-1 concentrations (GE, Amersham, Bucks, UK, specific activity 2200 Ci/mmol: $3x10^{-12}$-10^{-9} M, [total binding]) in 50mM tris HCl buffer, pH7.4, 5mM $MgCl_2$, 0.2% bovine serum albumin, 100 iu/ml aprotinin, 120min.

3. This was followed by rinsing (2x10min) in 50mM tris-HCl, 4^{o}C.

4. Non-specific binding (NSB) was established by incubating in the presence of 1µM unlabelled ET-1.
5. After incubation:

    (A) slides were briefly dipped into distilled water, 4^{o}C, to remove salts in the buffer, and dried under a stream of warm air (~15min) followed by cool air (15min) and stored overnight. Slides were post-fixed under vacuum in paraformaldehyde vapour, 2h, at 80^{o}C.

    (B) Homogenates were filtered and washed three times with buffer under vacuum through a cellulose GF/B filters attached to 12-well manifold chambers (both Millipore, Watford, UK). Homogenate bound ^{125}I-ET-1 retained by the filter paper was measured to establish total and non-specific binding.

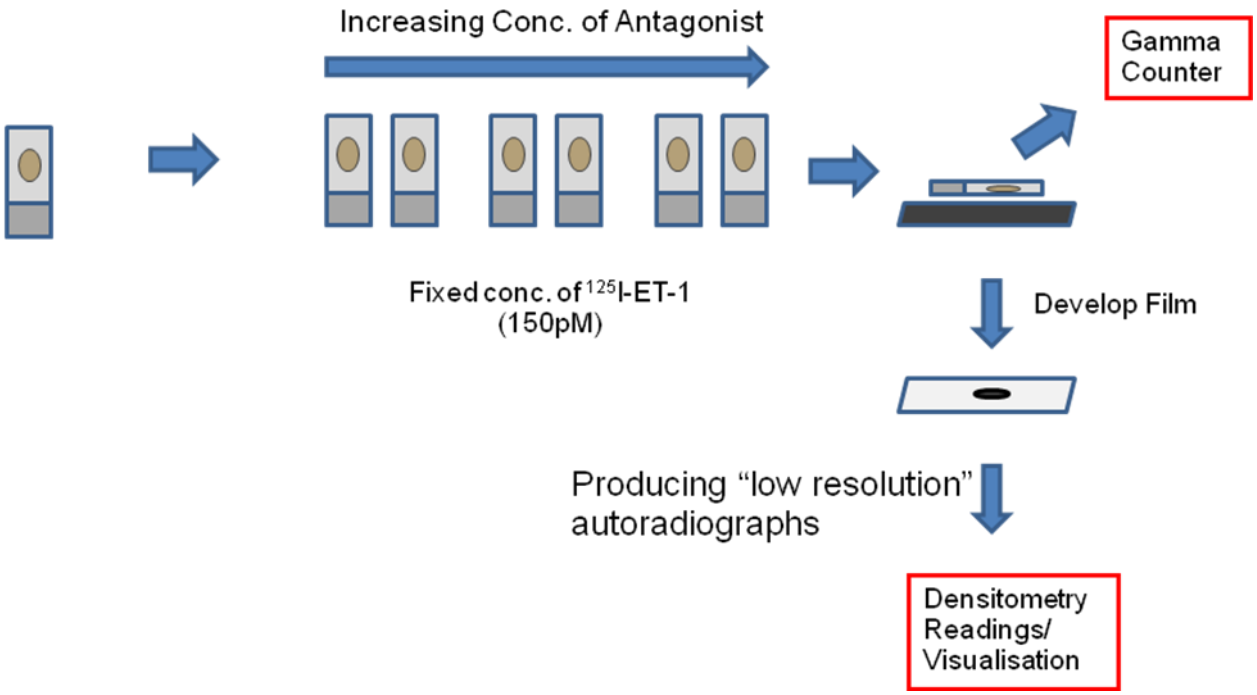
6. At the end of each set of incubations, ^{125}I scales were prepared where 50µl aliquots of each of the serial dilutions of radioligand were spotted onto filter paper or cellulose filters and then attached to microscope slides that were co-exposed to radio-sensitive film along with the cytospins.

7. Densitometric analysis of autoradiographic images 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 used.

8. Maximum receptor binding (B_{max}) and affinity (K_d) were obtained using GraphPad Prism™ software (GraphPad, Santa Barbara, CA).The same approach was used for analysing data from cells removed from slides with filter paper and measured in the gamma counter.

APPENDIX 15

Inhibition Analysis and Low Resolution Analysis



Appendix A.4. Schematic diagram showing the method used to analyse inhibition and produce low resolution autoradiographs.

Procedure

1. Unfixed cytospins and tissue homogenates were equilibrated to room temperature (~21^{o}C, 20min). A preliminary step was performed for cytospins, where slides were preincubated at room temperature in 50mM tris-HCl, pH7.4, 20 min, to reduce endogenous ET-1 levels.

2. Fixed ^{125}I-ET-1 concentrations (150pM; ~K_d value determined initially in the saturation studies) (GE, Amersham, Bucks, UK, specific activity 2200 Ci/mmol: 3x10^{-12}-10^{-9} M, [total binding]) in 50mM tris HCl buffer, pH7.4, 5mM $MgCl_2$, 0.2% bovine serum albumin, 100 iu/ml aprotinin, were made up and placed in individual tubes for increasing antagonist concentrations to be added.

3. Increasing concentrations ($3x10^{-9}$-$3x10^{-6}$M) of the ET_A receptor antagonists, BQ123, ZD4054, and the ET_B receptor antagonist BQ788 were made up and added to each tube containing ^{125}I-ET-1.

4. Solutions were added to Unfixed cytospins and tissue homogenates for 120 minutes.

5. Two fixed concentrations (high=25μM; low=5μM) for each antagonist were used for autoradiographs that were produced as described below.

6. Densitometric analysis of autoradiographic images 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).

7. Cells were removed from slides (NaOH digestion) with filter paper and activity measured in a gamma counter.

Low Resolution Autoradiographs:

1. Slide-mounted tissues and cytospins were placed in 24x30cm X-ray cassettes and apposed to Hyperfilm™MP (GE, Amersham) under dark-room conditions and exposure for 7-21 days, 4^{o}C.

2. In a dark-room, films were immersed in undiluted D19 developer (Kodak), 5min,

3. Briefly rinsed in tap water

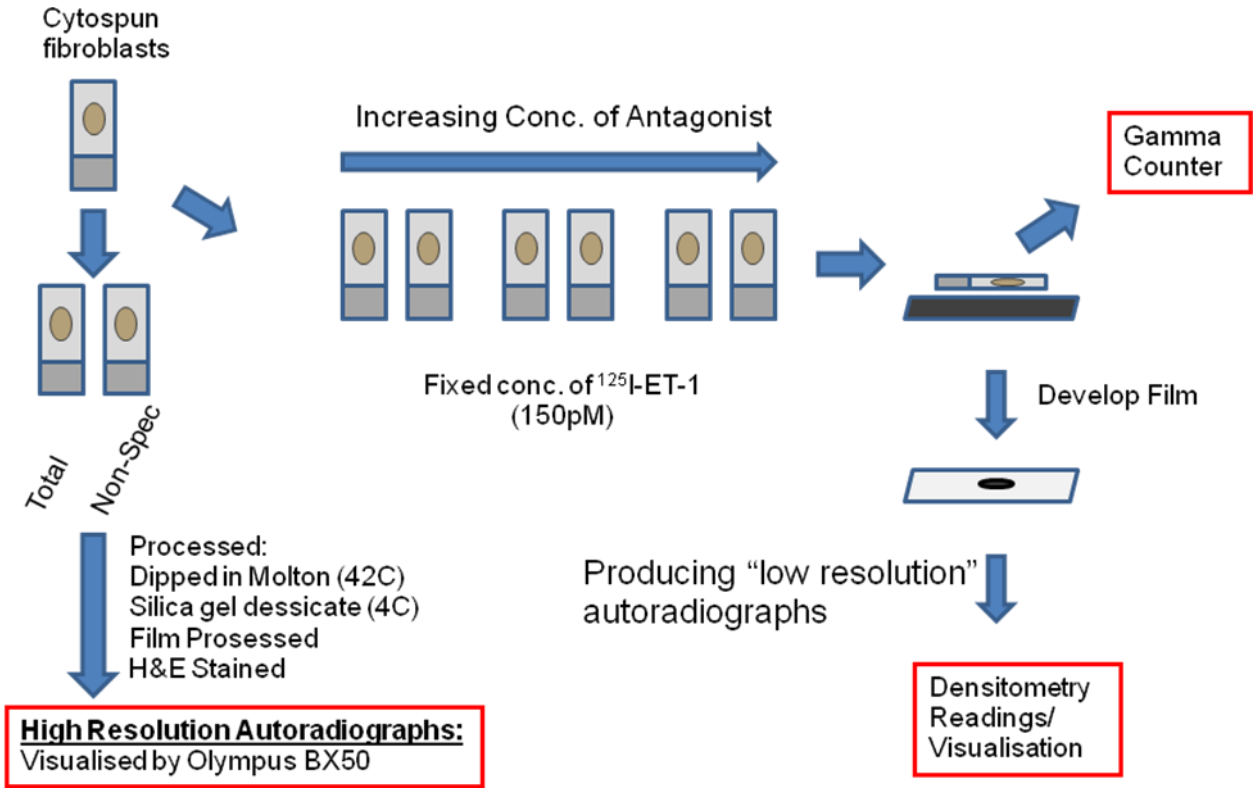
4. Immersed in Hypam™ fixative (Ilford, 1:3 in distilled water) for 5 minutes.

5. Washed in running tap water (~20minutes).

6. Films were dried and subsequently autoradiographs used for densitometry; representative images were photographed.

APPENDIX 16

IC_{50} Determination and Producing High and Low Resolution Autoradiographs



Appendix A.5. Schematic diagram showing the method used to analyse inhibition, IC50 and produce low and high resolution autoradiographs.

Procedure:

1. Calculation of IC_{50} was determined by carrying out the procedure as set out above for inhibition analysis.
2. Densitometry and gamma counter analysis was carried out as previously stated.
3. IC_{50} was determined by plotting graphs as set out in figures 5.18-5.19.

High Resolution Autoradiographs:

1. Tissues/cytospins were dipped in molten (42^{o}C) K2 emulsion (Ilford; 1:1 in 2% glycerol/distilled water) and allowed to dry overnight (dark-room).

2. Emulsion-coated slides were then placed in racks, stored in light-proof boxes containing silica gel dessicant (4^{o}C, 7-21 days).

3. For microautoradiographs, slides were immersed in D19 developer for 5 minutes.

4. Dipped in rapid stop solution (Ilford, 1:10 in distilled water).

5. Fixed in Hypam™ fixative (1:3 in distilled water, 10 minutes) and

6. Rinsed (3x10minutes) in distilled water.

7. Tissues/cells were stained with haematoxylin & eosin (H&E), dehydrated by immersion in increasing ethanol concentrations, de-waxed in Histo-clear™ (National Diagnostics, Hull, UK) and cover-slipped using DPX.

8. Slides were viewed under an Olympus BX50 microscope (autoradiographs under dark-field illumination; staining under bright-field illumination), photographed using a Zeiss Axiocam™ digital camera and images stored on a KS400 imaging system (Imaging Associates, Bicester, UK).

Chapter 9

References

Abe, M., Sogabe, Y., Syuto, T., Yokoyama, Y., and Ishikawa, O. (2007) Evidence that PI3K, Rac, Rho, and Rho kinase are involved in basic fibroblast growth factor-stimulated fibroblastcollagen matrix contraction. *J. Cell Biochem*

Ahmed SI, Thompson J, Coulson JM, Woll PJ. (2000). Studies on the expression of endothelin, its receptor subtypes, and converting enzymes in lung cancer and in human bronchial epithelium. *Am J Respir Cell Mol Biol.* **22,** 422-31.

Ali H, Loizidou M, Dashwood M, Savage F, Sheard C, Taylor I. (2000a). Stimulation of colorectal cancer cell line growth by ET-1 and its inhibition by ET(A) antagonists. *Gut .***47,** 685-8.

Ali H, Dashwood M, Dawas K, Loizidou M, Savage F, Taylor I. (2000b). Endothelin receptor expression in colorectal cancer. *J Cardiovasc Pharmacol.* **36,** S69-71

Alanen K, Deng DX, Chakrabarti S. (2000). Augmented expression of endothelin-1, endothelin-3 and the endothelin-B receptor in breast carcinoma. *Histopathology.* **36,** 161-7.

Almendro V, Ametller E, García-Recio S, Collazo O, Casas I, Augé JM, Maurel J, Gascón P. (2009). The role of MMP7 and its cross-talk with the FAS/FASL system during the acquisition of chemoresistance to oxaliplatin*. PLoS One.* **4(3),** e4728. Epub 2009 Mar 6.

Ametller E, García-Recio S, Pastor-Arroyo EM, Callejo G, Carbó N, Gascón P, Almendro V. (2011). Differential regulation of MMP7 in colon cancer cells resistant and sensitive to oxaliplatin-induced cell death. *Cancer Biol Ther.* **11(1),** 4-13. Epub 2011 Jan 1.

Armstrong AJ, Creel P, Turnbull J, Moore C, Jaffe TA, Haley S, Petros W, Yenser S, Gockerman JP, Sleep D, Hurwitz H, George DJ. (2008). A phase I-II study of docetaxel and atrasentan in men with castration-resistant metastatic prostate cancer. *Clin Cancer Res.* **14**, 6270-6.

Asham EH, Loizidou M, Taylor I. (1998) Endothelin-1 and tumour development. *Eur J Surg Oncol.* **24**, 57-60.

Asham E, Shankar A, Loizidou M, Fredericks S, Miller K, Boulos PB, Burnstock G, Taylor I. (2001). Increased endothelin-1 in colorectal cancer and reduction of tumour growth by ET(A) receptor antagonism. *Br J Cancer.* **85,**1759-63.

Assender JW, Irenius E, Fredholm BB. (1996). Endothelin-1 causes a prolonged protein kinase C activation and acts as a co-mitogen in vascular smooth muscle cells. *Acta Physiol Scand.* **157,** 451-60.

Arinami T, Ishikawa M, Inoue A, Yanagisawa M, Masaki T, Yoshida MC, Hamaguchi H. Chromosomal assignments of the human endothelin family genes: the endothelin-1 gene (EDN1) to 6p23-p24, the endothelin-2 gene (EDN2) to 1p34, and the endothelin-3 gene (EDN3) to 20q13.2-q13.3. (1991). *Am J Hum Genet.* (1991). **48**, 990-6.

Babic AM,Kireeva ML,Kolesnikova TV. (1998) CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. *Proc Natl Acad Sci USA*; **95**: 6355–60.

Bagnato A, Tecce R, Moretti C, Di Castro V, Spergel D, Catt KJ. (1995). Autocrine actions of endothelin-1 as a growth factor in human ovarian carcinoma cells. *Clin Cancer Res.* **1**,1059-66.

Bagnato A, Salani D, Di Castro V, Wu-Wong JR, Tecce R, Nicotra MR, Venuti A, Natali PG. (1999). Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. *Cancer Res.* **59,** 720-7.

Bagnato A, Spinella F, Rosanò L. (2005). Emerging role of the endothelin axis in ovarian tumor progression. *Endocr Relat Cancer.* **12**, 761-72.

Battistini B, Chailler P, D'Orléans-Juste P, Brière N, Sirois P. (1993). Growth regulatory properties of endothelins. *Peptides.* **14**, 385-99.

Beddy D, Mulsow J, Watson RW, Fitzpatrick JM, O'Connell PR. (2006). Expression and regulation of connective tissue growth factor by transforming growth factor beta and tumour necrosis factor alpha in fibroblasts isolated from strictures in patients with Crohn's disease. *Br J Surg.* **93**, 1290-6.

Bell E, Ivarsson B, Merrill C. (1979). Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl Acad Sci U S A.* **76**, 1274-8

Blank MA, Fuortes M, Nyrén O, Jaffe BM. (1991). Effect of endothelin-1 and vasoactive intestinal contractor on blood flow and output of vasoactive intestinal polypeptide in the feline colon. *Life Sci.* **48**, 1937-44

Blaukat A, Barac A, Cross MJ, Offermanns S, Dikic I. (2000). G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of Galpha(q) and Galpha(i) signals. *Mol Cell Biol.* **20,** 6837-48.

Böhm F, Pernow J, Lindström J, Ahlborg G. (2003). ETA receptors mediate vasoconstriction, whereas ETB receptors clear endothelin-1 in the splanchnic and renal circulation of healthy men. *Clin Sci (Lond)*. **104**, 143-51.

Bornstein P,Sage EH. Matricellular proteins: Extracellular modulators of cell function. *Curr Opin Cell Biol* 2002; **14**: 608–16.

Bradham DM, Igarashi A, Potter RL, Grotendorst GR. (1991). Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol.* **114,** 1285-94.

Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B, Attramadal H. (2000). Regulation and intracellular trafficking pathways of the endothelin receptors*. J Biol Chem.* **275,** 17596-604

Brigstock. (2002). Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* **5**: 153-165.

Bruewer M, Schmid KW, Krieglstein CF, Senninger N, Schuermann G. (2002). Metallothionein: early marker in the carcinogenesis of ulcerative colitis-associated colorectal carcinoma. *World J Surg.* **26(6),** 726-31. Epub 2002 Mar 26.

Burkhardt M, Barton M, Shaw SG. (2000). Receptor-and non-receptor-mediated clearance of big-endothelin and endothelin-1: differential effects of acute and chronic ETA receptor blockade. *J Hypertens.* **18**, 273-9.

Cancer Research UK. National Cancer Statistics. CancerStats UK 2006

Carducci MA, Saad F, Abrahamsson PA, Dearnaley DP, Schulman CC, North SA, Sleep DJ, Isaacson JD, Nelson JB. (2007). Atrasentan Phase III Study Group Institutions.A phase 3 randomized controlled trial of the efficacy and safety of atrasentan in men with metastatic hormone-refractory prostate cancer. *Cancer.* **110,**1959-66.

Cernacek P, Stewart DJ. (1989). Immunoreactive endothelin in human plasma: marked elevations in patients in cardiogenic shock. *Biochem Biophys Res Commun.* **161**,562-7.

Chang CC, Shih JY, Jeng YM, Su JL, Lin BZ, Chen ST, Chau YP, Yang PC, Kuo ML. (2004). Connective tissue growth factor and its role in lung adenocarcinoma invasion and metastasis. *J Natl Cancer Inst.* **96**, 364-75

Chang CC, Lin MT, Lin BR. (2006) Effect of connective tissue growth factor on hypoxia-inducible factor 1alpha degradation and tumor angiogenesis. *J Natl Cancer Inst*; **98**:984-95.

Chen CC,Mo FE,Lau LF. The angiogenic factor Cyr61 activates a genetic program for wound healing in human skin fibroblasts. *J Biol Chem* 2001; **276**: 47329–37.

Clarke JG, Benjamin N, Larkin SW, Webb DJ, Davies GJ, Maseri A. (1989). Endothelin is a potent long-lasting vasoconstrictor in men. *Am J Physiol.* **257**, (H2033-5.

Clozel M, Salloukh H. (2005). Role of endothelin in fibrosis and anti-fibrotic potential of bosentan. Ann *Med.* **37,** 2-12.

Cooke, M. E., Sakai, T., and Mosher, D. F. (2000) Contraction of collagen matrices mediated by alpha2beta1A and alpha(v)beta3 integrins. *J. Cell Sci.* **113**, 2375–2383

Cui TX, Iwai M, Hamai M, Shimazu T. (1999). Receptor subtype mediating the action of circulating endothelin on glucose metabolism and hemodynamics in perfused rat liver. *Regul Pept.* **83**, 117-22.

Curtis N, Howard Z, Brooks N, Curwen J. (2004). ZD4054 specifically inhibits endothelin A receptor-mediated anti-apoptotic effects, but not endothelin B receptor-mediated pro-apoptotic effects. *Eur J Cancer Suppl.* **2**: 27 (abstract 78).

Curtis N, Anderson E, Brooks N, Curwen J. (2005). ZD4054 blocks ET-1-stimulated phosphorylation of p44/42 mitogen-activated protein kinase and proliferation of osteoblast cells. *Proc Am Assoc Cancer Res.* **46**: 354 (abstract 1512)

Daub H, Weiss U, Wallasch C, and Ullrich A. (1996). Role of transactivation of the EGF receptor in signaling by G-protein-coupled receptors. Nature (1996), 379[557]: 460.

Davenport AP. (2002). International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. *Pharmacol Rev.* **54**, 219-26.

Del Bufalo D, Di Castro V, Biroccio A, Varmi M, Salani D, Rosanò L, Trisciuoglio D, Spinella F, Bagnato A. (2002). Endothelin-1 protects ovarian carcinoma cells against paclitaxel-induced apoptosis: requirement for Akt activation. *Mol Pharmacol.* **61**, 524-32.

Deng DX, Chakrabarti S, Waalkes MP, Cherian MG. (1998). Metallothionein and apoptosis in primaryhuman hepatocellular carcinoma and metastatic adenocarcinoma. *Histopathology*. **32**, 340–347.

Douglas ML, Richardson MM, Nicol DL. (2004). Endothelin axis expression is markedly different in the two main subtypes of renal cell carcinoma. *Cancer.* **100,** 2118-24

Dréau D, Karaa A, Culberson C, Wyan H, McKillop IH, Clemens MG. (2006). Bosentan inhibits tumor vascularization and bone metastasis in an immunocompetent skin-fold chamber model of breast carcinoma cell metastasis. Clin Exp Metastasis. 23(1):41-53. Epub

Ehrenreich H, Anderson RW, Fox CH, Rieckmann P, Hoffman GS, Travis WD, Coligan JE, Kehrl JH, Fauci AS. (1990). Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. *J Exp Med.*172, 1741-8.

Dreicer, N. Curtis, C. Morris, D. Wilson, A. Hughes, F. Le Maulf, Z. Howard, N. Brooks, J. Curwen. (2004). ZD4054 specifically inhibits endothelin A receptor-mediated effects, but not endothelin B receptor-mediated effects. *Proc ASCO Multidisciplinary Prostate Cancer Sympos.* **153,** 153 (abstract 237).

Eberle J, Fecker LF, Orfanos CE, Geilen CC. (2002). Endothelin-1 decreases basic apoptotic rates in human melanoma cell lines. *J Invest Dermatol.* **119**, 549-55.

Eddy AA, Kim H, López-Guisa J, Oda T, Soloway PD. (2000). Interstitial fibrosis in mice with overload proteinuria: deficiency of TIMP-1 is not protective. *Kidney Int.* **58**, 618-28.

Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer.* **2,** 161–174.

Egidy G, Juillerat-Jeanneret L, Korth P, Bosman FT, Pinet F. (2000). The endothelin system in normal human colon. *Am J Physiol Gastrointest Liver Physiol.* **279**, G211-22.

Eng FJ, Friedman SL. (2000). Fibrogenesis I. New insights into hepatic stellate cell activation: the simple becomes complex. *Am J Physiol Gastrointest Liver Physiol.* **279,** G7-G11.

Fabregat I, Rozengurt E. (1990). [D-Arg1,D-Phe5,D-Trp7,9,Leu11]substance P, a neuropeptide antagonist, blocks binding, Ca2(+)-mobilizing, and mitogenic effects of endothelin and vasoactive intestinal contractor in mouse 3T3 cells. *J Cell Physiol.* **145**, 88-94.

Fang Y, Lu ZH, Wang GQ, Pan ZZ, Zhou ZW, Yun JP, Zhang F, Wan DS. (2009). Elevated expressions of MMP7, TROP2, and surviving are associated with survival, disease recurrence, and liver metastasis of colon cancer. *Int J Colorectal Dis.* **24,** 875–884

Fang Y, Lu ZH, Wang F, Wu X, Li L, Zhang L, Pan Z, Wan D. (2010). Prognostic impact of ERβ and MMP7 expression on overall survival in colon cancer. *Tumor Biol.* **31**, 651–658

Fang Y, Lu ZH, Wang F, Wu X, Li L, Zhang L, Pan Z, Wan D. (2009). MMP7 expression regulated by endocrine therapy in ERβ-positive colon cancer cells. *Journal of Experimental & Clinical Cancer Research.* **28,** 132

Fearon ER, Vogelstein B. (1990). A genetic model for colorectal tumorigenesis. *Cell.* **61,**759-67.

Ferrari-Bravo A, Franciosi C, Lissoni P, Fumagalli L, Uggeri F. (2000). Effects of oncological surgery on endothelin-1 secretion in patients with operable gastric cancer. Int J Biol Markers. **15**, 56-7.

Fischer H, Stenling R, Rubio C, Lindblom A. (2001). Colorectal carcinogenesis is associated with stromal expression of COL11A1 and COL5A2. *Carcinogenesis.* **22,** 875-8.

Fodde R, Smits R, Clevers H. (2001). APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer.* **1,** 55-67.

Giuffre G, Barresi G, Sturniolo GC, Sarnelli R, D'Inca R, Tuccari G. (1996). Immunohistochemical expression of metallothionein in normal human colorectal mucosa, in adenomas and in adenocarcinomas and their associated metastases. *Histopathology*. **29,** 347–354.

Gohji K, Kitazawa S, Tamada H, Katsuoka Y, Nakajima M. (2001). Expression of endothelin receptor a associated with prostate cancer progression. *J Urol.* **165,**1033-6.

Grant K, Knowles J, Dawas K, Burnstock G, Taylor I, Loizidou M. (2007). Mechanisms of endothelin 1-stimulated proliferation in colorectal cancer cell lines. Br J Surg. **94,** 106-12.

Grimshaw MJ. (2007). Endothelins and hypoxia-inducible factor in cancer. *Endocr Relat Cancer.* **14,** 233-44.

Grotendorst GR. (1997). Connective tissue growth factor: A mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev*; **8**: 171–9.

Guidry, C., and Hook, M. (1991) Endothelins produced by endothelial cells promote collagen gel contraction by fibroblasts. *J. Cell Biol.* **115,** 873–880

Hague S, Zhang L, Oehler MK, Manek S, MacKenzie IZ, Bicknell R, Rees MC (2000) Expression of the hypoxically regulated angiogenic factor adrenomedullin correlates with uterine leiomyoma vascular density. Clin Cancer Res **3**: 2808 – 2814

Haque S, Bhalla A, Winslet M, **Clinical Management of Familial Adenomatous Polyposis (FAP) and Lynch Syndrome (Hereditary Non-Polyposis Colon Cancer – HNPCC).** *Oncology News Volume 3 Issue 3: October/November, 2008*

Haque S, Heetun M, Farooqui N, Loizidou M, Dashwood M, Shiwen X, Abraham D. (2009). Efficacy of the Specific ETA Receptor antagonist Zibotentan (ZD4054) in Cancer Cells and Fibroblasts from Colorectal Cancer. Mol. Cancer Ther; 8(12 Suppl):B190.

Haque S, Heetun M, Taylor I, Shiwen X, Winslet M, Loizidou M. (2009). Efficacy of the Specific ETA Receptor Antagonist Zibotentan in Colorectal Cancer Cells and Colorectal Fibroblasts. EJSO; **35** (11): 1206.

Hartel M, Di Mola FF, Gardini A, et al. (2004). Desmoplastic reaction influences pancreatic cancer growth behavior. *World J Surg.* **28**, 818e25.

Hawes BE, Luttrell LM, van Biesen T, Lefkowitz RJ. (1996). Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. *J Biol Chem.* **271,** 12133-6.

Hinsley EE, Kumar S, Hunter KD, Whawell SA, Lambert DW. (2012). Endothelin-1 stimulates oral fibroblasts to promote oral cancer invasion. Life Sci. 2012 Apr 13. [Epub ahead of print]

Hinson JP, Kapas S, Smith DM (2000) Adrenomedullin, a multifunctional regulatory peptide. Endocr Rev **21**: 138 – 167

Hirata Y, Takagi Y, Fukuda Y, Marumo F. (1989). Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis.* **78**, 225-8.

Hishikawa K, Oemar BS, Tanner FC, Nakaki T, Luscher TF, Fujii T. (1999). Connective tissue growth factor induces apoptosis in human breast cancer cell line MCF-7. *J Biol Chem***. 274**, 37461–6.

Hishikawa Y, Kohno H, Ueda S, Kimoto T, Dhar DK, Kubota H, Tachibana M, Koji T. Nagasue N. (2001). Expression of Metallothionein inColorectal Cancers and synchronous Liver Metastases Matrix Metalloproteinase 7 (MMP7). *Oncology*. **61**, 162–167

Hoosein MM, Dashwood MR, Dawas K, Ali HM, Grant K, Savage F, Taylor I, Loizidou M. (2007)Altered endothelin receptor subtypes in colorectal cancer. *Eur J Gastroenterol Hepatol.* **19**, 775-82.

Ihara M, Fukuroda T, Saeki T, Nishikibe M, Kojiri K, Suda H, Yano M. (1991). An endothelin receptor (ETA) antagonist isolated from Streptomyces misakiensis. *Biochem Biophys Res Commun.* **178**,132-7.

Ihara M, Ishikawa K, Fukuroda T, Saeki T, Funabashi K, Fukami T, Suda H, Yano M. (1992). In vitro biological profile of a highly potent novel endothelin (ET) antagonist BQ-123 selective for the ETA receptor. *J Cardiovasc Pharmacol.* **20** Suppl 12:S11-4.

Ivarsson M, McWhirter A, Black CM, Rubin K. (1993). Impaired regulation of collagen pro-alpha 1(I) mRNA and change in pattern of collagen-binding integrins on scleroderma fibroblasts. *J Invest Dermatol.* **101**, 216-21.

James ND, Caty A, Borre M, Zonnenberg BA, Beuzeboc P, Morris T, Phung D, Dawson NA. (2008). Safety and Efficacy of the Specific Endothelin-A Receptor Antagonist ZD4054 in Patients

with Hormone-Resistant Prostate Cancer and Bone Metastases Who Were Pain Free or Mildly Symptomatic: A Double-Blind, Placebo-Controlled, Randomised, Phase 2 Trial. *Eur Urol.* Nov 28.

James ND, Caty A, Borre M, Zonnenberg BA, Beuzeboc P, Morris T, Phung D, Dawson NA. (2009). Safety and Efficacy of the Specific Endothelin-A Receptor Antagonist ZD4054 in Patients with Hormone-Resistant Prostate Cancer and Bone Metastases Who Were Pain Free or Mildly Symptomatic: A Double-Blind, Placebo-Controlled, Randomised, Phase 2 Trial. Eur Urol. 2009 May; **55(5)**:1112-23

Jasani B, Campbell F, Navabi H, Schmid KW, Williams GT. (1998). Clonal overexpression of Metallothionein is induced by somatic mutation in morphologically normal colonic mucosa*. J Pathol.* **184**, 144–147.

Kaafarani I, Fernandez-Sauze S, Berenguer C, Chinot O et al. (2006). Targeting adrenomedullin receptors with systemic delivery of neutralizing antibodies inhibit tumor angiogenesis and suppresses growth of humen tumor xenografts in mice. FASEB **23**. (10) 3424-3435

Kamoi K, Sudo N, Ishibashi M, Yamaji T. (1990). Plasma endothelin-1 levels in patients with pregnancy-induced hypertension. *N Engl J Med.* **323,**1486-7.

Kar S, Yousem SA, Carr BI. (1995). Endothelin-1 expression by human hepatocellular carcinoma. *Biochem Biophys Res Commun*. 216, 514-9.

Kashiwabara T, Inagaki Y, Ohta H, Iwamatsu A, Nomizu M, Nishikori K. (1989). Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect. *FEBS Lett.* 247:73–76.

Kar S, Yousem SA. (1995). Carr BI. Endothelin-1 expression by human hepatocellular carcinoma. Biochem Biophys Res Commun. **216**, 514-9.

Kefford R, Beith JM, Van Hazel GA, Millward M, Trotter JM, Wyld DK, Kusic R, Shreeniwas R, Morganti A, Ballmer A, Segal E, Nayler O, Clozel M. (2007). A phase II study of bosentan, a dual endothelin receptor antagonist, as monotherapy in patients with stage IV metastatic melanoma. *Invest New Drugs.* **25,** 247-52.

Kernochan LE, Tran BN, Tangkijvanich P, Melton AC, Tam SP, Yee HF Jr. (2002). Endothelin-1 stimulates human colonic myofibroblast contraction and migration *Gut.* **50,** 65-70.

Khan R, Sheppard R. (2006). Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. *Immunology.* **118,** 10-24.

Khodorova A, Navarro B, Jouaville LS, Murphy JE, Rice FL, Mazurkiewicz JE, Long-Woodward D, Stoffel M, Strichartz GR, Yukhananov R, Davar G. (2003). Endothelin-B receptor activation triggers an endogenous analgesic cascade at sites of peripheral injury. *Nat Med.* **9,** 1055-61.

Kikuchi K, Nakagawa H, Kadono T, Etoh T, Byers HR, Mihm MC, Tamaki K. (1996). Decreased ET(B) receptor expression in human metastatic melanoma cells. *Biochem Biophys Res Commun.* **219,** 734-9.

Kim TH, Xiong H, Zhang Z, Ren B. (2005). beta-Catenin activates the growth factor endothelin-1 in colon cancer cells. *Oncogene, **24**: 597-604.*

King-VanVlack CE, Mewburn JD, Chapler CK. (1999). Receptor-mediated vascular and metabolic actions of endothelin-1 in canine small intestine. Am J Physiol. **276,** G1131-6.

Kita H, Hikichi Y, Hikami K, Tsuneyama K, Cui Z, Osawa H, Ohnishi H, Mutoh H, Hoshino H, Bowlus C, Yamamoto C, SuganoK. (2006). Differential gene expression between flat adenoma and normal mucosa in the colon in a microarray analysis. *J Gastroenterol.* **41**,1053–1063

Kiyohara T, Okuno M, Nakanishi T, Shinomura Y, Matsuzawa Y. (1993). Effect of endothelin 1 on ion transport in isolated rat colon. *Gastroenterology.* **104,** 1328-36.

Kmieć Z. Cooperation of liver cells in health and disease. (2001). Adv Anat Embryol Cell Biol. **161**, III-XIII, 1-151.

Knowles JP, Shiwen X, Haque S, Bhalla A, Dashwood MR, Yang S, Taylor I, Winslet MC, Abraham DJ, Loizidou M. (2011). Endothelin-1 stimulates colon cancer adjacent fibroblasts. Int. J. Cancer. Mar 28. doi: 10.1002/ijc.26090.

Kojima K, Nihei Z. (1995). Expression of endothelin-1 immunoreactivity in breast cancer. *Surg Oncol.* **4,**309-15.

Kolesnikova TV,Lau LF. Human CYR61-mediated enhancement of bFGF-induced DNA synthesis in human umbilical vein endothelial cells. *Oncogene* 1998; **16:** 747–54.

Koliopanos A, Friess H, di Mola FF. (2002). Connective tissue growth factor gene expression alters tumor progression in esophageal cancer. *World J Surg*. 26, 420e7.

Komuro I, Kurihara H, Sugiyama T, Yoshizumi M, Takaku F, Yazaki Y. (1988). Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. *FEBS Lett.* **238**, 249-52.

Kreig A, Rankin EB, Chan D, Razorenova O, Fernandez S, Giaccia AJ (2010). Regulation of the Histone Demethylase JMJD1A by hypoxia inducible factor 1 enhances hypoxic gene expression and tumor growth. *Molecular and cellular biology* **30** (10). 344-353

Kubo M, Kikuchi K, Nashiro K, Kakinuma T, Hayashi N, Nanko H, et al. *(1998).* Expression of fibrogenic cytokines in desmoplastic malignant melanoma.*Br J Dermatol.* **139,** 192–7.

Kurihara Y, Kurihara H, Oda H, Maemura K, Nagai R, Ishikawa T, Yazaki Y. (1995). Aortic arch malformations and ventricular septal defect in mice deficient in endothelin-1. *J Clin Invest.* **96,** 293-300.

Kurokawa S, Arimura Y, Yamamoto H, Adachi Y, Endo T, et al. (2005) Tumour matrilysin expression predicts metastatic potential of stage I (pT1) colon and rectal cancers. *Gut.* **54,** 1751–1758.

Kusuhara M, Yamaguchi K, Nagasaki K, Hayashi C, Suzaki A, Hori S, Handa S, Nakamura Y, Abe K. (1990). Production of endothelin in human cancer cell lines. *Cancer Res.* **50,** 3257-61.

Ladwa R, Pringle H, Kumar R, West K. (2011). Expression of CTGF and Cyr61 in colorectal cancer. *J Clin Pathol*; 64:58e64.

Lauffenburger DA, Horwitz AF. (1996). Cell migration: a physically integrated molecular process. *Cell.* **84,** 359-69.

Lahav R, Heffner G, Patterson PH. (1999). An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo. *Proc Natl Acad Sci* U S A. **96,** 11496-500.

Latinkic BV,O'Brien TP,Lau LF. Promoter function and structure of the growth factor-inducible immediate early gene cyr61. Nucleic Acids Res 1991; **19**: 3261–7.

Li Y, Lacerda DA, Warman ML, Beier DR, Yoshioka H, Ninomiya Y, Oxford JT, Morris NP, Andrikopoulos K, Ramirez F, et al. (1995). A fibrillar collagen gene, Col11a1, is essential for skeletal morphogenesis. *Cell.* **80,** 423-30.

Lin BR, Chang CC, Che TF, et al. (2005). Connective tissue growth factor inhibits metastasisand acts as an independent prognostic marker in colorectal cancer. *Gastroenterology.* **128**, 9-23.

Martinez A, Vos M, Guedez L, Kaur G, Chen Z, Garayoa M, Pio R, Moody T, Stetler-Stevenson WG, Kleinman HK, Cuttitta F (2002) The effects of adrenomedullin overexpression in breast tumor cells. *J Natl Cancer Inst* **94**(16): 1226 – 1237

Masaki T, Ninomiya H, Sakamoto A, Okamoto Y. (1999). Structural basis of the function of endothelin receptor. Mol Cell Biochem. **190**, 153-6.

Maurel J, Nadal C, Garcia-Albeniz X, Gallego R, Carcereny E, et al. (2007) Serum matrix metalloproteinase 7 levels identifies poor prognosis advanced colorectal cancer patients*. Int J Cancer.* **121,** 1066–1071.

Mendler M, Eich-Bender SG, Vaughan L, Winterhalter KH, Bruckner P. (1989). Cartilage contains mixed fibrils of collagen types II, IX, and XI. *Cell Biol.* **108**, 191-7.

Mickley EJ, Gray GA, Webb DJ. Activation of endothelin ETA receptors masks the constrictor role of endothelin ETB receptors in rat isolated small mesenteric arteries. *Br J Pharmacol.* **120,** 1376-82.

Miller EJ, Gay S. (1987). The collagens: an overview and update. *Methods Enzymol.* **144**, 3-41.

Miyauchi T, Yanagisawa M, Tomizawa T, Sugishita Y, Suzuki N, Fujino M, Ajisaka R, Goto K, Masaki T. (1989). Increased plasma concentrations of endothelin-1 and big endothelin-1 in acute myocardial infarction. *Lancet.* 1989. **2,** 53-4.

Moraitis S, Langdon SP, Miller WR. (1997). Endothelin expression and responsiveness in human ovarian carcinoma cell lines. *Eur J Cancer.* **33**(4):661-8.

Moraitis S, Miller WR, Smyth JF, Langdon SP. (1999). Paracrine regulation of ovarian cancer by endothelin. *Eur J Cancer.* **35**, 1381-7.

Moummi C, Gullikson GW, Gaginella TS. (1992). Effect of endothelin-1 on guinea pig gallbladder smooth muscle in vitro. *J Pharmacol Exp Ther.* **260,** 549-53.

Moussad EE,Brigstock DR. Connective tissue growth factor:What's in a name? Mol Genet Metab 2000; **71**: 276–92.

Mukudai Y, Kubota S, Takigawa M. (2003). Conserved repressive regulation of connective tissue growth factor/hypertrophic chondrocyte-specific gene 24 (ctgf/hcs24) enabled by different elements and factors among vertebrate species. *Biol Chem.* **384**,1–9.

Nagel WW, Vallee B (1995). Cell cycle regulation of metallothionein in human colonic cancer cells. *Proc Natl Acad Sci USA*. **92,** 579–583.

Nakamuta M, Ohashi M, Tabata S, Tanabe Y, Goto K, Naruse M, Naruse K, Hiroshige K, Nawata H. (1993). High plasma concentrations of endothelin-like immunoreactivities in patients with hepatocellular carcinoma. *Am J Gastroenterol.* **88,** 248-52.

Nastase A, Pâslaru L, Niculescu AM, Ionescu M, Dumitraşcu T, Herlea V, Dima S, Gheorghe C, Lazar V, Popescu I. (2011). Prognostic and predictive potential molecular biomarkers in colon cancer*. Chirurgia (Bucur).* **106(2),**177-85.

Nelson JB, Hedican SP, George DJ, Reddi AH, Piantadosi S, Eisenberger MA, Simons JW. (1995). Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med.* **1**,944-9.

Nelson JB, Chan-Tack K, Hedican SP, Magnuson SR, Opgenorth TJ, Bova GS, Simons JW. (1996). Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. *Cancer Res.* **56**,663-8.

Nelson JB, Nguyen SH, Wu-Wong JR, Opgenorth TJ, Dixon DB, Chung LW, Inoue N. (1999). New bone formation in an osteoblastic tumor model is increased by endothelin-1 overexpression and decreased by endothelin A receptor blockade. *Urology.* **53,** 1063-9.

Nelson J, Bagnato A, Battistini B, Nisen P. (2003). The endothelin axis: emerging role in cancer. *Nat Rev Cancer.* **3,** 110-6.

Nelson JB, Love W, Chin JL, Saad F, Schulman CC, Sleep DJ, Qian J, Steinberg J, Carducci M. (2008). Atrasentan Phase 3 Study Group. Phase 3, randomized, controlled trial of atrasentan in patients with nonmetastatic, hormone-refractory prostate cancer. *Cancer.* **113,** 2478-87.

Nikitenko LL, Fox SB, Kehoe S, Rees MCP, Bicknell R (2006). Adrenomedullin and tumour angiogenesis. *British Journal Cancer*. **94** (1), 1-7.

Oehler MK, Hague S, Rees MC, Bicknell R (2002) Adrenomedullin promotes formation of xenografted endometrial tumors by stimulation of autocrine growth and angiogenesis. *Oncogene* **21**: 2815– 2821

Öfner D, Maier H, Riedmann B, Bammer T, Rumer A, Winde G, Böcker W, Jasani B, Schmid KW (1994). Immunohistochemical metallothionein expression in colorectal adenocarcinoma: Correlation with tumour stage and patient survival. *Virchows Arch.* **425**, 491–497.

Okazawa M, Shiraki T, Ninomiya H, Kobayashi S, Masaki T. (1998). Endothelin-induced apoptosis of A375 human melanoma cells. *J Biol Chem.* 1998. **273,** 12584-92.

Oliver MH, Harrison NK, Bishop JE, Cole PJ, (1989) Laurent GJ. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *J Cell Sci.* **92,** 513-8.

Opgenorth TJ, Wu-Wong JR, Shiosaki K.Rubin SA, Levin ER. (1992). Endothelin-converting enzymes. *Clin endocr metabol FASEB J.* **6,** 2653-9.

Ouafik L, Sauze S, Boudouresque F, Chinot O, Delfino C, Fina F, Vuaroqueaux V, Dussert C, Palmari J, Dufour H, Grisoli F, Casellas P, Brunner N, Martin PM (2002) Neutralization of adrenomedullin inhibits the growth of human glioblastoma cell lines in vitro and suppresses tumor xenograft growth in vivo. *Am J Pathol* **160**(4): 1279– 1292

Oyama T, Takei H, Hikino T, Iino Y, Nakajima T. (1996). Immunohistochemical expression of Metallothionein in invasive breast cancer in relation to proliferative activity, histology and prognosis. *Oncology*. **53**, 112–117.

Peduto Eberl L, Bovey R, and Juillerat-Jeanneret L. (2003). Endothelin receptor antagonists are pro-apoptotic and antiproliferative in human colon cancer cells. *British Journal of Cancer*, **88**:788– 795.

Perbal B. The CCN family of genes: A brief history. *Mol Pathol 2001; **54**: 103–4.*

Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, Muff R, Fischer JA, Foord SM (2002) International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev* **54**:233 – 246

Pribnow D, Muldoon LL, Fajardo M, Theodor L, Chen LY, Magun BE. (1992). Endothelin induces transcription of fos/jun family genes: a prominent role for calcium ion. *Mol Endocrinol.* **6**, 1003-12.

Remuzzi G, Perico N, Benigni A. (2002). New therapeutics that antagonize endothelin: promises and frustrations. *Nat Rev Drug Discov.* **1,** 986-1001.

Rockey DC. (2003). Vascular mediators in the injured liver. *Hepatology.* **37**, 4-12.

Rosanò L, Varmi M, Salani D, Di Castro V, Spinella F, Natali PG, Bagnato A. (2001). Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. Cancer Res. **61,** 8340-6.

Rosanò L, Di Castro V, Spinella F, Decandia S, Natali PG, Bagnato A. (2006). ZD4054, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma cell proliferation. *Exp Biol Med* **231**, 132-5.

Rosanò L, Di Castro V, Spinella F, Tortora G, Nicotra MR, Natali PG, Bagnato A. (2007a). Combined targeting of endothelin A receptor and epidermal growth factor receptor in ovarian cancer shows enhanced antitumor activity. *Cancer Res.* **67**, 6351-9.

Rosanò L, Di Castro V, Spinella F, Nicotra MR, Natali PG, Bagnato A. (2007b). ZD4054, a specific antagonist of the endothelin A receptor, inhibits tumor growth and enhances paclitaxel activity in human ovarian carcinoma in vitro and in vivo. *Mol Cancer Ther.* **6**, 2003-11.

Rubin SA, Levin ER. (1994). The endocrinology of vasoactive peptides: synthesis to function. *J Clin Endocrinol Metab.* **78,** 6-10.

Sakamoto A, Yanagisawa M, Sakurai T, Takuwa Y, Yanagisawa H, Masaki T. (1991). Cloning and functional expression of human cDNA for the ETB endothelin receptor. *Biochem Biophys Res Commun.* **178,** 656-63

Sakamoto A, Yanagisawa M, Takuwa Y, and Masaki T. (2001). Cloning and functional expression of human cDNA for the ETB endothelin receptor. Biochemical and Biophysical Research Communications **178**, 656-663.

Sakurai T, Yanagisawa M, and Masaki T. (1992). Molecular characterisation of endothelin receptors. *Treads Pharmacol. Sci.* **13**, 103-108

Salani D, Di Castro V, Nicotra MR, Rosanò L, Tecce R, Venuti A, Natali PG, Bagnato A. (2000). Role of endothelin-1 in neovascularization of ovarian carcinoma. *Am J Pathol.* **157,** 1537-47.

Shichiri M, Kato H, Marumo F, Hirata Y. (1997). Endothelin-1 as an autocrine/paracrine apoptosis survival factor for endothelial cells. *Hypertension.* **30**, 1198-203.

Schmetterer L, Dallinger S, Bobr B, Selenko N, Eichler HG, Wolzt M. (1998). Systemic and renal effects of an ET(A) receptor subtype-specific antagonist in healthy subjects. *Br J Pharmacol.* **124,** 930-4.

Shankar A, Loizidou M, Aliev G, Fredericks S, Holt D, Boulos PB, Burnstock G, Taylor I. (1998). Raised endothelin 1 levels in patients with colorectal liver metastases. *Br J Surg*. **85,** 502-6.

Shimo T, Nakanishi T, Nishida T, Asano M, Sasaki A, Kanyama M, Kuboki T, Matsumura T, Takigawa M. (2001). Involvement of CTGF, a hypertrophic chondrocyte-specific gene product, in tumor angiogenesis. Oncology. 2001;**61**(4):315-22.

Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, Leask A, Abraham DJ. (2004). Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol Biol Cell.* **15**, 2707-19.

Shi-Wen X, Renzoni EA, Kennedy L, Howat S, Chen Y, Pearson JD, Bou-Gharios G, Dashwood MR, du Bois RM, Black CM, Denton CP, Abraham DJ, Leask A. (2007).

Endogenous endothelin-1 signaling contributes to type I collagen and CCN2 overexpression in fibrotic fibroblasts. *Matrix Biol.* **26,** (8):625-32.

Shome K, Rizzo MA, Vasudevan C, Andresen B, Romero G. (2000). The activation of phospholipase D by endothelin-1, angiotensin II, and platelet-derived growth factor in vascular smooth muscle A10 cells is mediated by small G proteins of the ADP-ribosylation factor family. *Endocrinology.* **141,** 2200-8

Simonson MS, Wann S, Mené P, Dubyak GR, Kester M, Dunn MJ. (1989). Endothelin-1 activates the phosphoinositide cascade in cultured glomerular mesangial cells. *J Cardiovasc Pharmacol.***13** Suppl 5, S80-3

Simpson RA, Dickinson T, Porter KE, London NJ, Hemingway DM. (2000). Raised levels of plasma big endothelin 1 in patients with colorectal cancer. *Br J Surg.* **87,** 1409-13.

Smith, K. D., Wells, A., and Lauffenburger, D. A. (2006) Multiple signaling pathways mediate compaction of collagen matrices by EGF-stimulated fibroblasts. *Exp. Cell Res.* **312,** 1970–1982

Sone M, Takahashi K, Totsune K, Murakami O, Arihara Z, Satoh F, Mouri T, Shibahara S. (2000). Expression of endothelin-1 and endothelin receptors in cultured human glioblastoma cells. *J Cardiovasc Pharmacol.* **36,**S390-2.

Spinella MJ, Malik AB, Everitt J, Andersen TT. (1991). Design and synthesis of a specific endothelin 1 antagonist: effects on pulmonary vasoconstriction. *Proc Natl Acad Sci U S A.* **88,**7443-6

Spinella F, Rosano L, Di Castro V, et al. (2002). Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1alpha in ovarian carcinoma cells. J Biol Chem, **277**:27850–27855.

Spinella F, Rosano L, Di Castro V, Nicotra MR, Natali PG, and Bagnato A. (2004). Inhibition of cyclo-oxygenase-1 and -2 expression by targeting the endothelin A receptor in human ovarian carcinoma cells. Clinical Cancer Research, **10**:4670–4679.

Stannard C, Lehenkari P, Godovac-Zimmermann J. (2003). Functional diversity of endothelin pathways in human lung fibroblasts may be based on structural diversity of the endothelin receptors. *Biochemistry.* **42,** 13909-18

Sutoh I, Kohno H, Nakashima Y, Hishikawa Y, Tabara H, Tachibana M, Kubota H, Nagasue N. (2000). Concurrent expressions of metallothionein, glutathione S-transferase-pi, and P-lycoprotein in colorectal cancers. *Dis Colon Rectum*. **43**, 221–232.

Suzuki T, Hoshi N, Watanabe K, Kasukawa R, Suzuki T. (1998). Immunohistochemical localization of endothelin-1/big endothelin-1 in normal liver, liver cirrhosis and hepatocellular carcinoma. *Fukushima J Med Sci.* **44,** 93-105.

Tangkijvanich P, Tam SP, Yee HF Jr. Wound-induced migration of rat hepatic stellate cells is modulated by endothelin-1 through rho-kinase-mediated alterations in the acto-myosin cytoskeleton. *Hepatology*. **33,** 74-80.

Takuwa N, Takuwa Y, Yanagisawa M, Yamashita K, Masaki T. (1989). A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J Biol Chem.* **264,** 7856-61.

Tingstrom, A., Heldin, C. H., and Rubin, K. (1992) Regulation of fibroblast-mediated collagen gel contraction by platelet derived growth factor, interleukin-1 alpha and transforming growth factor-beta 1. *J. Cell Sci.* **102**, 315–322

Tsukada S, Parsons CJ, Rippe RA. (2006). Mechanisms of liver fibrosis. *Clin Chim Acta.* **364,** 33-60.

Usune S, Katsuragi T, Furukawa T. (1991). Involvement of K(+)-channel opening in endothelin-1 induced suppression of spontaneous contractions in the guinea pig taenia coli. *Can J Physiol Pharmacol.* **69,** 1908-13.

Valentich JD, Popov V, Saada JI, Powell DW. (1997). Phenotypic characterization of an intestinal subepithelial myofibroblast cell line. *Am J Physiol.* **272**, C1513-24.

Vasen HFA, Moslein G, Alonso A. (2008). Guidelines for the clinical management of familial adenomatous polyposis (FAP). Gut 2008;**57**:704-13.

Vuurmans JL, Boer P, Koomans HA. (2004). Effects of endothelin-1 and endothelin-1-receptor blockade on renal function in humans. *Nephrol Dial Transplant.* **19,** 2742-6.

Wagenseil JE, Wakatsuki T, Okamoto RJ, Zahalak GI, Elson EL. (2003). One-dimensional viscoelastic behavior of fibroblast populated collagen matrices. *J Biomech Eng.* **125**, 719-25.

Wang WS, Chen PM, Wang HS, Liang WY, Su Y (2006) Matrix metalloproteinase-7 increases resistance to Fas-mediated apoptosis and is a poor prognostic factor of patients with colorectal carcinoma. *Carcinogenesis.* **27**, 1113–1120.

Wenger C, Ellenrieder V, Alber B, Lacher U, Menke A, Hameister H, Wilda M, Iwamura T, Beger HG, Adler G, Gress TM. (1999). Expression and differential regulation of connective tissue growth factor in pancreatic cancer cells. *Oncogene.* **28,**1073-80.

Wiklund NP, Wiklund CU, Cederqvist B, Ohlén A, Hedqvist P, Gustafsson LE. (1991). Endothelin modulation of neuroeffector transmission in smooth muscle. *J Cardiovasc Pharmacol.* **17,** S335-9.

Wilkins PL, Suchovsky D, Berti-Mattera LN. (1997). Immortalized schwann cells express endothelin receptors coupled to adenylyl cyclase and phospholipase C. *Neurochem Res.* **22,** 409-18.

Wülfing P, Kersting C, Tio J, Fischer RJ, Wülfing C, Poremba C, Diallo R, Böcker W, Kiesel L. (2004). Endothelin-1-, endothelin-A-, and endothelin-B-receptor expression is correlated with vascular endothelial growth factor expression and angiogenesis in breast cancer. *Clin Cancer Res.* **10,** 2393-400.

Wu JJ, Eyre DR. (1995). Structural analysis of cross-linking domains in cartilage type XI collagen. Insights on polymeric assembly. *J Biol Chem.* **270,** 18865-70.

Wu-Wong JR, Chiou WJ, Wang J. (2000). Extracellular signal-regulated kinases are involved in the antiapoptotic effect of endothelin-1. *J Pharmacol Exp Ther.* **293**, 514-21.

Xie D, Nakachi K, Wang H, Elashoff R, Koeffler HP. (2001). Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. *Cancer Res.* **61,** 8917-23.

Xie D, Yin D, Wang HJ, et al. (2004). Levels of expression of CYR61 and CTGF are prognostic for tumor progression and survival of individuals with gliomas. *Clin Cancer Res.* **10,** 2072e81.

Xie D, Nakachi K, Wang H, et al. (2004). Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. *Cancer Res.* **61**, 8917e23.

Xu SW, Howat SL, Renzoni EA, Holmes A, Pearson JD, Dashwood M, Bou-Gharios G, Denton CP, du Bois RM, Black CM, Leask A, and Abraham DJ. (2004). Endothelin-1 induces expression of matrix-associated genes in lung fibroblasts through MEK/ERK. Journal of Biological Chemistry 279[**22**], 23098-23103.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332,** 411-5.

Yang Z, Kraznici N, and Luscher TF. (1999)Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ETA and ETB receptor blockade. Circulation **100**, 5-8.

Yohn JJ, Smith C, Stevens T, Hoffman TA, Morelli JG, Hurt DL, Yanagisawa M, Kane MA, Zamora MR. (1994). Human melanoma cells express functional endothelin-1 receptors. *Biochem Biophys Res Commun*. **201,** 449-57

Yousufzai SY, Abdel-latif AA. (1997). Endothelin-1 stimulates the release of arachidonic acid and prostaglandins in cultured human ciliary muscle cells: activation of phospholipase A2. *Exp Eye Res.* **65,** 73-81.

Ziche M, Morbidelli L, Donnini S, Ledda F. (1995). ETB receptors promote proliferation and migration of endothelial cells. *J Cardiovasc Pharmacol.* **26,** S284-6.

Zudaire E, Martinez A, Cuttitta F (2003) Adrenomedullin and cancer. *Regul Pept* **112**: 175 – 183