The Mechanisms Underlying the Anti-Cancer Activity of Substance P Analogues

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I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Catherine Mary Waters.
18/9/00

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Abstract

The unrestrained growth of many cancers is maintained by autocrine and paracrine loops of neuropeptide growth factors. These growth factor loops present an important target for therapeutic intervention. Analogues of Substance P are currently undergoing clinical trials as potential novel therapies for small cell lung cancer (SCLC). Such analogues have been shown to inhibit mitogenic signal transduction by a range of neuropeptides, acting in a broad spectrum manner. They inhibit the *in vitro* growth of cancer cells within a physiologically obtainable concentration range and are also capable of inhibiting the growth of tumour xenografts in nude mice. They also stimulate apoptosis *in vitro*.

This study explores the range of tumour types which are sensitive to substance P analogues. It was found that a wider range of tumour types than had been previously demonstrated were sensitive to growth inhibition by the substance P analogue, [D-Arg^{6},D-Trp^{7,9},N^{me}Phe^{8}]-Substance P (6-11) (antagonist G). Sensitive tumour types included SCLC, NSCLC, colo-rectal and ovarian tumour cell lines with a mean IC_{50} value of 33μM for 11 tumour cell lines of various origins. The mechanism of action of the growth inhibitory effects of substance P analogues is unclear. Using RT-PCR this study shows that sensitivity of tumour cell lines to antagonist G is dependent upon the expression of neuropeptide receptors, in particular the bombesin / gastrin releasing peptide receptor, and not dependent on tumour type. This is confirmed by the demonstration of increased sensitivity of Rat-1 fibroblasts transfected with the bombesin receptor when compared to the parent cell line.

Substance P analogues have been previously thought to act as neuropeptide receptor antagonists but more recently they have been shown to have some agonist effects including the activation of c-Jun –N-terminal kinase (JNK). Further studies using fibroblast cell lines transfected with the bombesin receptor show that substance P analogues are potent activators of MAPK and that this occurs via activation of the bombesin / GRP receptor. It was found that the substance P analogue [D-Arg^{1},D-Phe^{5},DTrp^{7,9},Leu^{11}]-substance P (6-11) (antagonist D) inhibits bombesin stimulated calcium flux by preventing the activation of Gq stimulated downstream effectors and preferentially activates Gi downstream effectors, resulting in MAPK activation. It is demonstrated that MAPK activation by antagonist D is integral in the pro-apoptotic effects of this compound. Thus, the combined effects of substance P analogue stimulated, discordant MAPK and JNK activation, together with the inhibition of neuropeptide stimulated calcium flux, results in growth inhibition and apoptosis in cell types which express neuropeptide receptors.

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
ADH	Antidiuretic hormone
Ala	Alanine
AMP	Ampicillin
ANF	Atrial natriuretic factor
Ant D	Antagonist D
Ant G	Antagonist G
APS	Ammonium persulphate
Arg	Arginine
Asp	Aspartine
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BK	Bradykinin
BK2R	Bradykinin 2 receptor
BM	Bone marrow
BN	Bombesin
BOP	N-nitrosobis (2-oxyproply) amine
bp	Base pair
BRDU	Bromodeoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CaCl	Calcium Chloride
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CCKB	Gastrin
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CPP32	Caspase 3
CRC	Cancer research campaign

DAG	Diacylglycerol
DMSO	Dimethylsulphoxide
DNA	Deoxynucleic acid
DNAase	Deoxyribonuclease
dNTP	Deoxynuclotide triphosphate
DTT	Dithiotreitol
ECL	Enhanced chemiluminescence
ECL	Enterochromaffin-like
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
FACS	Fluorescence activated cell sorter
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
FTS	S-farnesylthiosaicyclic acid
FURA-2AM	Fura-2-tetraacetoxymethlyester AME
G418	Geneticin
Gly	Glycine
GM CSF	Granulocyte macrophage colony stimulating factor
GMP	Guanine monophosphate
GPCR	G protein coupled receptor
GRP	Gastrin releasing peptide
GRP R	Gastrin releasing peptide receptor
GST	Glutathione S-transferase.
GTP	Guanine triphosphate
HBE	Human bronchial epithelial
HBSS	Hanks balanced salt solution
HITES	Hydrocortisone,insulin,transferrin,estradiol,sodium selinite
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase

IC50	Inhibitory concentration 50%
ICRF	Imperial cancer research fund
IGF	Insulin like growth factor
IP3	Inositol 1,4,5 Trisphosphate
IPTG	Isopropylthio-β-D-galctoside
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
LB	Liquid broth
Leu	Leucine
LN	Lymph node
LPA	Lysophosphatidic acid
LRP	Lung resistance protein
MAPK	Mitogen activated protein kinase
MARCKS	Myrisolated alanine rich C kinase substrate
MDR	Multidrug resistant
Met	Methionine
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid.
MRP-1	Multidrug resistance protein -1
NaCl	Sodium chloride
NaF	Sodium flouride
NBT	Nitro-blue tetrazolium
NE	Neuroendocrine
NHS	National health service
NK	Neurokinin
NMB	Neuromedin B
NSCLC	Non-small cell lung cancer
NSE	Neuron specific enolase
NT	Neurotensin
OD	Optical density
PAGE	Polyacrylamide gel elecrophoresis
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE	Pleural effusion
PGE2	Prostaglandin E2
PKC	Protein kinase C
PKD	Protein kinase D
PLA_2	Phospholipase A_2
PLC	Phospholipase C
pMAPK	phosphorylated MAPK
Pro	Proline
PTX	Pertussis toxin
RPM	Revolutions per minute
RTK	Receptor tyrosine kinases
RT-PCR	Reverse transcriptase polymerase chain reaction
SCLC	Small cell lung cancer
SDS	Sodium dodecylsulphate
SITA	Selenium, Insulin, Transferrin, Albumin
SP	Substance P
SQCC	Squamous cell carcinomas
TCA	Tetrachloroacetic acid
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta.
Thr	Threonine
TM	Transmembrane region
Trp	Tryptophan
VIAR	Vasopressin 1A receptor
VP	Vasopressin
WHO	World health organisation

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CHAPTER 1: INTRODUCTION

1.1 Neuropeptide Signal Transduction.

The regulation of normal cell proliferation is central to many physiological processes, including embryogenesis, growth and development, haemopoeisis, tissue repair and immune responses. Cell proliferation is controlled by a plethora of growth factors as well as cellular interactions with extracellular matrix proteins in their immediate environment. Differentiated cells are generally maintained in a non-proliferating state but can be stimulated by external factors, to undergo mitosis.

Cancer cells are able to override the normal signals from their surrounding environment, and acquire complete or partial independence from mitogenic control signals through different mechanisms(Westermark and Heldin, 1991; Cross and Dexter, 1991). This process of transformation from a normal cell to a cancer cell and finally to a cancer cell which is able to metastasise, is a multi-step process. Tumour cells are characterised by many mutations which are found to accumulate as the cancer progresses. These mutations appear to occur sequentially and often, but not always, involve tumour suppressor genes and oncogenes. They can occur spontaneously or as a result of exposure to mutagens. In lung cancer, frequent mutations include the genes for the oncogene myc, tumour suppressor protein p53, retinoblastoma protein and the cell cycle inhibitor $p16^{INK}$ (for review see, Braithwaite et al, 1999).

Several cancer cell types can produce growth factors that stimulate unrestrained growth via receptors present on the same cells (autocrine) or on adjacent cells (paracrine). This hypothesis is supported by the observation that cancer cells require fewer exogenous growth factors than normal cells and are able to proliferate in serum free medium. Cancer cells may escape normal regulatory controls by alterations in the number or structure of cellular receptors and changes in the activity of post-receptor signalling pathways. (Seger, 1989). This is supported by the discovery of increasing numbers of oncogenes coding for growth factors, their receptors and post-receptor signalling molecules.

Thus, increased understanding of the growth factors and signal transduction mechanisms which mediate cancer cell growth and the development of drugs that can inhibit these processes are vital if we are to succeed in controlling the disease.

It has become apparent that cell proliferation can be stimulated through multiple, independent signal-transduction pathways which act synergistically (Rozengurt, 1986). At least two major growth factor mediated signal transduction pathways initiate cascades of molecular events leading to proliferation of cultured cells: one involves polypeptide growth factors that bind to receptors with intrinsic tyrosine kinase activity while the other involves receptors coupled to guanine nucleotide binding regulatory proteins (G-proteins). The two pathways are subject to considerable cross talk and transactivation of one pathway by another is a frequent phenomenon.

1.1.1 Neuropeptides

In recent years an increasing number of small regulatory peptides or neuropeptides have been discovered in the neural and neuroendocrine cells of the gastrointestinal tract and central nervous system (Walsh, 1987). In the central and peripheral nervous system they are synthesised and stored in presynaptic neurones and act as fast acting neurotransmitters, while in peripheral neuroendocrine cells they act both systemically as hormones circulating through the blood stream and locally in a paracrine or autocrine fashion. Multiple peptides are found in both neuronal and endocrine cells, and can modulate each other's effects, e.g. bombesin/gastrin-releasing peptide (GRP), stimulates the release of other biologically active peptides and galanin inhibits GRP induced insulin release from the pancreas. The role of these peptides as fast-acting neuro-hormoral signallers has been expanded by the discovery that they also stimulate cell proliferation (Zachary et al., 1987; Rozengurt, 1991). In addition, these neuropeptides are increasingly implicated as growth factors in human cancer. Consequently, it is very important to understand in detail the receptors and signal transduction pathways that mediate the mitogenic action of neuropeptides because they may provide novel targets for therapeutic intervention.

Swiss 3T3 cells are mouse embryonic fibroblasts that have been used as a model system to identify the molecular pathways by which neuropeptides stimulate mitogenesis. These cells readily become quiscent (G_1/G_0 phase of the cell cycle) and can be stimulated by serum or mitogenic factors, to reinitiate DNA synthesis and cell division. Much of the early work of screening neuropeptides for growth promoting activity was carried out in these cells. The list of neuropeptides that

can act as mitogens has now grown considerably and includes bombesin, gastrin releasing peptide, galanin, gastrin, cholycystokinin, bradykinin, endothelin and vasopressin (Woll and Rozengurt, 1989a; Rozengurt, 1990; Rozengurt and Sinnett-Smith, 1990; Sethi et al., 1992).

1.1.2 G protein couped receptors

Neuropeptides signal via G-protein coupled receptors (GPCRs). Approximately 1-2% of coding regions in the mammalian genome belongs to the superfamily of G protein coupled receptors (GRCRs) (reviewed in (Probst et al., 1992)). This family consists of three major sub-types; receptors related to the rhodopsin receptor (Type A), receptors related to the calcitonin receptor (Type B) and receptors related to the metabotrophic receptors (Type C). Of these, the rhodopsin family is the largest and includes β-adrenergic receptors, dopamine receptors, seratonin receptors and receptors for several other neuropeptides. These receptors couple to, and transduce signals via, heterotrimeric GTP binding proteins. The predicted protein structures contain seven stretches of 20-30 hydrophobic amino acids, which are believed to form membrane spanning α-helices. These helices are referred to as transmembrane domains 1-7 (TM1-7). The proteins have extracellular amino termini and cytoplasmic carboxyl termini (Fig 1.1).

The areas of greatest homology among the GPCRs are in the seven transmembrane regions. Proline residues in TM 4, 5, 6, and 7 introduce kinks in the α-helices and may be important in the formation of the binding pocket. Overall there is little sequence homology among the receptors in the first

extracellular domain. However, GPCRs have single conserved cysteine residues in each of the first two extracellular loops that are believed to form a disulphide bond that stabilises the functional protein. The resulting structure is represented in figure 1.2. Different sub-classes of GPCRs bind ligand by different mechanisms, but it seems that all involve the ligand interacting with a binding pocket which is created due to the specific three dimensional arrangement of the receptor protein within the plasma membrane.

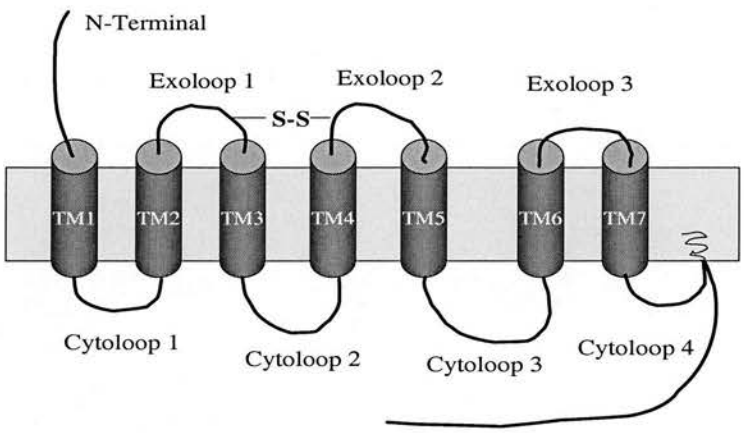


Fig 1.1 Schematic representation of the structure of a G protein coupled receptor. The receptor consists of a single polypeptide chain containing 7 hydrophobic α-helices, which create 7 membrane spanning segments (TM1-7). The remainder of the polypeptide consists of four intracellular, cytoplasmic loops and three extracellular loops. The receptors have an extracellular N-terminal and an intracellular C-terminal.

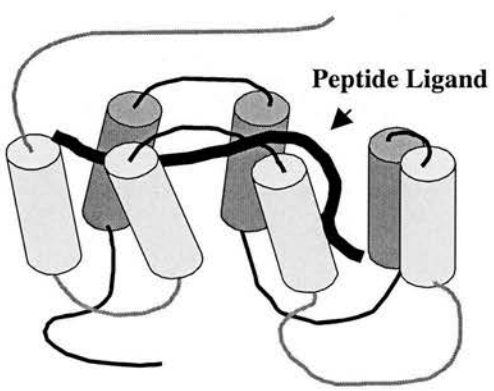


Fig 1.2 Schematic representation of the 3D structure of a GPCR receptor.
The conformation of the 7 transmembrane domains within the membrane provides a binding pocket for peptide ligand interaction.

The endogenous ligands for many GPCRs are peptides. Catecholamines and related biogenic amines are small molecules that bind primarily within the transmembrane domain of their receptors (Strader et al., 1995) but peptide ligands are considerably larger and it is thought that their binding sites may involve extracellular domains in addition to the transmembrane region. Site directed mutagenisis of the substance P (NK1) and bradykinin receptors has revealed that the binding site involves residues of both the extracellular and transmembrane regions (Fong et al., 1992). Electrostatic forces between specific receptor residues and peptide ligands are thought to initially attract the ligand towards the receptor, thus increasing the effective concentration of ligand and therefore increasing the binding probability. Additional interactions occur upon ligand binding and together result in high affinity interaction of the ligand to GPCR. The binding of an agonist to a GPCR, results in a conformational change in the receptor, leading to the formation of a high affinity agonist-receptor-G-protein complex which initiates downstream signalling.

Agonists induce conformational changes upon their receptors. It has been proposed that receptor activation involves protonation of amino acid residues. In many GPCRs, a highly conserved Arg resides in a hydrophilic pocket formed by TM1,2 and 7. The protonation of an Asp residue causes Arg shift out of the pocket leading to the exposure of G-proteins, to previously buried sequences in the second and third intracellular loops. This hypothesis has been backed up by both experimental data and computer simulations (Scheer and Cotecchia, 1997).

In several receptor types it is apparent that GPCRs are maintained in an inactive conformation by stabilising intramolecular interactions. It has been proposed that

important conformational restraints maintain the receptor in an inactive state and that these constraints are released upon agonist binding causing key sequences to be exposed to the G –protein. There are several theories to further explain the mechanics of agonist activation of GPCRs. Perhaps the most widely accepted is the ternary complex model, which accounts for co-operative interactions between agonist, receptor and G-protein (De Lean et al., 1980). The model proposes that a receptor can exist in an equilibrium of functionally distinct states inactive **R**, which does not interact with G-proteins, and active **R***, which activates G proteins and initiates signalling events. The level of basal receptor activity, in the absence of ligand is determined by the equilibrium between **R** and **R***. The efficacy of ligands is determined by their ability to alter this equilibrium. Agonists bind to and stabilise the **R*** form of the receptor, thus increasing its presence, whereas antagonists bind to and stabilise **R**, thus reducing the formation of **R*** (Fig 1.3).

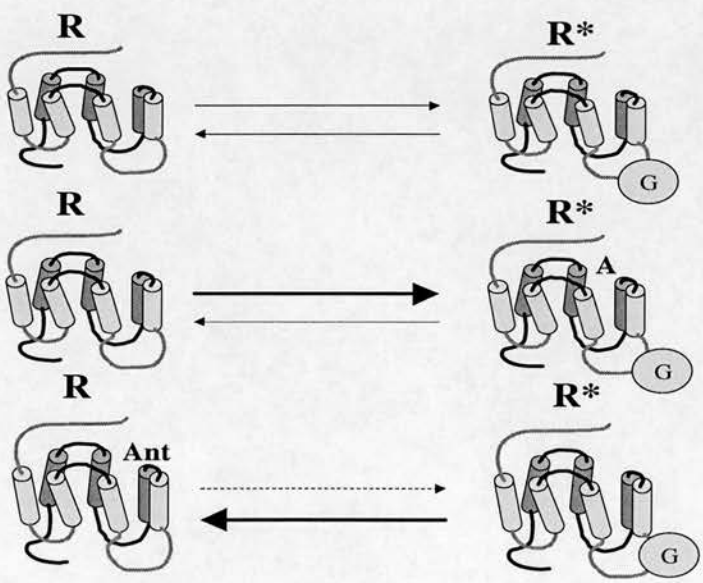


Fig1.3 Ternary complex model of GPCR activation.
Receptors exist in equilibrium between inactive R and active R*. Agonists (A) bind to and stabilise R* allowing increased interaction with G-Proteins. Antagonists (Ant) bind to and stabilise R, decreasing interactions with G-Proteins.

1.1.3 G-PROTEINS

G proteins constitute a large family of highly homologous proteins. The proteins are heterotrimers consisting of α-, β- and γ-subunits. The α-subunit appears to be the most diverse and has traditionally been used to define the purified heterotrimeric proteins. The α–subunit can often account for the primary activity of the G protein. Thus $G_s\alpha$ stimulates adenylate cyclase and transducin α ($G_t\alpha$) activates a cyclic GMP-dependent phosphodiesterase. The G_i proteins were identified as inhibitory regulators of adenylate cyclase. More recently, the G_i and G_o proteins have been implicated in the regulation of several ion channels and G_i has been associated with activation of MAPK pathways and tyrosine kinase pathways. The G_t, G_i and G_o proteins are substrates for pertussis toxin induced ADP-ribosylation; attenuation of hormone action by this toxin implicates the participation of one or more of these proteins. In the basal state the α-subunit contains bound GDP, and association of α- and βγ-subunits is highly favoured. Stimulation of the G protein results when it binds GTP rather than GDP. Receptors interact most efficiently with the heterotrimeric form of the G protein and accelerate activation by increasing the rate of dissociation of GDP and potentially enhancing association of GTP. When activated, the affinity between the α and βγ-subunits of the G protein is decreased. This increases the likelihood of dissociation of subunits and the generation of two potential pathways (α(GTP) and free βγ-subunits) for downstream regulation. Finally, the G protein α-subunit has an intrinsic hydrolytic activity that converts GTP to GDP and returns the G protein to it's inactive form (Fig 1.4)

A mutation has been characterised that interrupts this GTP-driven cycle in the α-chain of G_S, the G-protein that stimulates adenyl cyclase. The mutation inhibits GTPase activity and results in a constitutively activated adenyl cyclase mediated cyclic adenosine monophosphate (cAMP) production. These mutations are commonly found in growth hormone secreting pituitary adenomas (Landis et al., 1989).

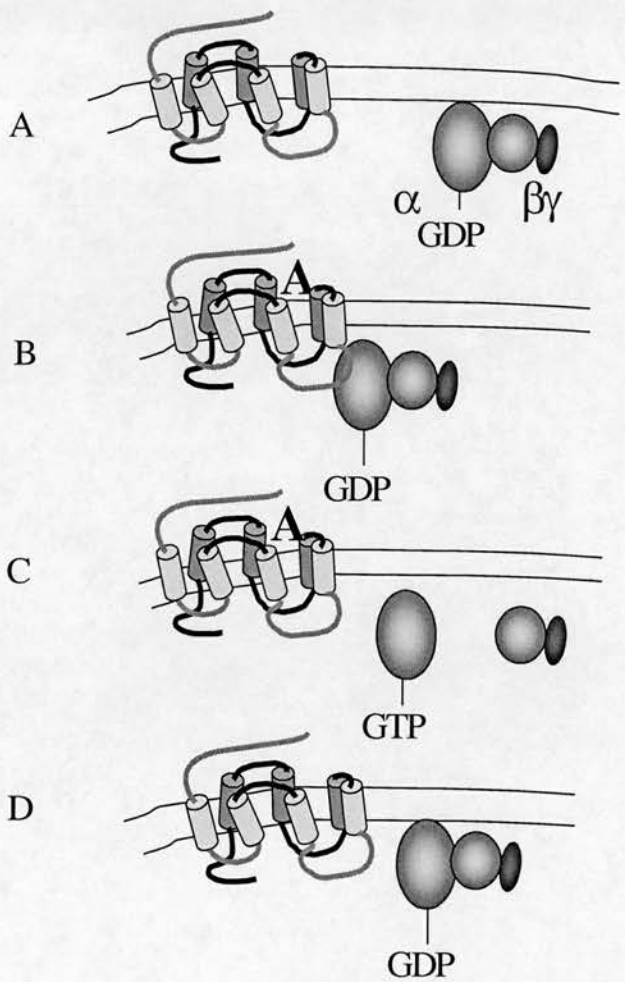


Fig 1.4 Schematic Representation of the Activation of G-proteins by Ligand-Bound Receptor.
A) Without agonist stimulation the receptor state favours no interaction with the G-protein.
B) Binding of the agonist (**A**) induces a conformational change in the receptor which presents previously unexposed residues to the G-protein and promotes G-protein receptor coupling.
C) This event promotes the catalytic exchange of GDP for GTP on the α subunit of the G-protein and results in the dissociation of the heterotrimer into α and β/γ subunits. The free subunits can then interact with various downstream effectors.
D) Intrinsic GTPase activity of the α subunit reverts the α subunit back to its GDP bound form and promotes re-association of the heterotrimer.

1.1.4 BOMBESIN

As discussed, studies in murine Swiss 3T3 fibroblasts have identified many neuropeptides as mitogens. Neuropeptides have been shown to stimulate colony formation in semi-solid media and can induce DNA synthesis and mitogenic signal transduction in various cell types.

The first neuropeptide to be identified as a mitogen was bombesin/GRP. GRP has 9/10 N-terminal amino acids identical to bombesin. Studies with synthetic bombesin-like peptides have demonstrated that full biological activity requires more than 7 but no more than 9 N-terminal amino acids (Heimbrook et al., 1988). Further bombesin-like peptides have been isolated from porcine brain and spinal cord, designated neuromedins (Table 1.1). The human GRP gene is located on chromosome 18 at 18q21 (Naylor et al., 1987), whereas the human neuromedin B gene lies on the long arm of chromosome 15 (Krane et al 1988).

Bombesin-like peptides function both as neurotransmitters and gut hormones (Moody et al., 1983). They are localised to neurones and neuroendocrine cells in the central and peripheral nervous systems (Panula, 1984), although autoradiographic studies in gut suggest that their receptors have a wider distribution.

Infused GRP has a plasma half-life of 2.8 minutes (Knigge et al., 1984). It causes secretion of gastrin, pancreatic polypeptide, insulin, glucagon, cholecystokinin and gastric inhibitory peptide, leading to amylase and gastric acid secretion (Knigge et al., 1984). GRP and its receptor are abundant in the hypothalamus, and this has lead to speculation that it may regulate pituitary hormone secretion.

In support of this, GRP infusion has been shown to stimulate the secretion of ACTH, cortisol and endorphin in normal subjects (Knigge et al., 1987).

Table 1.1 Amino-acid sequence of bombesin like peptides.

MAMMALIAN															
GRP(1-27) Human		Pro	Leu	Pro	Ala	Gly	Gly	Gly	Thr	Val	Leu	Thr	Lys	---	
-----	Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
GRP(1-27) Porcine	Ala	Pro	Val	Ser	Val	Gly	Gly	Gly	Thr	Val	Leu	Ala	Lys	---	
-----	Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
GRP(14-27) Porcine	Met	Tyr	Pro	Arg	Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
GRP10 (NeuromedinC)					Gly	Asn	His	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
Neuromedin B					Gly	Asn	Leu	Trp	Ala	Thr	Gly	His	Leu	Met	NH_2
AMPHIBIAN															
Bombesin	pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
Bombesin(8-14)								Trp	Ala	Val	Gly	His	Leu	Met	NH_2
Ranatensin				pGlu	Val	Pro	Gln	Trp	Ala	Val	Gly	His	Leu	Met	NH_2
Litorin						pGlu	Gln	Trp	Ala	Val	Gly	His	Leu	Met	NH_2

1.1.5 BOMBESIN/GRP SIGNAL TRANSDUCTION.

In serum-free medium bombesin stimulates DNA synthesis and cell division in the absence of other growth-promoting agents. The ability of bombesin, like platelet-derived growth factor (PDGF), to act as a sole mitogen for these cells contrasts with other neuropeptide growth factors which are active only in synergistic combinations (Rozengurt, 1986). The mitogenic effects of bombesin are markedly potentiated by insulin, which both increases the maximal response and reduces the bombesin concentration required for half-maximal effect (Rozengurt and Sinnett-Smith, 1983). The cause-effect relationships and temporal organisation of the early signals and molecular events induced by bombesin provide a model for the study of other mitogenic neuropeptides and illustrate the activation and cross talk of a variety of signalling pathways (Rozengurt, 1998).

1.1.5.1 Bombesin/GRP Receptors

Bombesin and GRP bind to a single class of high affinity receptors. The bombesin/GRP receptor has been cloned and sequenced (Battey et al., 1991). The deduced amino acid sequence predicts a polypeptide core of Mr 43000 and demonstrates that it belongs to the super-family of G protein coupled receptors.

1.1.5.2 Inositol Phospholipid Turnover, Ca^{2+} Mobilisation and Activation of Protein Kinase C.

Binding of bombesin/GRP to its receptor initiates a cascade of intracellular signals (Fig 1.5) culminating in DNA synthesis 10-15 h later. One of the earliest events to

occur after the binding of bombesin to its specific receptor is the activation of the heterotrimeric G-protein, Gq. The released GTP bound Gqα subunit activates phospolipase C β (PLC-β) which catalyses the hydrolysis of the membrane phospholipid phosphatidyl inositol 4,5 bisphosphate (PIP2) to produce the second messengers, inositol 1,4,5- trisphosphate (IP3) and diacylglycerol (DAG) (Exton, 1996). IP3 stimulates the release of calcium from intracellular stores in the endoplasmic reticulum via the binding of ligand-gated Ca^{2+} channels (Mikoshiba, 1997) and DAG directly activates several isoforms of protein kinase C (Nishizuka, 1995). These two events are implicated in the stimulation of DNA synthesis in several cell types (Charlesworth and Rozengurt, 1994). Both the mobilisation of intracellular calcium and the production of DAG activate PKC and as a result, bombesin stimulates the translocation of the PKC substrate myrisolated alanine rich C kinase substrate (80K/MARCKS) from the membrane to the cytoplasm (Zachary and Rozengurt, 1986). 80K/MARCKS binds calmodulin in a Ca^{2+} dependent manner, which can be prevented by PKC phosphorylation of 80K/MARCKS (the calmodulin-binding domain turned out to be identical to the phosphorylation site domain). Activation of PKC and increases in $[Ca^{2+}]_i$ could regulate the interaction of 80K with both the cytoskeleton and the plasma membrane, and 80K/MARCKS may play a suppressor role in the control of cell proliferation. (reviewed in (Blackshear, 1993). Bombesin stimulation of PKC also results in the activation of the PKC substrate, protein kinase D (PKD) (Matthews, 1997). The activation of PKD represents a novel kinase cascade downstream of PKC.

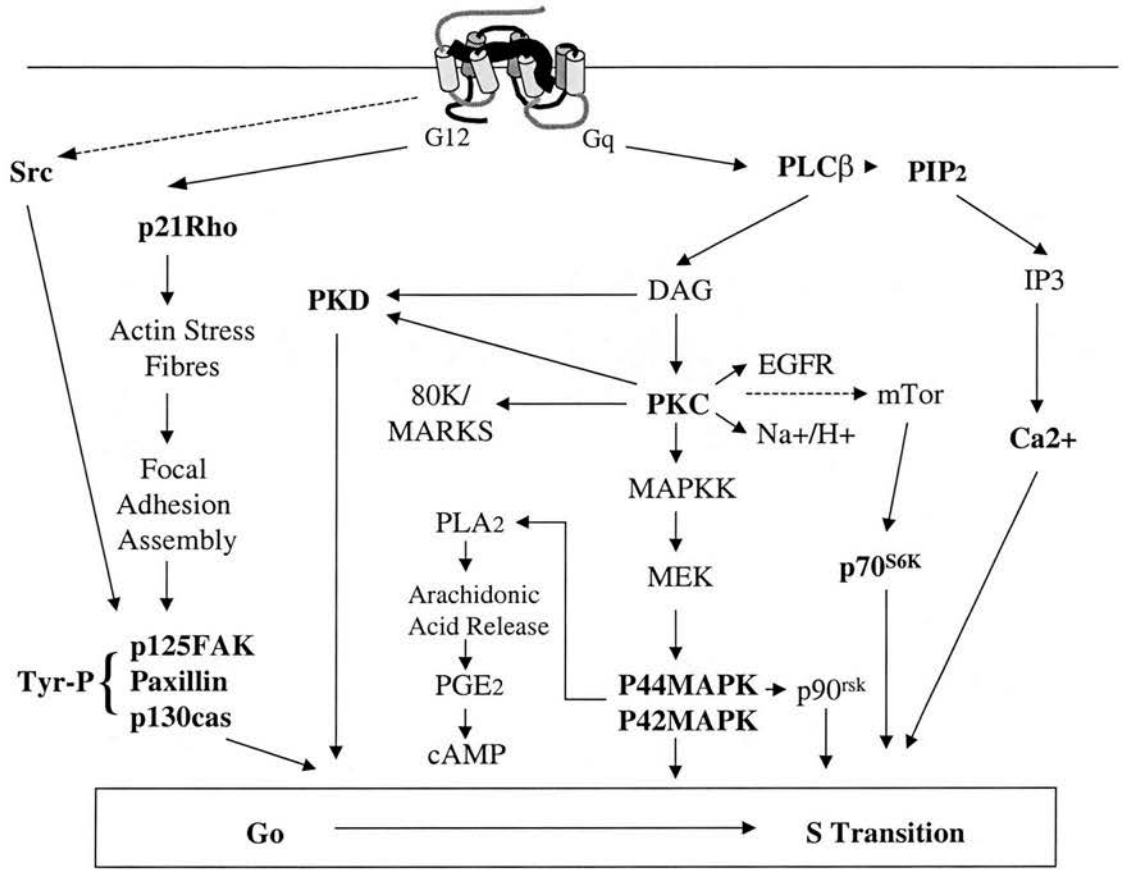


Fig 1.5 Bombesin Mediated Mitogenic Signal Transduction Pathways. See text for details and abbreviations.

1.1.5.3 Activation of MAPK by Bombesin.

PKC plays a role in transducing neuropeptide signals into activation of serine/threonine protein kinase cascades including p42MAPK/p44MAPK and protein kinase D (PKD)

The activation of the MAPK cascade relays mitogenic signals to the nucleus (Khokhlatchev et al., 1998) resulting in the activation of transcription factors and cell cycle progression. The activated MAP kinase directly phosphorylates transcription factor regulators which results in the increased expression of c-*fos*

and c-*myc* (Treisman, 1992). However, neither direct activation of PKC by phorbol esters nor addition of vasopressin stimulate a maximal increase in c-*fos* mRNA levels. It is likely that the induction of c-*fos* by bombesin is mediated by the co-ordinated effects of PKC activation, Ca^{2+} mobilisation and an additional pathway dependent on arachidonic acid release (Rozengurt, 1991).

P42 (Erk2) and P44 (Erk1) mitogen activated protein kinases (MAPK) are activated by phosphorylation of both tyrosine and threonine residues by the cytoplasmic dual specific kinase, MEK (Seger and Krebs, 1995). The MAPK cascade can be activated by a wide range of mitogenic stimuli including the activation receptor tyrosine kinases and the activation of G-protein coupled receptors. Bombesin has been shown to potently stimulate MAPK via its Gq coupled receptor in several cell types. In Swiss 3T3 cells this MAPK activation has been shown to be via the activation of PKC and does not appear to involve Ras (Mitchell et al., 1995; Seufferlein and Rozengurt, 1996b). This distinguishes bombesin mediated MAPK activation from that via Gi and tyrosine kinase receptors. The mechanisms by which MAPK is activated by GPCRs is dependent more so, upon cell type than on the nature of the stimuli (Della Rocca et al., 1997). Accordingly, it has been found that in Rat-1 fibroblasts transfected with the bombesin receptor, MAPK is activated by bombesin via a PKC independent and Ras dependent pathway (Charlesworth and Rozengurt, 1997). This demonstrates that the same GPCR can activate MAPK via different pathways depending on cellular context.

In addition to the above pathways leading to MAPK activation, it has also been shown that GPCRs are able to transactivate EGF receptor tyrosine kinases which

leads to the Ras dependent activation of Raf and subsequent MAPK stimulation(Daub et al., 1997).

1.1.5.4 Bombesin stimulation of p70 S6 Kinase.

The activation and phosphorylation of the serine, threonine kinase, p70 S6 kinase occurs in response to many mitogenic stimuli. Bombesin stimulates the activation of p70 S6 kinase via a PKC dependant pathway (Withers et al., 1997) although the precise mechanism is unknown. It is thought that the activation of p70 S6 Kinase by bombesin is dependent upon the activity of the RAFT1/FRAP/mTor complex (Thomas and Hall, 1997). RAFT1 shares homology with the catalytic domain of PI3K and is implicated in the regulation of mitogenisis. The drug rapamycin, blocks the activity of the RAFT1 containing complex and inhibits DNA synthesis by bombesin in Swiss 3T3 cells (Withers et al., 1997).

1.1.5.5 Bombesin Stimulation of Arachidonic Acid Release and Prostaglandin Synthesis.

While bombesin/GRP stimulates DNA synthesis in the absence of other factors, vasopressin is mitogenic for Swiss 3T3 cells only in synergistic combination with other factors e.g. insulin (Rozengurt et al., 1979; Rozengurt and Sinnett-Smith, 1983). Binding of vasopressin to its G-protein coupled receptor in quiescent cultures of Swiss 3T3 cells, causes a rapid production of $Ins(1,4,5)P_3$, mobilisation of Ca^{2+} from intracellular stores and sustained activation of PKC (Rozengurt, 1991). Independent signal-transduction pathways act synergistically

in the initiation of DNA synthesis, hence, the ability of bombesin to act as a sole mitogen could be due to activation of a signalling pathway not stimulated by vasopressin.

Bombesin, but not vasopressin, has been shown to induce a marked, biphasic release of arachidonic acid into the extracellular medium (Millar and Rozengurt, 1990). A first phase involves rapid activation of phospholipase A_2 (PLA_2). The major phase of arachidonic acid mobilisation begins 20 min after the addition of ligand. The stimulation of arachidonic acid release by bombesin is likely to contribute to bombesin-induced mitogenesis because arachidonic acid has been shown to potentiate mitogenesis induced by agents that stimulate polyphosphoinositide breakdown but not arachidonic acid release, e.g. vasopressin. Arachidonic acid released by bombesin is converted into E-type prostaglandins which enhance cAMP accumulation in the cell (Millar and Rozengurt, 1990). Since elevated cAMP levels constitute a mitogenic signal for Swiss 3T3 cells (reviewed in (Rozengurt, 1991)) it is likely that part of the mitogenic effects of bombesin are mediated via this pathway.

1.1.5.6 Bombesin Stimulation of Tyrosine Kinase Activity

The receptor for peptides of the bombesin family does not possess intrinsic tyrosine kinase activity. However, bombesin has been shown to rapidly increase tyrosine phosphorylation of multiple substrates in intact quiescent Swiss 3T3 cells (Zachary et al., 1991). Vasopressin bradykinin, gastrin, CCK and endothelin elicit a similar response (Seufferlein and Rozengurt, 1995).

One of the prominent tyrosine phosphorylated proteins in bombesin and other neuropeptide stimulated Swiss 3T3 cells is the non-receptor protein tyrosine kinase focal adhesion kinase (p125FAK) (Zachary and Rozengurt, 1992). Phosphorylated p125FAK presents a high affinity binding site for the SH2 domains of the Src family kinases which further activates p125FAK and results in the binding of other signalling or scaffolding molecules (Parsons and Parsons, 1997). The focal adhesion proteins paxillin and p130cas (Sakai et al., 1994) are adapter proteins which promote the assembly of, p125FAK containing, signalling complexes. Neuropeptides have been shown to induce the phosphorylation of paxillin and p130cas and promote the formation of a complex between p130cas and the proto-oncogene c-Crk (Zachary et al., 1993). This event may be integral in the mitogenic effects of neuropeptides as c-Crk can activate a number of signalling molecules, including Rap-1, which is a small GTP-binding protein which can stimulate mitogenisis in Swiss3T3 cells (Casamassima and Rozengurt, 1997). Accordingly it has been shown that inhibitors of tyrosine kinases can inhibit bombesin stimulation of DNA synthesis in Swiss 3T3 cells by 60% (Seckl and Rozengurt, 1993).

Neuropeptides stimulate the tyrosine phosphorylation of FAK via a pathway that is largely independent of PKC and intracellular calcium flux (Sinnett-Smith et al., 1993). This suggests that bombesin stimulation of tyrosine phosphorylation occurs via a pathway which is independent of the Gq mediated PLC pathway. The prevention of focal adhesion assembly via disruption of the actin cytoskeleton by cytochalesin D, prevents bombesin mediated phosphorylation of p125FAK (Rozengurt et al., 1995) indicating that focal adhesion assembly is

upstream of bombesin mediated p125FAK phosphorylation. GPCR agonists promote the formation of actin stress fibres and focal adhesions and this is thought to be via the small G protein, Rho (Tapon and Hall, 1997). Inhibition of Rho by treatment with *C. botulinum* C3 exoenzyme inhibits bombesin stimulation of p125FAK, paxillin and p130Cas (Rankin et al., 1994; Zachary et al., 1993).

It has been suggested that the mechanism by which GPCRs stimulate the formation of focal adhesions and subsequent tyrosine phosphorylation of focal adhesion proteins, involves the coupling of the receptor to the G12 family of heterotrimeric G-proteins (Buhl et al., 1995). In other words, the bombesin receptor couples to both a Gq mediated PLC pathway involving calcium mobilisation and PKC activation, and to a G12 mediated pathway which stimulates focal adhesion formation via the activation of Rho.

In addition to the stimulation of tyrosine kinases via focal adhesion associated proteins, bombesin and other neuropeptides have been shown to modulate tyrosine kinase receptor activity. Classical receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) receptor are single transmembrane proteins that dimerise and transphosphorylate upon ligand binding. The mechanisms by which GPCRs transactivate are unclear but it has been suggested that the resulting phosphorylated EGFR, recruits SHC to the plasma membrane where this adaptor protein is tyrosine phosphorylated by Src family kinases resulting in the activation of the MAPK cascade (Zwick et al., 1999). It has been shown that, in COS7 cells, bombesin is capable of stimulating the tyrosine phosphorylation of SHC via EGF receptor activation (Daub et al., 1997).

1.1.6 Other Neuropeptide Growth Factors.

1.1.6.1 Vasopressin.

Vasopressin or antidiuretic hormone (ADH) is a cyclic nonapeptide synthesised in the supra-optic nucleus of the hypothalamus, ADH containing vesicles pass down the neural axons to the posterior pituitary before being secreted into the circulation. Vasopressin is thus found in the brain and peripheral organs. Two types of vasopressin receptor have been distinguished functionally and pharmacologically (Huffman et al., 1988). Stimulation of the renal V2 receptor by vasopressin results in H_2O reabsorption and induction of cAMP in collecting ductules. The majority of the other functions of vasopressin are mediated via the V1A receptor. These functions include blood vessel constriction, liver glycogenolysis, platelet adhesion and various brain functions. The V1B receptor mediates stimulation of pituitary corticotrophin release. Reviewed in (Zingg, 1996)

The V_1 receptors mediate their vascular and hepatic effects of vasopressin by activating inositol phosphate turnover. The V2 receptors are coupled to adenylate cyclase and mediate antidiuretic responses of the kidney. Vasopressin has been demonstrated to act as a mitogen for Swiss 3T3 cells (Rozengurt et al., 1979). It has been shown, in this context, to bind to specific high affinity receptors (Collins and Rozengurt, 1984) of the V_1 type (Zachary and Rozengurt, 1986). Although vasopressin alone does not stimulate cell proliferation in serum free medium, it acts synergistically with insulin, serum, EGF and PDGF at nanomolar concentrations. Like bombesin, vasopressin causes a rapid production of Ins(1,4,5)P3, mobilisation of Ca^{2+} from intracellular stores and sustained activation of PKC via a G-protein linked transduction pathway (reviewed in

(Rozengurt, 1991). Vasopressin has also been shown to rapidly increase tyrosine phosphorylation of multiple substrates including p125FAK, in quiescent Swiss 3T3 cells (Zachary et al., 1991).

1.1.6.2 Bradykinin.

The nonapeptide bradykinin is generated in the plasma or tissues from large molecular weight precursors (kininogens) by the action of kallikreins, which are activated during proteolysis and clotting. Bradykinin is usually present in plasma at very low concentrations, due to its rapid degradation by carboxypeptidase N and angioconverting enzyme. Bradykinin is one of the most potent pain producing substances and receptors have been localised to the nocioceptive sensory pathways, where it acts as a neurotransmitter (Steranka et al., 1989). Bradykinin is implicated in smooth muscle contraction, vasodilatation and vascular permeability. There are at least two bradykinin receptor subtypes: B_1 and B_2. Bradykinin in the presence of insulin is mitogenic for Swiss 3T3 cells and this is mediated through a B_2 receptor (Woll and Rozengurt, 1988). Bradykinin rapidly mobilises $[Ca^{2+}]_i$ and stimulates phosphorylation of 80K/MARCKS. However, in contrast to bombesin and vasopressin the stimulation of 80K/MARCKS phosphorylation by bradykinin is slower, reaching a maximum after 1 min of incubation, and then rapidly decreases to almost basal levels. Furthermore, bradykinin does not induce PKC mediated events or enhancement of cAMP accumulation. Bradykinin induces rapid accumulation of total inositol phosphates, but in contrast to bombesin and vasopressin which stimulates a linear increase in inositol phosphate accumulation over a 10-min

period (Issandou and Rozengurt, 1990). In neuronal cells, bradykinin has been shown to induce transactivation of the EGF receptor and subsequent Ras/MAPK activation via a calcium dependent mechanism (Zwick et al., 1997).

1.1.6.3 Neurotensin.

Neurotensin is a 13 amino acid polypeptide which is present in the hypothalamus and in the mucosal endocrine cells of the ileum. It is a neuromodulator of dopamine transmission and of anterior pituitary hormone secretion. It exerts potent hypothermic and analgesic effects in the brain (Vincent et al., 1999). Neurotensin, in the presence of EGF, stimulates DNA synthesis in hepatocytes (Carr et al., 1992) and has been implicated as a growth factor in several cancers (discussed later).

1.1.6.4 Gastrin and Cholecystokinin.

Gastrin and cholecystokinin (CCK) share a common C-terminal pentapeptide and bind to at least two different receptors (Jensen et al., 1989). The CCKB/Gastrin receptors, which are found mainly in the central nervous system and in the gastrointestinal tract, bind both CCK and gastrin with equal affinity whereas the CCKA receptors preferentially bind CCK, with a 400 fold increase in affinity for CCK than for gastrin (Jensen et al., 1989).

CCK exerts trophic effects on normal pancreas and human pancreatic cancers via the mobilisation of intracellular calcium (Smith et al., 1991) and has mitogenic effects in other cell types. Gastrin exists in multiple forms including penta-gastrin (G14), big-gastrin (G34) and the most common gastrin –1 (G17). Gastrins are found in the

proximal duodenum and gastric antrum where they are secreted by neuroendocrine G-cells. Gastrin release stimulates gastric acid secretion, gastric motility and contraction of the lower oesophageal sphincter. In addition gastrin is found in the hypothalamus and pituitary where it may act as a neurotransmitter (Walsh, 1987). Gastrin exerts growth promoting effects in the digestive tract and exocrine pancreas (Johnson, 1984). Gastrin has been identified as a growth factor for several cancers (discussed later).

1.1.6.5 Galanin

Galanin is a 29 amino acid peptide first isolated from porcine intestine (Tatemoto et al., 1983) and is widely distributed in central and peripheral neurones (Rokaeus, 1987). Its biological responses include the modulation and release of several hormones (Fisone et al., 1987), the stimulation of smooth muscle contractility and inhibition of neuronal excitation (Ekblad et al., 1985). In the endocrine pancreas, galanin inhibits the release of insulin (Ahren et al., 1988) and blocks the mobilisation of intracellular calcium via a pertussis sensitive G-protein (Dunne et al., 1989). It has been shown that galanin receptors couple negatively to adenylate cyclase through this G protein (Ahren et al., 1988). Galanin can also act as a mitogen. This was demonstrated by its ability to stimulate the mobilisation of intracellular calcium and proliferation in SCLC cells (Woll and Rozengurt, 1989b; Sethi and Rozengurt, 1991b; Sethi and Rozengurt, 1991a). Galanin has subsequently been shown to activate MAPK in SCLC cells (Seufferlein and Rozengurt, 1996a).

1.1.6.6 Tachykinins.

Table 1.2 Amino Acid sequences of tachykinins.

Substance P	Arg	Pro	Lys	Pro	Gln	Gln	Phe	Phe	Gly	Leu	Met	NH_2
Substance K: (neurokinin A)		His	Lys	Thr	Asp	Ser	Phe	Val	Gly	Leu	Met	NH_2
Neurokinin K: (neurokinin B)		Asp	Met	His	Asp	Phe	Phe	Val	Gly	Leu	Met	NH_2

The three mammalian tachykinins illustrated above (Table 1.2) have similar biological activities associated with their shared carboxy-terminal sequence, but bind to distinct receptors. Substance P and substance K are derived from the same gene by alternative splicing. The tachykinins are widely distributed in the brain (hypothalamus, trigeminal ganglion, caudate nucleus and olfactory bulb), the spinal cord and gut neurones. The best studied is the undecapeptide substance P, which is synthesised in the dorsal root ganglia and transported axoplasmically to be stored in the sensory nerve endings. A variety of stimuli lead to its release, causing local pain, smooth muscle spasm and vasodilatation (Payan, 1989).

The tachykinin receptors have been characterised pharmacologically (Regoli et al., 1989; Snider et al., 1991). The receptors for substance P, substance K and neuromedin K have been designated as NK-1 (substance P), NK-2 (substance K) and NK-3 (neuromedin K).

Substance P has been shown to have mitogenic effects on T lymphocytes, at concentrations as low as 100pM, mediated through it's specific receptor (Payan et

al., 1984). Tachykinins have been shown to stimulate growth of human skin fibroblasts, arterial smooth muscle cells and keratinocytes (Nilsson et al., 1985)

1.2 Neuropeptides as Cancer Growth Factors

1.2.1 Neuropeptides as Growth Factors in Lung Cancer

The neuroendocrine nature of SCLC has been long recognised on morphological grounds (presence of neurosecretory granules), as well as biochemical grounds. The first peptide documented to be produced by SCLC was bombesin/GRP. The current list is extensive (Table 1.3) and can explain the many symptoms of ectopic hormone production that small cell lung cancer patients suffer.

In addition many of these bioactive peptides can be produced by normal lung (Becker, 1985). Many of these peptides are synthesised as pro-hormones that acquire biological activity only after specific post-translational modifications (Quinn et al., 1991).

The effects of a range of peptides were investigated by (Woll and Rozengurt, 1990). The ability of 32 neuropeptides and hormones to stimulate mobilisation of intracellular calcium was examined in 5 small cell lung cancer cell lines. (Table 1.4)

Table 1.3: Peptides and Hormones Secreted by SCLC.

ACTH	(Becker et al., 1984)
Atrial natriuretic peptide	(Bliss et al., 1990; Gross et al., 1993)
Calcitonin gene related product	(Bepler et al., 1988)
CCK	(Moody et al., 1988; Rehfeld et al., 1989)
Chorionic gonadotrophin	(Sorenson et al., 1981; Gazdar and Carney, 1984)
FSH	(Sorenson et al., 1981)
GRP	(Moody et al., 1981; Erisman et al., 1982)
Gastrin	(Gazdar and Carney, 1984; Rehfeld et al., 1989)
GM CSF	(Abe et al., 1984)
Growth hormone	(Sorenson et al., 1981)
Glucagon	(Sorenson et al., 1981; Bepler et al., 1988)
IGF-I	(Macaulay et al., 1990; Reeve, 1991)
Lipotrophin	(Sorenson et al., 1981; Abe et al., 1984)
Neuromedin B	(Giaccone et al., 1992; Cardona et al., 1991)
Neurotensin	(Bepler et al., 1988; Moody et al., 1985a)
Opioid peptides	(Roth and Barchas, 1986)
Oxytocin	(Maurer, 1985; Sorenson et al., 1981)
Parathyroid hormone	(Yoshimoto et al., 1989; Sorenson et al., 1981)
Physalaemin	(Lazarus and Hernandez, 1985)
Prolactin	(Sorenson et al., 1981)
Serotonin	(Sorenson et al., 1981)
Somatostatin	(Wood et al., 1981)
Vasopressin	(North et al., 1980; Gross et al., 1993)

Table 1.4: The effect of multiple peptide hormones and neuropeptides on [Ca2+]i mobilisation in SCLC cell lines (adapted from (Woll and Rozengurt, 1990)).

EFFECTIVE	NON EFFECTIVE
Bradykinin	ACTH
Cholecystokinin	Angiotensin I, II and III
Gastrin	Calcitonin
Galanin	Chorionic Gonadotrophin
Bombesin	Dynorphin
Gastrin Releasing Peptide	α-endorphin
Neurotensin	Endothelin
Vasopressin	Epinephrine
	Follicle Stimulating Hormone
	GHRH
	GIP
	Glucagon
	5-Hydroxytryptamine
	Leu-enkephalin
	Neuropeptide-Y
	Parathyroid Hormone
	Substance K
	Substance P
	TRH

1.2.1.1 Gastrin-releasing peptide.

Willey et al (Willey et al., 1984) reported that bombesin and GRP acted as growth factors for cells derived from explants of normal human bronchial epithelium cells. Embryonic mouse lungs express bombesin receptors during

branching morphogenesis. Treatment with bombesin increases lung branch formation and can be inhibited by [Leu^{13}-psi(CH_2N_2)Leu^{14}]-bombesin, a specific bombesin antagonist (Wang et al., 1996; Aguayo et al., 1994). In a rhesus monkey model of lung development GRP mRNA was detected in embryos between 63 and 80 days of gestation. Immunoreactivity for bombesin-like peptides is usually localised to neuroendocrine cells but during this stage of gestation GRP was also detected in cells of budding airways. In this study treatment with bombesin resulted in increased size and number of airways and cultured airway epithelial cells could be stimulated to undergo DNA synthesis by bombesin treatment (Li et al., 1994).

In the normal, adult lung, secretion of GRP by pulmonary neuroendocrine cells occurs in response to alterations in pulmonary oxygenation, such as those associated with birth (increased oxygenation) or with chronic obstructive airways disease (decreased oxygenation) (Schuller et al., 1991). Many of these pulmonary diseases are associated with smoking. Increased secretion of GRP is accompanied by pulmonary neuroendocrine cell hyperplasia (Schuller, 1994). Siegfried et al (Siegfried et al., 1993) investigated the responses of human bronchial epithelial (HBE) cells from 13 donors. It was found that GRP/bombesin stimulated colony formation in 8 of the cell lines. The same group later investigated expression of bombesin receptor subtypes in HBE cells and found that increased expression of these receptors correlated to a history of cigarette smoking. Cell lines from donors that smoked for more than 25 pack-years (number of years smoking multiplied by the number of packs smoked per day) could be stimulated to proliferate in response to bombesin (Siegfried et al.,

1997). In support of this hypothesis, transfection of GRP-R into a human bronchial epithelial cell line resulted in the ability of bombesin to stimulate mitogenesis (al Moustafa et al., 1995). Elevated levels of GRP have been found in broncho-alveolar lavages of normal smokers compared to non-smokers (Aguayo et al., 1990). The high incidence of SCLC in smokers with a history of chronic obstructive lung disease, and the induction of bombesin-producing carcinoids in hamsters by simultaneous exposure to hyperoxyia and carcinogenic nitrosamines (Schuller et al., 1991), implies a role for GRP and abnormal oxygenation in the initiation of smoking related tumours. Curiously, in a study of schizophrenic patients, who are often very heavy smokers, it was found that plasma bombesin levels in smokers were significantly lower than levels found in non-schizophrenic smokers. In fact schizophrenic patients had lower serum bombesin levels than non-schizophrenic patients, irrespective of smoking history. These findings may help explain the lower incidence of lung cancer seen in schizophrenic smokers (Olincy et al., 1999).

Bombesin-like peptides were first detected in SCLC in the early 1980s (Moody et al., 1981). Demonstration of pre-pro GRP mRNA, pro-bombesin C-terminal peptide and multiple GRP gene associated peptides in SCLC confirm that the GRP and related peptides originate in these tumours (Sunday et al., 1991; Sausville et al., 1986; Cuttitta et al., 1988). GRP secretion by SCLC can be stimulated by treatment with secretin or vasoactive intestinal peptide (Korman et al., 1986).

Currently a major factor in diagnosis of SCLC is detection of bombesin-like immunoreactivity as well as other markers of neuroendocrine differentiation. In

a recent study an enzyme immunoassay was developed to detect ProGRP, the more stable precursor of GRP. Sera of 87 SCLC patients were tested for the presence of proGRP. Using this assay 45% of SCLC samples were correctly identified with no false positives in a reference group of 75 sera samples of patients with benign lung disease (Stieber et al., 1999).

In addition to secreting bombesin-like peptides, SCLC also express bombesin receptors, as demonstrated by specific binding of [^{125}I-Tyr^{4}] bombesin to SCLC (Moody et al., 1985a). This was the first suggestion that GRP may act as an autocrine growth factor for SCLC. Subsequently, several studies have confirmed the presence of bombesin receptors on SCLC cells. The presence on lung cancer cells of three structurally and pharmacologically distinct bombesin receptor subtypes has been demonstrated. These subtypes are GRPR, neuromedin B receptor (NMB-R) and the bombesin receptor subtype 3 (BRS-3) (Fathi et al., 1996; DeMichele et al., 1994b). Toi-scott et al (Toi-Scott et al., 1996) showed the presence of GRP-R in 17/20 SCLC cell lines. 11/20 cell lines expressed Neuromedin B receptor and 5/20 expressed bombesin receptor subtype-3. The various subtypes of this family of receptors shall be further discussed in another section.

In a recent study by Shriver et al the authors compared GRP-R expression in airway cells and tissues of males and females. They discovered that GRP-R expression was significantly higher in samples from non-smokers and short term smoking females than in corresponding males (75% compared to 20%). Additionally, female smokers showed expression of GRP-R earlier in their smoking history than male smokers (37 compared to 59 pack-years). This may

be due to the fact that the gene for GRP-R is located on the X chromosome and is not susceptible to X inactivation. This means that females possess 2 copies of the gene whereas men only have one. These findings may explain the increased incidence of smoking related lung cancer in women (Shriver et al., 2000).

GRP/bombesin has been shown to act as growth factors in SCLC *in vitro* and *in vivo*. Weber et al (Weber et al., 1985) reported that GRP enhanced DNA synthesis in 2 SCLC cell lines. In another study, colony formation in 9/10 SCLC cell lines was stimulated up to 150-fold by GRP, with maximal effects at 50nM (Carney, 1987). However, there was no correlation between amounts of GRP secreted, response to exogenous GRP, and the number of binding sites in individual cell lines. Growth of SCLC xenografts (NCI-H69) was reported to be increased 77% above control in nude mice treated three times daily with intraperitoneal injections of bombesin (Alexander et al., 1988).

The hypothesis of autocrine growth stimulation by GRP in SCLC was tested by Cuttitta et al. (Cuttitta et al., 1985), using a monoclonal antibody to [Lys^3] bombesin (2A11). It inhibited the clonal growth of 2 SCLC cell lines in serum free medium, and retarded the growth of one growing as xenografts in nude mice. This antibody has recently been in a phase 1 clinical trial and showed no dose limiting toxicity and tumour uptake in 11/12 patients (Chaudry et al., 1999).

GRP has been shown to activate PLC in SCLC, both in metabolically labelled whole cell and membranous preparations (Trepel et al., 1988). This activation is accompanied by an elevation of $[Ca^{2+}]_i$ (Heikkila et al., 1987). GRP stimulation of $InsP_3$ and increase in $[Ca^{2+}]_i$ were inhibited by prior treatment with PKC activator phorbol 12-myristate 13-acetate, suggesting that PKC might exert

negative feedback regulation on this response. Because non-hydrolysable GTP analogues could modulate PLC activation in response to GRP (Sharoni et al., 1990; Trepel et al., 1988), it was concluded that the GRP receptor in SCLC was coupled to PLC by a G-protein. The GRP receptor and neuromedin B receptors have been cloned from SCLC cells and two consensus PKC phosphorylation sites are conserved in these receptors (Corjay et al., 1991). Bombesin has been shown to potently activate MAPK in SCLC with maximal activation at 6 minutes (Seufferlein and Rozengurt, 1996). Draoui et al showed that stimulation of 2 SCLC cells with 10nM bombesin induced expression of c-fos and c-jun transcription factors indicating that bombesin can regulate nuclear oncogene expression (Draoui et al., 1996). It has also been shown that bombesin can stimulate tyrosine phosphorylation and tyrosine kinase activity in SCLC cell lines (Tallett et al., 1996). Phosphorylation of a 120kd protein which was identified as focal adhesion kinase (FAK) was observed. The same effects were observed with stimulation using other neuropeptides (bradykinin, gastrin and neurotensin) indicating a common signalling pathway in SCLC which is utilised by multiple neuropeptides. (Tallett et al., 1996).

It seems likely that SCLC may be stimulated to grow by the normal intracellular signals evoked by ligand-dependent activation of bombesin peptide receptors. However, in a study by Woll and Rozengurt (Woll and Rozengurt, 1989b) GRP did not stimulate an increase in $[Ca^{2+}]_i$ in all SCLC cell lines, which suggests that in some SCLC cell lines, unrestrained growth is due to other factors and not a GRP mediated autocrine growth loop.

1.2.1.2 Vasopressin.

In 1985 vasopressin, in addition to bombesin, was identified as a chemoattractant of SCLC (Ruff et al., 1985) suggesting a role for vasopressin in SCLC metastasis. Subsequent work suggested a role for vasopressin as a growth factor for SCLC cells. Vasopressin does not seem to be a growth factor for human bronchial epithelial (HBE) cells. In a study by Franknell et al 0/6 HBE cells responded by mobilisation of intracellular calcium to vasopressin stimulation (Frankel et al., 1994).

Vasopressin is secreted by up to 65% of SCLC (North et al., 1980; Sorenson et al., 1981) although some studies have suggested a lower frequency of expression. Verbeeck et al (Verbeeck et al., 1992) detected vasopressin mRNA in only 3/26 SCLC lines and only one expressed both vasopressin and vasopressin receptor mRNA suggesting that autocrine growth loops involving vasopressin may not be as common as those involving bombesin/GRP. Vasopressin at nanomolar concentrations increased $[Ca^{2+}]_I$, in all SCLC cell lines tested, through V_1 receptors (Woll and Rozengurt, 1989b; Bunn et al., 1992). The presence of V1A receptors in SCLC has been confirmed recently by North et al (North et al., 1997) and in addition, V1B and V2 receptors were detected. The presence of multiple subtypes of vasopressin receptors indicates a multifaceted role for this peptide in SCLC.

Vasopressin has been shown to stimulate mobilisation of intracellular calcium in SCLC cells. This calcium mobilisation can be blocked by specific vasopressin antagonists. Vasopressin activates MAPK and promotes colony formation in responsive SCLC cells (Sethi and Rozengurt, 1991a; Sethi et al., 1992b; Bunn et

al., 1992; Seufferlein and Rozengurt, 1996). This can be blocked by the MEK antagonist PD098059 and the PKC antagonist GF109203X (Bunn et al., 1994; Seufferlein and Rozengurt, 1996).

Interestingly, variant SCLC cell lines do not seem to proliferate in response to vasopressin but functional, structurally normal receptors have been detected on both classical and variant cell lines. Stimulation of these receptors in both cell types produce an increase in inositol phosphate production but in variant lines, no corresponding calcium mobilisation is seen. This indicates regulation of the cellular response to vasopressin at a post receptor level (North et al., 1997).

As discussed, both mitogenic vasopressin and vasopressin receptors are present in SCLC tumours indicating that this neuropeptide is an autocrine growth factor for SCLC cells.

1.2.1.3 Bradykinin.

Bradykinin is usually present in plasma at very low concentrations, due to its rapid degradation by carboxypeptidase N and angioconverting enzyme. However, lung tumours are frequently surrounded by areas of tissue and tumour necrosis and local concentrations of bradykinin may be significantly higher. Bradykinin is indeed present in normal lung (Said et al., 1980). In a study by Franknel et al, 6/6 human bronchial epithelial cells were able to mobilise intracellular calcium in response to bradykinin stimulation (Frankel et al., 1994). Thus bradykinin was identified as having a potential role in lung physiology and may also play a role in the growth of lung tumours. Bradykinin at nanomolar concentrations increases $[Ca^{2+}]_i$ in all SCLC cell lines tested through B_2

receptors (Woll and Rozengurt, 1989a). In a panel of 18 human lung cancer cell lines BK caused calcium mobilisation and DNA synthesis in more cell lines than any other neuropeptide tested and the responses were of a greater magnitude than those achieved with any other neuropeptide (Bunn et al., 1992; Bunn et al., 1992). Bradykinin has been shown to stimulate colony growth in SCLC cell lines (Sethi and Rozengurt, 1991b), activate MAPK (Seufferlein and Rozengurt, 1996) and stimulate both tyrosine kinase activity and tyrosine phosphorylation (Tallett et al., 1996).

Although the presence of both bradykinin and bradykinin receptors has never been demonstrated on the same SCLC cell line or tumour sample, it is fair to hypothesise that bradykinin may be an autocrine growth factor for SCLC.

1.2.1.4 Galanin.

In the mammalian lung the distribution of galanin seems to be confined to smooth muscle, seromucous glands and blood vessels as well as airway nerves (Cheung et al., 1985; Ghatei et al., 1987; Luts et al., 1989). It elicits a variety of rapid biological responses including modulation of the release of several hormones (Fisone et al., 1987), stimulation of smooth muscle contractility and inhibition of neuronal excitability (Ekblad et al., 1985). Galanin also inhibits neurokinin stimulated airway mucous production (Wagner et al., 1995; Wagner et al., 1995).

In several contexts galanin opposes the Ca^{2+} signals and modulates the action of other neuropeptides in various cell systems (see previous chapter), it is interesting that in SCLC cells, galanin can stimulate mobilisation of intracellular

calcium at nanomolar concentrations (Woll and Rozengurt, 1989a). Subsequently this calcium mobilisation was found to be via a pertussis toxin insensitive G protein and was precluded by accumulation of inositol phosphates. Activation of PKC by phorbol esters attenuated the ability of galanin to stimulate calcium mobilisation (Sethi et al., 1992b). In several separate studies galanin was found to stimulate colony formation in SCLC cell lines. It was found that the mitogenic capacity of galanin was dependant upon the activation of MAPK and in one study, dependant upon the activation of PKC (Sethi and Rozengurt, 1991a; Sethi et al., 1992b; Seufferlein and Rozengurt, 1996).

There is therefore considerable evidence implicating galanin as a potential growth factor in SCLC however, there is little evidence to suggest that it is an autocrine growth factor. It has not been detected in SCLC tumour samples or cell lines, in fact, in a panel of 35 endocrine tumours of the lung, pancreas and gastrointestinal tract, there was no expression of galanin whereas expression was more prevalent in tumours of various brain regions (Sano et al., 1991).

1.2.1.5 Gastrin and Cholecystokinin

Low level CCK immunoreactivity has been detected in pulmonary neuroendocrine cells (Wang and Cutz, 1993) and trace levels of gastrin has been demonstrated in non-neoplastic lung tissue (Rehfeld et al., 1989), although in a study by Frankel et al 0/3 human bronchial epithelial cells responded to gastrin or CCK by mobilising intracellular calcium (Frankel et al., 1994). In a study by Hansen et al (Hansen et al., 1980) serum gastrin levels were found to be marginally elevated in 20% of patients with SCLC and in another study the

presence of gastrin was detected in one SCLC tissue sample (Hattori et al., 1979). A significant increase in the presence of gastrin was found between limited and extensive stage SCLC (Dowlati et al., 1996). The expression of gastrin and CCK peptides has been demonstrated in SCLC cell lines (Rehfeld et al., 1989; Geijer et al., 1990; Denyer et al., 1994). Other studies have reported receptors for CCK and gastrin in some ,but not all ,SCLC cell lines (Yoder and Moody, 1987; Lin et al., 1989; Sethi and Rozengurt, 1992; Sethi et al, 1993; Denyer et al., 1994). Gastrin receptor has been detected on between 42% and 100% of SCLC cell lines in various studies (Matsushima et al., 1994; Matsumori et al., 1996; Reubi et al., 1997).

The presence of both gastrin/CCK peptides and corresponding receptors in SCLC cells implicated these neuropeptides as potential autocrine growth factors in SCLC. Stimulation of SCLC cells with gastrin or CCK induces mobilisation of intracellular calcium (Staley et al., 1989b; Woll and Rozengurt, 1989b; Bunn et al., 1992; Sethi and Rozengurt, 1992; Sethi et al., 1993) This mobilisation of calcium was found to be via a G protein (Kaufmann et al., 1995). CCK and gastrin were also shown to stimulate clonal growth of various SCLC lines (Sethi and Rozengurt, 1992; Sethi et al., 1993). It was found that the activities of CCK and gastrin could be blocked by both specific CCK and Gastrin receptor antagonists indicating that CCK and gastrin are both capable of acting at the same receptor in SCLC cells (Staley et al., 1990; Sethi et al., 1993). More recently, stimulation of SCLC cells with gastrin has been shown to stimulate tyrosine kinase activity and tyrosine phosphorylation (Tallett et al., 1996).

There is considerable evidence suggesting that CCK and gastrin may be autocrine growth factors in SCLC. Receptor expression, particularly of the gastrin receptor, is frequent in SCLC tissue and cell lines however tissue expression of gastrin and CCK peptides seem to be of relatively low occurrence so this autocrine growth loop may be of less importance than those involving other neuropeptides.

1.2.1.6 Neurotensin.

Neurotensin is found at relatively low concentrations in the normal lung (Goedert et al., 1984) but there is responsiveness to neurotensin in some human bronchial epithelial cells (Frankel et al., 1994). Neurotensin immunoreactivity has been demonstrated in benign pulmonary carcinoid tumours (Farhangi et al., 1987)and neurotensin has been shown to stimulate calcium mobilisation in embryonic lung fibroblasts (Gupta et al., 1994). It is apparent that neurotensin may play a role in normal lung physiology but more interestingly neurotensin and neurotensin receptors are upregulated in some lung neoplasms. High neurotensin immunoreactivity has been detected in SCLC cell lines in comparison to normal lung tissue (Goedert et al., 1984) and in other studies up to 50% of SCLC cell lines have been shown to produce neurotensin (Wood et al., 1981; Luster et al., 1985; Moody et al., 1985b). Neurotensin binding sites have also been demonstrated in SCLC cells (Allen et al., 1988; Reubi et al., 1999). Neurotensin is now known to stimulate mobilisation of intracellular calcium in SCLC cell lines (Woll and Rozengurt, 1989b; Davis et al., 1989; Staley et al., 1989a; Sethi and Rozengurt, 1991b; Sethi et al., 1992a; Bunn et al., 1992) and induce clonal

growth of SCLC cells in semi-solid media (Davis et al., 1989; Sethi and Rozengurt, 1991b; Sethi et al., 1992a). Subsequent studies have shown that neurotensin activates MAPK via a PKC dependent pathway (Seufferlein and Rozengurt, 1996) and stimulates tyrosine kinase activity (Tallett et al., 1996). Thus, it is extremely likely that neurotensin acts as an autocrine growth factor for some SCLC tumours.

It is evident that multiple autocrine and paracrine growth stimulatory loops involving several neuropeptides and their receptors, exist in SCLC and potentially fuel the growth of this aggressive cancer. Some of these neuropeptide autocrine loops, in particular those involving bombesin/GRP, seem to be upregulated as normal lung undergoes the multistep process of carcinogenisis. Neuropeptides and neuroendocrine cells are common throughout the body and neuropeptide growth loops are therefore not confined to tumours of pulmonary origin. The remainder of this section shall discuss the importance of neuropeptides in other tumour types.

1.2.2 Neuropeptides as Growth Factors for Non-Small Cell Lung Cancer.

There is considerable evidence that bombesin/GRP is a potent growth factor for NSCLC tumours, as it is for SCLC tumours. Positive immunoreactivity for bombesin-like peptides has been demonstrated in 21% of NSCLC tumour samples (Kiriakogiani-Psaropoulou et al., 1994) and it has been shown that GRP stimulates colony formation in NSCLC cells in semi-solid media (DeMichele et al., 1994a). In another study it was found that bombesin treatment significantly increased the

growth rate in 1/3 NSCLC xenografts (Kruczynski et al., 1993). Several subsequent studies have detected the presence of bombesin/GRP receptors on NSCLC cell lines and tissues. The GRP receptor was detected by RT-PCR in 84% of NSCLC cell lines (Toi-Scott et al., 1996) and using the same technique, Siegfried et al showed that the GRP receptor was expressed in 9/14 (64%) NSCLC cell lines and receptors for the bombesin-like peptide Neuromedin B (NMB) were expressed in 14/14 (100%) of the same NSCLC cell panel. Using an HPLC assay to detect bombesin-like peptides in conditioned media, it was found that 7/9 NSCLC cell lines produced NMB and 7/9 produced GRP. Interestingly, in the cells that produced both peptides, 7-15 times the amount of NMB was produced in comparison to GRP. In the same study, Siegfried et al showed that stimulation of three NSCLC cell lines with GRP or NMB increased colony formation and increased BRDU incorporation – an indicator of DNA synthesis (Siegfried et al., 1999). Bombesin has been shown to stimulate mobilisation of intracellular calcium in NSCLC cells and in addition causes translocation of PKC from the cytoplasm to the membrane. In this study it was also shown that bombesin stimulated increased colony formation in semi-solid media in a NSCLC cell line (Moody et al., 1996). It has also recently been shown that stimulation of a NSCLC cell line with bombesin results in the phosphorylation of MAPK (Koh et al., 1999).

It is apparent that bombesin/GRP and neuromedin B can both act as autocrine growth factors in NSCLC cells and that the expression of this autocrine loop is not uncommon in this type of pulmonary tumour.

Neuropeptides as Growth Factors for Gastric Cancer and Colonic Cancer.

There is considerable evidence implicating gastrin as an autocrine growth factor in gut cancers. The major physiological roles of gastrin include stimulation of acid secretion, proliferation of gastric mucosa and regulation of the mucosal cell lineage (Kidd et al., 1998).

Gastrin precursor mRNA has been detected in several gastric and colonic cancer cell lines (Baldwin et al., 1990; Baldwin and Zhang, 1992; Baldwin, 1995; Kelly et al., 1998) and the expression of the gastrin receptor (CCKB-R) has been demonstrated. In one study, gastrin was expressed by 5/5 gastric and 7/8 colonic cell lines and it was found that in the same cell panel, 3/5 gastric and 5/8 colonic cell lines co-expressed the truncated gastrin receptor, delta CCKB (McWilliams et al., 1998). Gastrin/CCK specific antagonist L365,260 inhibited DNA synthesis and proliferation of a gastric adenocarcinoma cell line which had been shown to express gastrin (Remy-Heintz et al., 1993). Additionally, inhibition of a gastric cancer cell line has been demonstrated using the non-selective gastrin/CCK receptor antagonists proglutamide and benzotript, thus providing more evidence implicating gastrin as an autocrine growth factor for such cancers (Baldwin, 1994).

Colorectal and gastric tumour cell lines, when grown as xenografts in nude mice can be stimulated to proliferate in response to exogenously administered human gastrin (Watson et al., 1989). In a study by Baldwin et al, gastrin was shown to stimulate the growth of 50% of gastric and colorectal tumours, but consistent proliferative effects on cell lines were not shown. (Baldwin and Whitehead, 1994).

Gastrin is synthesised and secreted mostly by neuroendocrine calls of the gastric antrum. Transcriptional regulation of gastrin is positively regulated by transforming

growth factor alpha (TGFα) and inhibited by somatostatin. Gastrin precursors must undergo several post-translational processing steps and aberrations in the processing steps may produce incompletely processed forms including glycosylated progastrin which has been implicated as an autocrine growth factor for gut neoplasms (Kidd et al., 1998). Gastrin release is stimulated by luminal aromatic amino acids, a decrease in luminal pH and other factors including gastrin releasing peptide (GRP). Hypergastrinemia is a condition characterised by increased serum presence of gastrin and its precursors. It is often caused by an increase of luminal pH caused by pharmacological agents or excessive gastrin release resulting from helicobacter infections (Konturek et al., 1997). This increase in gastrin levels has been shown to cause proliferation of gastrin dependant fundic enterochrommaffin-like (ECL) cells in some cases resulting in the formation of hyperplastic lesions which may evolve into neoplastic lesions (Baldwin, 1995) (reviewed in (Bordi et al., 1997)). It has been shown that gastrin has a promoting but not transforming role in the development of gastric cancers and is likely to require genetic changes such as loss of the MEN-1 suppressor gene or over-expression of BCL-2 for transformation to occur. Indeed, in humans, ECL cell dysplasia and gastric carcinoids have only been demonstrated in patients who show loss of the MEN-1 suppressor gene from chromosome 11q13. Gastric dependence of gastrointestinal adenocarcimona has not been proven and epidemiological evidence does not support gastrin dependence of carcinomas of the stomach, pancreas or colon (reviewed in (Creutzfeldt, 1994)).

Gastrin releasing peptide (GRP) has also been implicated as an autocrine growth factor in gut cancers. High affinity binding sites for GRP have been identified on

gastric and colonic cell lines (Bold et al., 1998b; Halmos et al., 1994; Preston et al., 1994). Bombesin binding to these binding sites was both saturatable and reversible. Stimulation of these receptors result in activation of a G-protein and mobilisation of intracellular calcium (Qin et al., 1994). In addition, bombesin stimulation of several colonic tumour cell lines results in accumulation of inositol phosphates, and increased DNA synthesis (Narayan et al., 1992; Williams and Schonbrunn, 1994). It has been shown that, in a gastric cancer cell line, bombesin increased the expression of transcription factors c-Jun and JunB as well as increasing the binding affinity of AP1 transcription factors (Kim et al., 1996). In another gastric cancer cell line (S11A) bombesin stimulated both intracellular calcium mobilisation and tyrosine phosphorylation. Specific bombesin antagonists blocked calcium mobilisation but not tyrosine phosphorylation. Bombesin caused proliferation in this cell line but this was unaffected by the bombesin antagonist and blocked by tyrphostin, an antagonist of tyrosine phosphorylation (Bold et al., 1994).

Resected gastric and colonic tumours have been shown to express GRP receptors. In one study 12/13 gastric tumours expressed binding sites for GRP and only one uninvolved mucosal sample was found to contain GRP binding sites. This patient suffered from Menetrier's disease which is a pre-malignant disease of mucosa (Preston et al., 1993). Another study demonstrated GRP receptors in 27/29 colon cancer cell lines but no expression in normal colonic epithelium. High expression of GRP receptor correlated with tumours that had undergone lymphatic vessel invasion (Saurin et al., 1999). Carroll and colleagues have shown that in an extensive panel of resected tumour samples, 84% of colon cancers expressed GRP or its receptor. Interestingly 62% expressed both GRP and GRP receptors and this group showed

high expression of proliferating cell mitogenic antigen indicating that GRP is indeed an autocrine growth factor and has important clinical relevance in cancers of the colon (Carroll et al., 1999).

Receptors for other neuropeptides have been detected on colonic and gastric cancer cell lines but the physiological significance of these findings are not clear. It is apparent that gastrin and GRP play an important role in these cancers and that the clinical use of antagonists of these peptide growth factors may be an important step in finding novel therapies.

Neurotensin is a product of gastrointestinal endocrine cells (Solcia et al., 1987) and its release is stimulated by intraluminal fats. It is trophic for normal bowel and colonic mucosa. Expression of neurotensin is localised to the mucosa of the adult small bowel but is expressed in foetal colon. Ectopic neurotensin expression is found in colon cancers suggestion a reversion to foetal phenotype (Evers et al., 1996). Neurotensin may function as an autocrine growth factor in colon cancers.. This hypothesis has been confirmed by other studies. In one study, mRNA for neurotensin was detected in 4/4 cell lines, 2/6 freshly resected human colon tumours and 1/9 human colon cancer xenografts in nude mice. No neurotensin expression was detected in normal colonic mucosa. Neurotensin receptor was identified in 2/3 cell lines and elevates $[Ca^{2+}]_i$ in 2/3 cell lines (Evers et al., 1992). In a study of 19 human colon carcinoma cell lines and normal colon epithelia, over 40% of the tumour cell lines expressed neurotensin in comparison with 0% of the normal epithelia (Maoret et al., 1994). This work strongly suggested that neurotensin expression occurs in colonic cells upon the development of malignancy. Neurotensin

also stimulated the growth of a mouse colon cancer and human colon cancer cell line *in vivo* (Yoshinaga et al., 1992). Two recent studies have provided more evidence implicating neurotensin as an autocrine growth factor. Maoret et al found that neurotensin stimulated the growth of 5/6 colon cancer cell lines in culture. Further experiments with one of these cell lines showed that neurotensin stimulated colony growth in semi-solid media and xenograft growth in nude mice (Maoret et al., 1999). A study by Ehlers et al noted that stimulation of a colon cancer cell line with neurotensin resulted in the mobilisation of intracellular calcium and the activation of MAPK. Neurotensin also increased cellular levels of c-fos in the same cell line (Ehlers et al., 1998).

It is highly likely therefore, that neurotensin plays an important role as a paracrine and autocrine growth factor in colon cancers and also perhaps in other gut cancers too.

1.2.4 Neuropeptides as Growth Factors for Breast Cancer

Bombesin-like immunoreactivity and the expression of bombesin/GRP has been demonstrated in several breast cancer cell lines (Burns et al., 1999) and the plasma concentration of bombesin in breast cancer patients has been shown to be significantly higher than normal control patients (Milewicz et al., 1994). Various studies have attempted to determine the importance of bombesin as a growth factor in breast cancer. Bombesin/GRP receptors have been detected in resected tissue from breast cancer patients. In three studies the frequency of bombesin receptor expression in breast cancer tissue ranged from 33-61% and in one study, high GRP receptor expression correlated with high expression of receptors for the sex steroids,

oestrogen and progestin. Lymph node metastasis biopsy samples were also positive for GRP receptor expression. No receptor expression was demonstrated in non-neoplastic breast samples or in normal lymphoreticular tissue surrounding lymph node metastases (Giacchetti et al., 1990; Halmos et al., 1995; Gugger and Reubi, 1999). In one study, the level of bombesin receptor expression was seen to increase following cell line exposure to radiotherapy (Aalto et al., 1998).

In oestrogen independent and dependent breast cancer cell lines, bombesin signals through a pertussis toxin insensitive G protein (Schrey et al., 1992) resulting in intracellular calcium mobilisation(Bold et al., 1998a) via production of inositol phosphates (Patel and Schrey, 1990). Interestingly bombesin has also been shown to stimulate endothelin release in a breast cancer cell line via a pertussis toxin sensitive G protein (Schrey et al., 1992). Bombesin has been shown to stimulate DNA synthesis in breast cancer cell lines and this can be inhibited by the specific bombesin antagonist RC3095 (Yano et al., 1992).

Several groups have investigated the effects of bombesin antagonists on the growth of breast cancer xenografts in nude mice. Treatment with specific bombesin antagonists RC3095 and RC3940II significantly inhibited xenograft growth in several studies (Yano et al., 1994; Szepeshazi et al., 1992; Miyazaki et al., 1998).

The current success in breast cancer therapy is largely due to the sensitivity of these cancers to the drug tamoxifen. Unfortunately, tamoxifen is a successful treatment only in oestrogen dependent breast cancers. The prognosis for patients suffering from oestrogen independent breast cancer is still unsatisfactory. It is apparent that bombesin/GRP plays an important role as a growth factor for both oestrogen dependant and independent breast cancer and the development of drugs that can

block the mitogenic effects of bombesin are important in the search for new therapies.

It has been demonstrated that vasopressin level in the neoplastic breast are higher than level of vasopressin in normal circulation (Taylor et al., 1990). In a study by North et al, it was found that 12/12 breast cancer tissue samples contained vasopressin gene related products, including vasopressin and its pre-cursors (North et al., 1995). The same author then demonstrated the presence of all three subtypes of the vasopressin receptor, including an abnormal "diabetic" form of the V2 receptor, on a breast cancer cell line (North et al., 1999). It is highly likely, especially as it seems that at least the majority of breast cancers produce vasopressin peptide, that vasopressin is an autocrine growth factor for breast cancer cells and it is surprising that more research has not been carried out to further clarify the situation.

1.2.6 Neuropeptides as Growth Factors for Ovarian Cancer

Ectopic gastrin production by ovarian cancers was previously considered to be unusual and was associated with rare cases of, ovarian neoplasm induced, Zollinger-Ellison syndrome (Primrose et al., 1988; Maton et al., 1989). More recently gastrin secretion by ovarian tumours has been shown to be commonplace. In a study by Vansolinge et al it was shown that 16/16 ovarian tumours secreted forms of gastrin (amidated gastrin, glycine extended gastrin and progastrin) and that peptide levels were higher than levels found in benign tumours and normal ovarian tissue suggesting that the synthesis and processing of progastrin increases during the progression of ovarian malignancy (Van Solinge et al., 1993). In another gastrin

expression was demonstrated in 1/5 ovarian cancer cell lines and in this cell line gastrin stimulated mobilisation of intracellular calcium (Kim et al., 1998). The presence of gastrin receptors in ovarian cancer tissues has been confirmed by Reubi et al. In this study 100% of stromal ovarian tissues were found to express gastrin receptors (Reubi et al., 1997).

In addition, ovarian cancers have been shown to express gastrin-releasing peptide (GRP). 49/100 ovarian mucinous tumours showed immunoreactivity to the N-terminal epitope of GRP (Tenti et al., 1992) and an ovarian cancer cell line has been shown to express GRP receptors which were shown to be linked to a intracellular calcium mobilising pathway (Kim et al., 1998).

It is probable that gastric type differentiation is common in ovarian tumours but there has been no work carried out to show that either gastrin or gastrin releasing peptide act as growth factors in ovarian cancers. However, in light of these peptides mitogenic activity in other tumour types, this possibility is not unlikely.

1.2.6 Neuropeptides as Growth Factors for Prostate Cancer

Prostate cancer cells are most often androgen dependent and can be successfully treated with current therapies. Relapsed prostate cancer is often androgen independent and resistant to treatment. Several studies have shown that as these tumours progress from androgen dependence to androgen independence, they gain increased neuroendocrine differentiation. This increased expression of neuroendocrine markers, such as chromogrannin A and neuron specific enolase, is associated with poor prognosis and tumour progression (Abrahamsson, 1996;

Abrahamsson, 1999). Cussenot et al found that 50% of malignant prostate tumours contain neuroendocrine cells (Cussenot et al., 1996). It can be postulated that, as neuroendocrine cells do not express androgen receptors, these cells will be selected for during anti-androgen therapy and form the basis of the relapsed tumour.

Elevated bombesin levels have been noticed in the serum of prostate cancer patients (Cussenot et al., 1996). It has also been shown that bombesin can stimulate the mobilisation of intracellular calcium in prostate cancer cell lines (Aprikian et al., 1996; Wasilenko et al., 1996). The presence of bombesin receptors of prostate cancer cell lines and tissues has been established by several groups (Aprikian et al., 1996; Bartholdi et al., 1998). In a recent study, GRP receptors were detected in 30/30 invasive prostatic carcinomas, 26/26 cases of pre-malignant prostatic intra-epithelial neoplasias and 4/7 androgen independent bone marrow metastasis. In this study, well differentiated tumours had higher GRP receptor expression than poorly differentiated tumours. GRP receptors were detected at a much lower density and frequency in hyperplastic and normal prostate. This study strongly suggests that GRP receptor expression is increased during malignant progression of prostate cancer (Markwalder and Reubi, 1999).

Treatment with bombesin stimulates mobilisation of intracellular calcium via a G-protein in prostate cancer cell lines (Reile et al., 1994). In addition, bombesin was shown to stimulate the growth of two prostate carcinoma cell lines in culture (Bologna et al., 1989). Stimulation of in vivo growth by bombesin has not yet been demonstrated but several studies have shown that specific bombesin antagonists, including the compound Hca^{6},$Leu^{13}\Psi[CH_2N]Tac^{14}$-Bombesin(6-14) (RC3904-II),

inhibit the growth of various prostate cancer xenografts in nude mice (Pinski et al., 1993; Pinski et al., 1994; Jungwirth et al., 1997). Bombesin has also been shown to enhance migration of an androgen-independent prostate cancer cell line. This phenomenon was dependent upon the activation of PKC, protein tyrosine kinases and focal adhesion kinase (FAK). It was also noted that bombesin stimulated the tyrosine phosphorylation of integrin associated proteins in prostate cancer cells (Aprikian et al., 1997). Another group demonstrated that bombesin increased the invasion of a prostate cancer cell line through a reconstituted basement membrane (Hoosein et al., 1993).

It is likely therefore, that bombesin has both autocrine mitogenic effects on cancers of the prostate and can mediate the migration and invasion of these tumour cells. Bombesin seems to be an important growth factor in androgen-independent prostate cancer. Development of compounds, which target the actions of bombesin in these cancers, may provide novel therapies for relapsed prostate cancer, which currently has a very poor prognosis.

Several studies have demonstrated the presence of neurotensin receptors on both androgen dependent and androgen independent prostate cancer cell lines (Sehgal et al., 1994; Seethalakshmi et al., 1997; Carraway and Mitra, 1998). Neurotensin production by prostate cancer cell lines has also been demonstrated (Moody et al., 1998; Seethalakshmi et al., 1997). Neurotensin can stimulate calcium mobilisation and DNA synthesis in prostate cancer cell lines (Carraway and Mitra, 1998; Seethalakshmi et al., 1997; Mitra and Carraway, 1999). In a study by Sehgal et al it was shown that, in androgen sensitive LNCaP prostate cancer cells, neurotensin

secretion followed androgen withdrawal. This cell line expressed neurotensin receptors but a growth response to neurotensin could only be demonstrated when the cells were deprived of androgens. This group found that androgen stimulation of LNCaP cells induced the production of a metalloprotease that was active towards neurotensin (Sehgal et al., 1994). This work provides a potential mechanism whereby anti-androgen therapy can produce cells which are stimulated to growth by an alternative, neuropeptide growth stimuli.

1.2.7 Neuropeptides as Growth Factors for Pancreatic Cancer

It has long been known that bombesin can stimulate the growth of normal pancreas *in vitro* and *in vivo* (Evers et al., 1990; Hajri et al., 1992). The production of bombesin by pancreatic cancer cells has been demonstrated in a study by Wang et al who found that 4/4 ductal pancreatic carcinoma cell lines secreted bombesin (Wang et al., 1996). Numerous studies have detected bombesin/GRP high affinity binding sites on pancreatic tumour cell lines and tissues (Logsdon et al., 1987; Singh et al., 1990; Avis et al., 1993; Wang et al., 1996; Farre et al., 1994). It has been shown that bombesin stimulation of receptor bearing pancreatic carcinoma cell lines causes increased mobilisation of intracellular calcium (Simeone et al., 1995), accumulation of inositol phosphates (Avis et al., 1993) activation of MAPK (Douziech et al., 1999) and increased DNA synthesis (Avis et al., 1993). Bombesin stimulation of growth in pancreatic cancer cell lines in vitro, has been demonstrated (Avis et al., 1993; Wang et al., 1996; Douziech et al., 1999) but the evidence for bombesin stimulated growth in vivo is somewhat conflicting. Hajri et al showed that bombesin treatment stimulated the growth of pancreatic cancer xenografts in rats (Hajri et al., 1992).

However, in a study by Alexander et al, it was found that chronic bombesin treatment of a human ductal pancreatic cancer xenograft, reduced tumour growth but stimulated pancreatic growth in the same animals (Alexander et al., 1988). Similar results have been obtained in other studies. Meijers et al found that bombesin treatment reduced the formation of N-nitrosobis(2-oxoproply)amine (BOP) induced pre-neoplastic pancreatic lesions in hamsters (Meijers et al., 1991). In another study, the growth of a bombesin receptor expressing pancreatic cancer xenograft was reduced by bombesin treatment. Again, normal pancreatic growth was observed (Farre et al., 1994). Studies using specific bombesin antagonists, such as RC3095 and RC3940-II, showed that these compounds inhibit the growth of pancreatic cancer xenografts *in vivo* (Szepeshazi et al., 1991; Qin et al., 1994; Qin et al., 1995) but interestingly, in one study both bombesin and the bombesin antagonist RC-3095 reduced the growth of BOP induced pancreatic carcinomas and treatment with bombesin and RC3095 together had a synergistic effect on tumour inhibition.

It is clear that in some circumstances bombesin can act as growth factor for pancreatic cancer cells but it seems that the *in vivo* situation is considerably more complex. The mechanisms of action of bombesin in pancreatic cancer are unclear and it is apparent that there is heterogenicity in the cellular response to bombesin.

Gastrin and CCK have been implicated as growth factors for pancreatic cancer cells. Gastrin production by pancreatic cancer cells has been demonstrated in vivo (Smith et al., 1996). Receptors for these neuropeptides have been demonstrated on pancreatic cancer cell lines *in vitro* (Smith et al., 1993). It is thought that both gastrin and CCK stimulate pancreatic cancer cells via the CCKB/gastrin receptor and not the CCKA receptor (Smith et al., 1995). The growth promoting activity of

gastrin/CCK in pancreatic cancer is controversial. In several studies these peptides have been shown to produce a proliferative response in cell lines and in tumour xenografts in nude mice (Smith et al., 1995). The growth of pancreatic tumour xenografts can be blocked by the addition of anti-sense oligonucleotides to gastrin (Smith et al., 1999) and by specific gastrin antagonists (Smith et al., 1996). However in one study it was shown that in two pancreatic cell lines transfected with the gastrin receptor, the addition of gastrin produced a growth inhibitory effect (Detjen et al., 1997) which raises the possibility that gastrin/CCK have a more complex role in pancreatic tumours that simply acting as autocrine growth factors.

1.3 Lung Cancer

Cancer is diagnosed in over a quarter of a million people each year in the UK and is the cause of one in four deaths. Lung cancer is the most common fatal malignancy in the developed world and over 40,000 cases were diagnosed in the UK alone in 1995. In men, lung cancer is the most common form of malignancy and caused 22,760 UK deaths in 1996. Lung cancer mortality in UK women is approaching the mortality levels caused by breast cancer. However, in Scotland and Northern England lung cancer mortality in women now exceeds that from breast cancer and may soon do likewise in the rest of the UK (Fig 1.6) (Cancer Research Campaign cancer statistics 1999). World wide, the incidence of pulmonary cancer is expected to rise over the next twenty years with an increasing number of cases unrelated to smoking (Osann, 1991).

The survival figures for lung cancer are extremely low in comparison to other tumour types. Figure 1.7 shows the age-standardised 5 year survival rates for lung cancer to be 5% in both men and women. Apart from cancer of the pancreas, this is the lowest survival rate for any cancer type.

Several genetic mutations are required to complete the transformation of normal lung cells into lung cancer cells. These genetic changes cause the activation of oncogenes and the deletion of tumour suppressor genes and result in pathological changes which, although the early stages are reversible, generally result in neoplastic progression. The resulting tumour arises from cells which have an increased proliferation rate which is often unchecked due to defects in their ability to initiate apoptosis (programmed cell death).

Lung tumours are carcinomas originating from respiratory epithelium and are classified on the basis of histological type (W.H.O. report 1982). The two main types are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma, and collectively accounts for 75% of all new cases. SCLC accounts for the remaining 25% of lung tumours and includes several subtypes which shall be discussed at length.

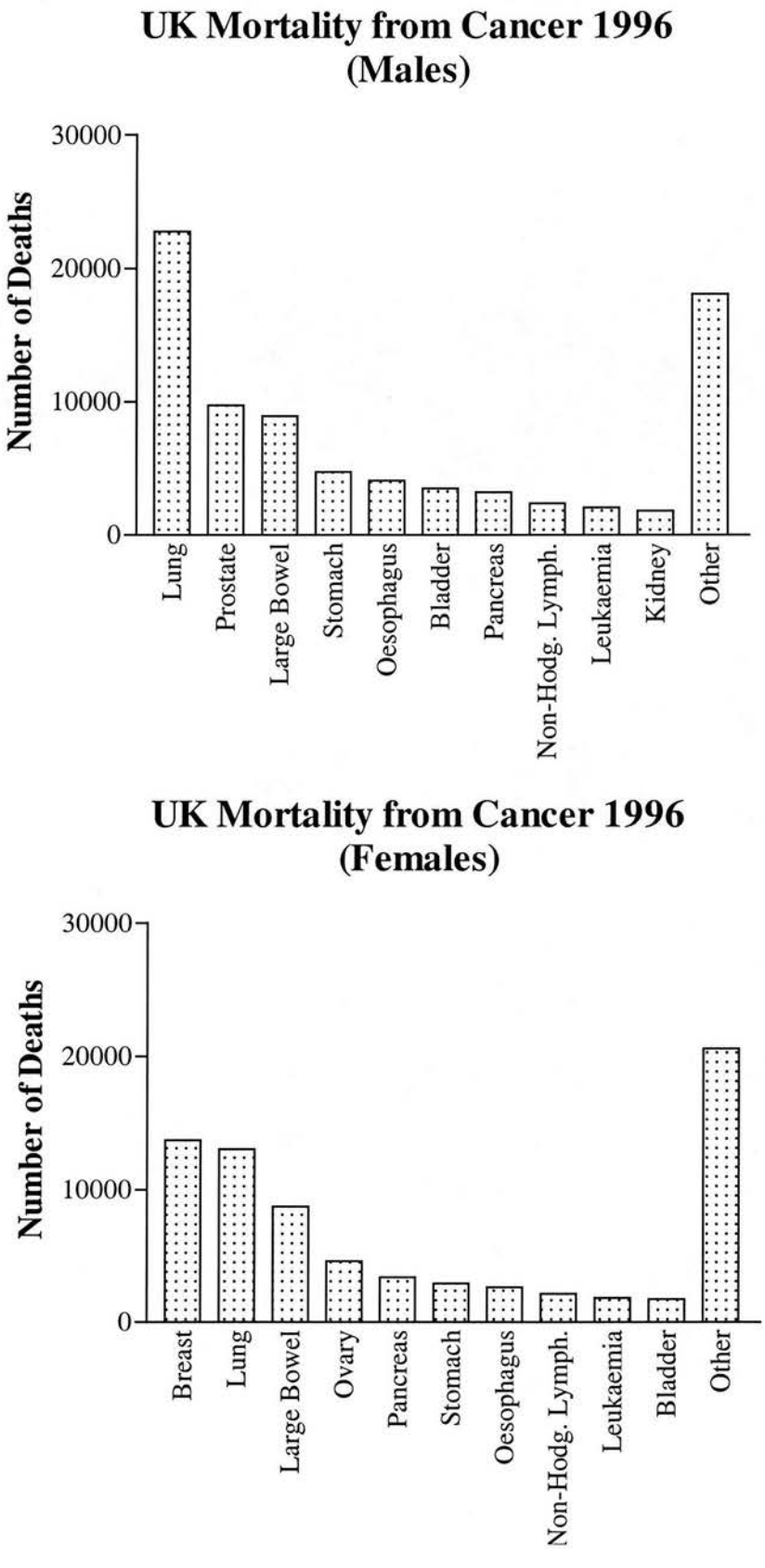


Fig 1.6 Mortality figures for men and women in the UK due to various types of cancer. Data from the Cancer Research Campaign Cancer Statistics 1999 (available on CRC website).

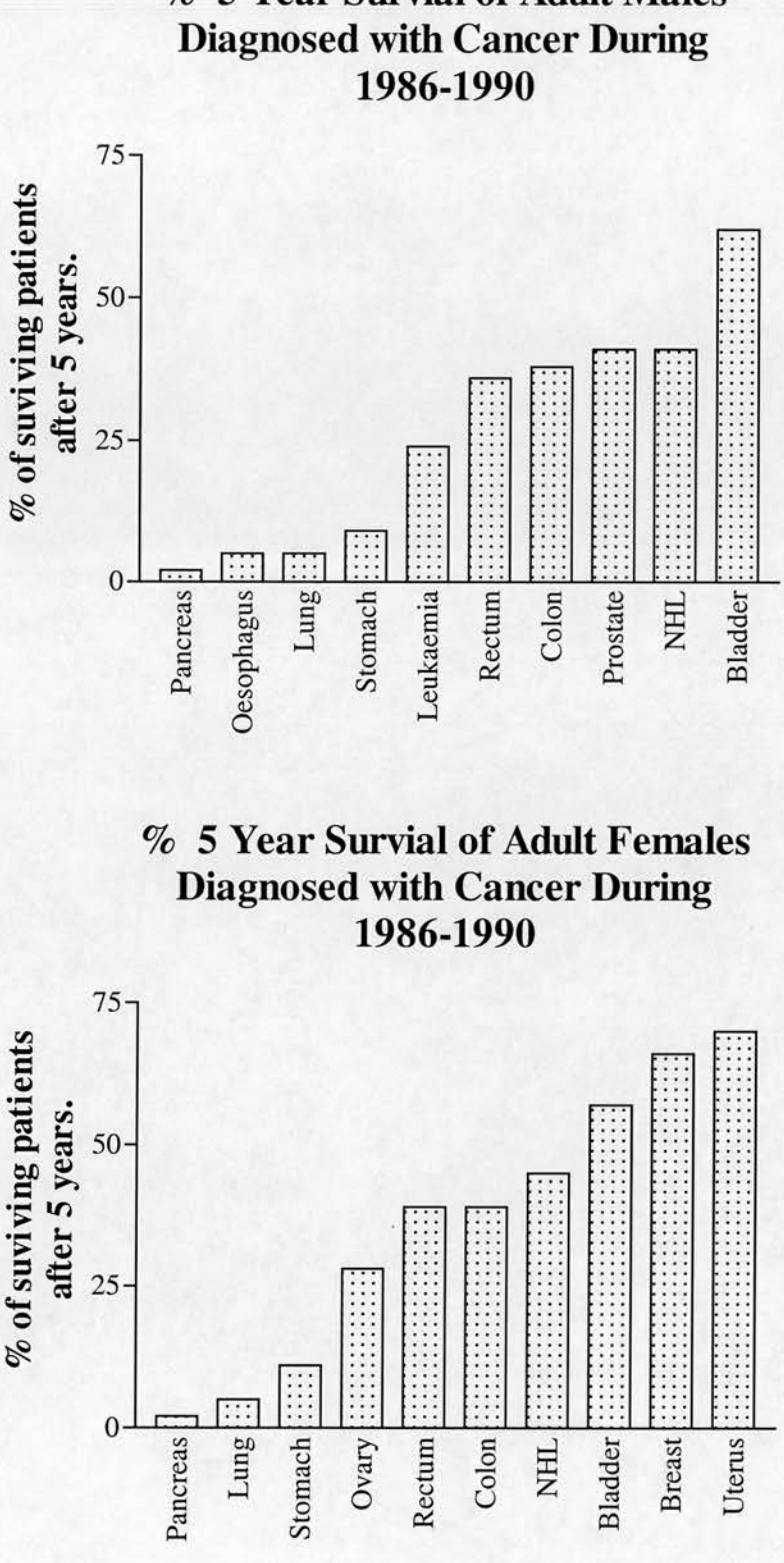


Fig 1.7 Five-year relative survival of adults diagnosed during 1986-1990 in England and Wales. (adapted from Cancer survival trends in England and Wales 1971-1995: deprivation and NHS Region)

1.3.1 Causative agents of Lung Cancer.

All types of bronchial carcinomas can be related to smoking with the exception of bronchial carcinoids. The association with smoking and bronchoalveolar carcinomas, a subgroup of adenocarcinomas, which arise from the terminal bronchioles and alveoli, is not as strong as that of the other carcinoma types. Smoking accounted for 91% of male lung cancer deaths in the USA in the 1970's (W.H.O. report 1982) and accounts for 30% of all cancer deaths in the UK.

Further avoidable causes of lung cancer include exposure to occupational hazards such as asbestos, arsenic, bischloromethyl ether, chromium, mustard gas, nickel, polycyclic hydrocarbons and ionising radiation. Although asbestos has now been removed from most work places it is still the UK's biggest cause of newly diagnosed occupational cancers because asbestos induced cancers can take a considerably longer time to develop. Radon is a naturally occurring radioactive gas which can seep into houses, especially in Cornwall and Devon. The National Radiological Protection Board estimate that Radon exposure may account for1 in 20 lung cancer cases in the UK.

There are synergistic interactions between different carcinogens, e.g. smoking and asbestos or radiation exposure.

1.3.2 Classification of Lung Cancers.

Bronchogenic carcinomas arise from the mucosa of the tracheo-bronchial tree (Gazdar and McDowell, 1988). Squamous cell carcinomas (SQCC) arise from metaplastic squamous epithelial cells. Cell lines demonstrating ultrastructural features of squamous differentiation include well and poorly differentiated

SQCC, adenosquamous and mucoepidermoid carcinomas. All these tumour types demonstrate upregulation of EGF receptor expression and markers characteristic of squamous differentiation (involucrin, transglutaminase activity, higher molecular weight keratins and cornified envelope formation).

Adenocarcinomas appear to arise from several cell types including surface epithelium (ciliated and mucus producing cells), giving rise to mucin-containing or mucin-secreting tumours, and the progenitor cells of the peripheral airways (Clara cells and Type II pneumocytes) giving rise to peripheral airway cell tumours (Gazdar and McDowell, 1988). The latter types often demonstrate morphological features of lepidic and papillary growth, and include the adenocarcinoma subtypes bronchioloalveolar and papillary.

Large cell carcinomas are predominantly undifferentiated tumours, which may represent their basal or stem cell origin.

SCLC were previously thought to arise from pulmonary neuroendocrine cells (Gazdar et al., 1985), however it now seems more likely that they arise from a common stem cell (see below).

Bronchial carcinoid tumours are a minor category of lung cancers which have neuroendocrine features. These tumours are thought to arise from submucosal glands and are not highly malignant. Atypical carcinoids lie between the SCLC and the carcinoid tumours.

As mentioned above each of the four major types of lung cancer reflects phenotypic features of the cell types that make up the normal bronchial epithelium. A new hypothesis that is becoming increasingly accepted is that the four types of lung cancer are related through a common differentiation pathway

in the bronchial epithelium (reviewed in (Mabry et al., 1991)). This proposal is supported by clinical evidence that demonstrates potential for transitions between the different tumours, in other words, tumours in patients can be mixtures of different phenotypes. Neuroendocrine (NE) features can be found in NSCLC tumours. This occurs in about 25% of adenocarcinomas and confers an initial sensitivity to chemotherapy and radiotherapy, but these tumours with NE features are faster growing and have a worse prognosis compared to adenocarcinomas without NE features. Furthermore, individual cells have been observed to manifest features of the SCLC and NSCLC phenotypes simultaneously. It is proposed that these transitions mimic normal cellular transitions in bronchial mucosa and are mediated by the consistent genetic abnormalities now being described for lung cancer. A cell culture model system for one of these transitions, SCLC to the large cell undifferentiated phenotype has been developed. In this *in vitro* model, the combination of two genetic abnormalities that can occur in lung cancer results in a transition of phenotype, where there are losses of NE markers, acquisition of drug resistance patterns typical of NSCLC. There is a concomitant fall in cellular production of GRP, and an increase in expression of EGF receptor on their surface and production of TGF-α. This model has been extended linking differentiation of adult normal bronchial epithelial cells and transitions between the lung cancer phenotypes (Mabry et al., 1991).

1.3.3 Small Cell Lung Cancer

SCLC constitutes 25% of all primary pulmonary cancers hence it is the sixth most common malignancy and the fourth leading cause of death from cancer.

Without treatment, SCLC has a more aggressive clinical course than any other pulmonary tumour with median survival from diagnosis only 2-4 months. SCLC is usually widely metastasised at the time of presentation and because of this, localised forms of treatment such as surgical resection and radiation therapy rarely result in long term survival (Prasad et al., 1989).

SCLC is usually initially responsive to cytotoxic chemotherapy, but survival is only prolonged for more than two years in less than 5% patients as it almost invariably relapses and is resistant to further treatment (Smyth et al., 1986). For those patients who survive in excess of 2 years from initial diagnosis, there is an increased risk of mortality from other types of lung carcinoma (Minna et al., 1985). This dismal prognosis has fuelled interest in SCLC pharmacology and encouraged research with the aim of providing a more detailed understanding of the fundamental biology of SCLC. It is hoped that this research will serve as a basis for developing urgently required, novel therapeutic approaches.

1.3.3.1 Cellular Classification of SCLC sub-types

The current classification of SCLC subtypes are : (Hirsch et al., 1988)

- Small cell carcinoma
- Mixed Small cell / large cell carcinoma.
- Combined small cell carcimoma (SCLC with neoplastic squamous or glandular components)

Neuroendocrine carcinomas represent a spectrum of disease. At one extreme is SCLC, which has a poor prognosis. At the other extreme are bronchial carcinoids which have a promising prognosis following surgical resection (Harpole et al., 1992). Between these two extremes is a sub-type called well-differentiated neuroendocrine carcinoma of the lung. Like SCLC it occurs primarily in smokers but it metastasises less frequently and the 5-year survival rate is often greater than 50%. The pathological diagnosis of well-differentiated neuroendocrine tumours from SCLC is difficult because both cell types are very similar.

The characteristics of classic SCLC cells are the presence of sparse cytoplasm and indistinct or absent nucleoli. Cells are 2-3 times the size of mature lymphocytes and their nuclei are darkly stained. Small dense-core granules are seen in the cytoplasm, and are a manifestation of the peptide secretion that is the hallmark of the neuroendocrine properties observed in SCLC, but relatively infrequently in other types of lung cancer.

SCLC cell lines grow in culture as tightly clustered spheroids in suspension, form colonies in soft agarose and can form tumours in nude mice. They have a doubling time ranging from 32-72 hours. In contrast NSCLC cell lines grow as attached monolayer cultures. They are also tumourigenic in nude mice and will form colonies in soft agarose.

SCLC has distinct neuroendocrine biochemistry, and the cells express several bio-markers of neuroendocrine differentiation including:

- L-Dopa decarboxylase
- Neuron Specific Enolase,
- The BB isoenzyme of Creatine Kinase,
- Chromogrannin A

Interestingly, 25% of NSCLC adenocarcinomas express endocrine markers L-Dopa Decarboxylase and Neuron specific enolase. These cells are faster growing, have a worse prognosis and are chemosensitive in contrast to other adenocarcinomas (Gazdar and McDowell, 1988). SCLC cells also express many peptide hormones (discussed in section 1.2) which are responsible for many clinical symptoms of SCLC and also stimulate the unregulated growth of these tumours.

In studies of a large number of SCLC cell lines it is clear that there is considerable heterogeneity of neuroendocrine marker expression, allowing sub-classification of SCLC into two distinct types (Carney et al., 1985): Classic cell lines, which express high levels of all 4 biomarkers and variant cell lines, in which there is selective loss of some of these biomarkers. *In vitro* studies have shown that variant cell lines have a more rapid growth rate and a higher cloning efficiency in soft agar and higher tumourgenicity in nude mice than classic cell lines. Variant cell lines have larger cell morphology, are more undifferentiated and are relatively chemo and radio resistant. Variant cell lines have been proposed to represent the *in vitro* counterpart of the poor prognosis, mixed Small cell/large cell SCLC subtype. 10% of SCLC cell lines are biochemical variants which lack DDC and other biochemical markers, another 20% are morphological

variants which grow as loose aggregates or monolayers with short doubling times and high cloning efficiencies. Although conversion from classic to variant phenotype might explain the clinical development of resistance to chemotherapy in some cases, other mechanisms are also likely to be operative in the change from responsive to non-responsive in SCLC, this is because only 10% of SCLC patients have tumours showing histology in which large cell admixtures are present . At post-mortem this figure has moderately increased to 13-28%, despite the majority of these tumours being resistant to treatment (Sehested et al., 1986). Several longitudinal studies show that the more extensive the disease the higher the incidence of positivity of endocrine markers (Johnson et al., 1991) and reviewed in Carney et al.(1991). Neuron Specific Enolase (NSE), is a highly specific marker for neurones. Its levels are more elevated in extensive disease. Sequential determinations of NSE levels are of value in monitoring the response to chemotherapy and the detection of relapse. Levels of Chromogrannin A, BB isoenzyme of Creatine kinase and Carcinoma embryonic antigen are also of prognostic value and showed a significant correlation with tumour response to cytotoxic therapy and relapse after therapy. Brambilla and his colleagues (Brambilla et al., 1991) have also showed in a longitudinal study (before and after the acquisition of resistance to chemotherapy and radiotherapy) involving 20 patients, an increase in neurosecretory granules in SCLC cells and these cells appear more differentiated after chemotherapy. However, these results though in agreement with those of Bepler et. al. (Bepler et al., 1987), are in contradiction with other observations of cell lines and biopsies from treated patients (Berendsen et al., 1988) and with long-term untreated SCLC cultures, in which it

has been shown that large cells exhibited a dedifferentiated phenotype with loss of NE markers. On balance it seems most likely that in the majority of cases of SCLC cells are of the classic phenotype and maintain this classic phenotype during chemotherapy and the acquisition of chemoresistance , in a minority of cases there may be conversion from classic to variant phenotype or a maintenance of variant phenotype during the course of the disease.

1.3.3.2 Clinical features of SCLC.

SCLC tends to infiltrate the submucosa. This causes bronchial obstruction with consequent cough, wheezing, chest discomfort, haemoptysis or post-obstructive pneumonia. Two-thirds of patients will have evidence of distant metastases, most often to the liver, bone, bone marrow, or brain, at the time of original diagnosis.

There are many paraneoplastic syndromes associated with SCLC and which may be confused with symptomatic metastases (Bunn and Ridgway, 1989). These syndromes are often due to the synthesis and release by the tumour of various hormones and peptides. Adrenocorticotrophic factor (ACTH) secretion causes ectopic Cushing's syndrome. Arginine vasopressin (AVP) and atrial natriuretic (ANF) (Bliss et al., 1990; Gross et al., 1993) release causes hyponatraemia and the syndrome of inappropriate antidiuretic hormone secretion.

Immune reactions to tumour antigens, often result in neurological syndromes such as the Eaton-Lambert myaesthenic syndrome, in which the presence of circulating antibodies react with voltage-gated calcium channels (de Aizpurua et al., 1988). Visual deficits produced by retinal degeneration and sensory

neuropathy are two other syndromes most commonly found in SCLC, in which serum antibodies reacting with both tumour and neuronal tissue are also observed (Bunn and Ridgway, 1989).

1.3.4 Treatment of Lung Cancer

1.3.4.1 Treatment of Small Cell Lung Cancer

The majority of patients present with extensive disease. That is thoracic disease involving more than one hemithorax or with metastatic spread. The presentation of limited disease involving only one hemithorax is less common.

Because of the frequency of metastatic disease, chemotherapy is the treatment of choice for SCLC patients. The majority of SCLC tumours show good sensitivity to cytotoxic drugs and initial response rates are high although tumours nearly always relapse and are subsequently often resistant to chemotherapy.

A variety of chemotherapy regimens (Table 1.5) have been used with response rates of up to 80% in unselected patients, and complete response rates of 50-60% in patients with limited disease (Minna et al., 1985).

In limited SCLC, combination chemotherapy produces results that are superior to single agent treatment and intensive doses are more effective than doses that only produce mild toxic effects. Randomized trials have suggested a modest but significant improvement in survival when chemotherapy is used in combination with radiotherapy (Murray et al., 1993). Combined etoposide and cisplatin chemotherapy with con-current chest radiation has achieved a 40-50% 2-year survival in pateints with limited disease (McCracken et al., 1990; Johnson et al., 1996).

The relative effectiveness of 2-5 drug regimens and different schedules of chest radiation therapy appear to be similar and the use of alternating chemotherapy regimens have not proven more effective than consistent administration of a single regimen. The optimum duration of chemotherapy for patients with limited stage SCLC is not clear, however, there is no improvement in survival after drug administration exceeds 3-6 months (Murray et al., 1993; Johnson et al., 1996).

Treatment for extensive stage SCLC, as for limited stage disease is multiple agent chemotherapy at moderate-high doses. At this stage of disease, chest irradiation does not improve survival compared with chemotherapy alone however radiation therapy is important in control of the symptoms from the primary tumour and of metastatic disease particularly brain, epidural and bone metastasis (Twelves et al., 1990). Many patients with extensive disease have a much poorer performance status at the time of presentation than limited stage patients. Such patients have a poorer prognosis and tolerate aggressive treatment regimens poorly. Low dose, single agent regimens have been developed for these patients although this is not as effective as normal treatment (James et al., 1996; Souhami et al., 1997).

The prognosis for SCLC that has progressed despite chemotherapy is extremely poor. Median survival is 2-3 months. However patients who are initially sensitive to chemotherapy and then relapse are more likely to respond to additional chemotherapy. Chemotherapeutics that have shown some limited success as second line treatments include; oral etoposide, etoposide/cisplatin, cylclophosphamide/doxorubicin/vincristine, lomustine/methotrexate and topotecan (Greco, 1993; Johnson et al., 1990; Evans et al., 1985).

Biological response modifiers are another form of treatment for SCLC. SCLC cells have reduced expression of specific cell surface antigens, specifically the class 1 major histocompatability antigens. These lack of these molecules may be relevant in the escape of SCLC cells from immune surveillance. Treatment of SCLC cells with leukocyte A interferon and γ-interferon can induce expression of these antigens and preliminary results show greater survival for patients receiving interferon maintenance therapy than those not receiving such therapy (Mattson et al., 1992).

Unfortunately the outlook for patients with SCLC is bleak. Relapse and chemotherapy resistance is usually the outcome irrespective of the treatments used. Over the last twenty years there has been no significant improvements in overall success of the available treatments for SCLC. It is obvious that novel approaches to the treatment of the disease are urgently required.

Table 1.5: Cytotoxic drugs for SCLC treatment.

Alkylating agents	Cyclophosphamide
	Ifosfamide
	Hexamethylmelamine
	Lomustine
Vinca alkaloids	Vincristine
	Vindesin
Podophyllotoxin derivatives	Etoposide
	Teniposide
Platinum analogues	Cisplatinum
	Carboplatin
Miscellaneous	Doxorubicin
	Metotrexate
	Mitomycin C

1.3.4.2 Treatment of Non-Small Cell Lung Cancer

Non-small cell lung cancer (NSCLC) consists of at least 3 distinct histological types of lung cancer including epidermoid or squamous carcinoma, adenocarcinoma, and large cell carcinoma. These histological types are often classified together because, when localized, all have the potential for cure with surgical resection. Systemic chemotherapy can produce partial responses and short term relief of symptoms in patients with advanced disease. Local control can be achieved with radiation in a large number of patients with un-operable disease. Unfortunately a cure is seen only in a small minority of patients as NSCLC tumours are less sensitive to chemotherapy than untreated SCLC tumours . Patients with good performance status, women, and patients with distant metastases confined to a single site appear to live longer than others (Albain et al., 1991).

Currently no single chemotherapy regimen is recommended for routine use. For operable patients, large tumour size and presence of the erbB-2 oncoprotein are negative prognostic factors (Albain et al., 1991).

Other factors that have been identified as adverse prognostic factors in non-small cell lung cancer include mutation of the K-ras gene, vascular invasion, and increased numbers of blood vessels in the tumor specimen (Harpole et al., 1995). Treatment is not satisfactory for almost all patients with NSCLC.

1.3.5 Mechanisms of Drug Resistance in Lung Cancer.

Clinical drug resistance is defined according to W.H.O. criteria which state that if a tumour shows less than a partial response to chemotherapy then it is classified as being resistant. SCLC is initially sensitive to chemotherapy but almost

invariably relapses and is resistant to further treatment. There are few drugs which show even a modest activity against NSCLC tumours. There are several mechanisms by which tumours can exhibit resistance to chemotherapeutic treatments. Several of these mechanisms shall be discussed.

Cellular drug accumulation can be modified by alteration of the influx or efflux of drugs. Most drugs enter the cell via passive diffusion but mechanisms of active transport also exist (Ishikawa, 1992). Typical multi-drug resistance is caused P-glycoprotein, encoded by the *MDR-1* gene, which is a cell surface ATP-binding cassette transporter which transports anti-tumour agents out of the cell. Overexpression of the *MDR-1* gene does not seem to correlate with clinical resistance in lung cancer so it is thought that this is not the major mechanism of drug resistance in such tumours (Lai et al., 1989). Other transporter proteins have been associated with clinical drug resistance in lung cancer, namely the non-P-glycoprotein, multidrug resistance protein-1 (MRP-1) which, like P-glycoprotein, actively transports drugs out of the cancer cell (Cole et al., 1992).

It has been reported that in doxorubicin and cisplatin resistant cell lines, changes in the intracellular distribution of anticancer drugs, contribute to clinical resistance. This mechanism is thought to be P-glycoprotein independent and results in the accumulation of drugs in extranulear vesicles as opposed to a nuclear distribution in chemotherapy sensitive cells (Takeda et al., 1991). This phenotype is reversible *in vitro* by tyrosine kinase inhibitors (Takeda et al., 1994). Lung resistance protein (LRP) is protein located in the cytoplasm and nuclear membrane and is though to induce drug resistance by acting on nuclear transport.

Another mechanism of drug resistance in lung cancer is thought to involve overexpression of glutathione-S-transferase (GST). This enzyme plays a role in the detoxification of free radical damage such as that caused by doxorubicin derivatives (Shen et al., 1997).

Metallothioneins have been shown to react with heavy metals and scavenge free radicals and are candidates drug resistance compounds (Kasahara et al., 1991). Some lung cancer cells, which show resistance to cadmium chloride treatment, have increased expression of metallothioneins (Endresen et al., 1983).

DNA topisomerase I and II inhibitors such as etoposide and doxorubicin are used to treat lung cancer patients. Increased expression of topoisomerases correlates with sensitivity to these drugs (Kasahara et al., 1992). Decreased expression or mutation in the topoisomerases is a mechanism of resistance to such drugs.

1.4 Blocking Growth Factor Action

The majority of currently used cancer therapies, are untargeted cytotoxic drugs which have devastating effects on all rapidly dividing cells. Side effects of these chemotherapeutics are expected but it is generally considered that the benefits of such treatments marginally outweigh the risk to the patient. Despite recent advances in cytotoxic drug development, it is a universal goal to develop novel cancer therapies, which are more specific to cancer cells, and produce minimal damage to non-malignant cells. One approach to realising this goal is the development of drugs that are able to block the growth promoting effects of cancer cell mitogens. As discussed earlier, small cell lung cancer cells, in addition to several other tumour types, proliferate in response to a range of neuropeptide growth factors and in many cases these neuropeptides are involved in autocrine and paracrine growth loops, that fuel the unrestrained proliferation of cancer cells. There has been considerable research carried out in order to identify compounds that are capable of breaking these growth loops. The most accessible methods of intervention are to neutralise the ligand or to prevent its interaction with the receptor. It is widely accepted that bombesin is an important growth factor for SCLC and several groups have designed specific bombesin antagonists with the aim of preventing bombesin mediated autocrine growth loops. Monoclonal antibodies have been developed against circulating bombesin and one such antibody, 2A11 has been shown to inhibit the growth of SCLC in vitro and also as xenografts in nude mice (Chaudry et al., 1999). This antibody has recently completed a phase 1 clinical trial that showed no dose limiting toxicity and produced a clinical remission of one patient with relapsed small

cell lung cancer. Although one patient had a complete response during treatment with A211, it is not clear how suitable this approach is for general clinical use. Antibodies are costly to manufacture and have relatively low tissue penetration compared to small peptide drugs. Peptide receptor antagonists have been the main focus of research in this drug development strategy. One such antagonist is [Leu^{13}-*psi*(CH_2NH)Leu^{14}]Bombesin, a pseudopeptide analogue of bombesin. It was shown to be a potent inhibitor of bombesin stimulated growth in Swiss 3T3 cells and blocks bombesin stimulated calcium mobilisation and clonal growth in soft agar (Mahmoud et al., 1991). Another bombesin antagonist [D-$Phe^{6,13}$ *psi*^{14},CH_2-NH,Cys^{14}]Bombesin-(6-14) (RC-3950-I) and a series of similar analogues have shown anti-tumour activity against a wide range of bombesin receptor expressing tumour types (Table1.6). Recently these bombesin antagonists have been used to target conjugated cytotoxic drugs such as doxorubicin to tumour sites with some evidence of anti-tumour activity (Kiaris et al., 1999).

Bombesin is only one of several neuropeptide tumour cell growth factors and it is likely that for maximal anti-tumour effect, the actions of more than one neuropeptide growth factor must be inhibited simultaneously. The following section will discuss the development of substance P analogues as broad spectrum neuropeptide antagonists.

Table 1.6: Tumour types sensitive to growth inhibition in xenograft models by RC3095 and related bombesin antagonists

Tumour Type	Reference
Small Cell Lung Cancer	(Halmos and Schally, 1997)
Prostate Cancer	(Milovanovic et al., 1992; Pinski et al., 1993; Jungwirth et al., 1997b; Jungwirth et al., 1997a)
Renal Adenocarcinoma	(Jungwirth et al., 1998)
Breast Cancer	(Yano et al., 1992; Szepeshazi et al., 1992; Shirahige et al., 1994; Miyazaki et al., 1998)
Colo-Rectal Cancer	(Radulovic et al., 1991; Radulovic et al., 1992)
Pancreatic Cancer	(Qin et al., 1994; Qin et al., 1995; Szepeshazi et al., 1999)
Gastric Cancer	(Pinski et al., 1994; Qin et al., 1994)
Malignant Glioblastoma	(Pinski et al., 1994; Kiaris et al., 1999b)

1.4.1 Substance P Analogues as a Novel Cancer Therapy

Substance P is an 11 amino acid peptide (Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met NH2) of the tachykinin family. Synthetic analogues of substance P (Table1.7) were initially identified as antagonists of various, substance P mediated, cellular effects and were subsequently found to also antagonise the cellular effects of structurally unrelated bombesin (Jensen et al., 1984). When tested in SCLC cell lines, it was found that several of these substance P analogues were able to inhibit the mobilisation of calcium stimulated by the neuropeptides; bombesin, bradykinin, gastrin, galanin, vasopressin, cholecystokinin and neurotensin (Woll and Rozengurt, 1988; Woll and Rozengurt, 1990; Langdon et al., 1992). They were also found to

inhibit mitogenisis by the same range of neuropeptides in both Swiss 3T3 cells and SCLC cells (Everard et al., 1992; Woll and Rozengurt, 1990; Woll and Rozengurt, 1988; Bepler et al., 1989). All neuropeptides which were sensitive to inhibition by substance P analogues, mediate their effects via receptors coupled to Gq. In contrast substance P analogues had no effect on the mitogenic effects of the neuropeptide, vasoactive intestinal peptide (VIP) which activates cAMP accumulation via Gs or on the signals stimulated by PDGF which signals through a receptor tyrosine kinase (Seckl and Rozengurt, 1998). Substance P analogues inhibit multiple mitogenic signals emanating from neuropeptide receptors. They have been shown to inhibit ligand binding at vasopressin, bradykinin and bombesin receptors (Woll and Rozengurt, 1988; Bepler et al., 1989) and in addition to inhibiting agonist stimulated mobilisation of intracellular calcium by these neuropeptides, they also inhibit PKC mediated 80K/MARCKS phosphorylation, PLCβ activation, MAPK activation (Seckl et al., 1996) and FAK tyrosine phosphorylation (Tallett et al., 1996) by bombesin. In addition to the inhibition of bombesin stimulated signalling events, substance P analogues also inhibit vasopressin stimulated 80K/MARCKS phosphorylation and tyrosine phosphorylation and both vasopressin and bradykinin stimulated DNA synthesis and MAPK activation (Seckl et al., 1996). It has been demonstrated that the inhibition of the above downstream signalling pathways by substance P analogues is reversible by the addition of high concentrations of neuropeptide agonists (Seckl et al., 1997).

Table 1.7: Comparison of structure/inhibitory activity of multiple substance P analogues on the growth of the H69 SCLC cell line in liquid culture on day12 (adapted from (Seckl and Rozengurt, 1998)).

Substance P Analogue Structure	% Growth inhibition at 25μM
Arg –D-Trp-MePhe-D-Trp-Leu-Met-NH_2	30 (a)
Arg –D-Trp-MePhe-D-Trp-Leu-D-Met-NH_2	32
Arg–D-Trp-MePhe-D-Trp-D-Leu-Met-NH_2	37
Arg–D-Trp-MePhe-D-Trp-D-Leu-Val-NH_2	27
Arg–D-Trp-MePhe-D-Trp-D-Leu-Gly-NH_2	19
Arg–D-Trp-MePhe-D-Trp-D-Leu-Met-OH	16
H-Arg–D-Trp-MePhe-D-Trp-Leu-OH	13
D-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2	61
D-Pro-Lys-Pro-D-Trp-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2	92 (b)
D-Pro-Lys-Pro-D-Trp-Gln-D-Trp-Phe-D-Trp-Leu-Val-NH_2	36
D-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Gly-NH_2	20
D-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-NH_2	21 (c)
Ac-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2	16
Ac-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Gly-NH_2	0
Arg-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-HN_2	39
Arg-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Gly-HN_2	42
D-Arg-Pro-Lys-Dphe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2	55 (d)
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2	0 (e)

(a) Antagonist G – currently entering a Phase II clinical trial.
(b) Most effective analogue at inhibiting SCLC growth.
(c) This analogue inhibits vasopressin but not bombesin stimulated signalling and mitogenisis.
(d) Antagonist D – (Woll and Rozengurt, 1990)
(e) Substance P

In addition to the *in vitro* growth inhibitory effects of substance P analogues, it has also been shown that these compounds can inhibit the growth of tumours in xenograft models in nude mice. Both antagonist D and antagonist G have been shown to inhibit the growth of the SCLC cell line WX332 *in vivo* (Langdon et al., 1992) and antagonist G inhibits the growth of the H69 cell line *in vivo* (Jones et al., 1997). Unpublished data from our laboratory has shown that other tumour cell lines including HT29 (colorectal cancer) and PEO4 (ovarian cancer) are sensitive to growth inhibition by antagonist G *in vivo*.

The nature of the growth inhibition by these peptides has recently been shown to be at least partly via the induction of apoptosis. In three SCLC cell lines, antagonist G induces apoptosis at similar concentrations to which it causes growth inhibition (Tallett et al., 1996; MacKinnon et al., 1999).

The exact mechanism by which these compounds exert their anti-tumour effects have been subject to considerable debate over the last decade. The fact that substance P analogues can reversibly inhibit agonist binding and subsequent signalling from multiple, structurally unrelated, neuropeptides lead to the hypothesis that they were acting as competitive antagonists of ligand binding, perhaps acting at a common domain shared by all the sensitive receptor types. Interestingly, the pro-apoptotic and growth inhibitory effects of substance P analogues are not reversible by exogenous neuropeptides (Mitchell et al., 1995; MacKinnon et al., 1999) which may point towards a mechanism which is independent of competitive neuropeptide antagonism. An alternative hypothesis suggested is that as these compounds are small hydrophobic peptides, they may insert into membranes and either disrupt membrane integrity in a non-specific manner or influence receptor / G-protein

coupling. The parent peptide, substance P, can insert into cell membranes and directly activate G-proteins in a receptor independent fashion resulting in the release of histamine from mast cells (Mousli et al., 1990). An argument against the proposal that substance P analogues can directly modulate G-protein activity involves the ability of two substance P analogues to differentially modulate the same signalling pathways from bombesin and vasopressin receptors. [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]Substance P inhibits both bombesin and vasopressin binding and signal transduction. A truncated version of this peptide, [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}]Substance P, showed activity against vasopressin but not against bombesin (Seckl and Rozengurt, 1998). This suggests that the truncated peptide acts as a vasopressin antagonist but not a bombesin antagonist which implies that these compounds block neuropeptide signal transduction at the receptor level.

However, several recent papers have highlighted the fact that the mechanism of action of substance P analogues may be more complex than was previously appreciated. Mitchell et al showed that at low bombesin concentrations (1-10nM) antagonist D blocked the activation of MAPK by bombesin, however, at higher bombesin concentrations (>10nM), antagonist D enhanced bombesin stimulated MAPK activation. Thus antagonist D is capable of differentially modulating neuropeptide signal transduction rather than simply acting as a competitive antagonist (Mitchell et al., 1995). Furthermore, our group have demonstrated that antagonist G can induce the sustained activation of c-Jun-N-terminal kinase (JNK) in SCLC cells and this JNK activation occurs in the absence of exogenously added neuropeptides. This JNK activation does not appear to be dependant upon competitive neuropeptide antagonism as it is not reversible by high concentrations of

neuropeptides (MacKinnon et al., 1999). Thus, antagonist G has neuropeptide independent agonist activity in addition to its previously described antagonist effects. The agonist activities of substance P analogues were further investigated by Jarpe et al. It was shown that antagonist D could also activate JNK, and that this JNK activation was dependent upon the presence of neuropeptide receptors – in this case the GRP receptor. JNK activation by GPCRs has been shown to be via the G12 family of G-proteins (Prasad et al., 1995). Jarpe et al proposed the novel terminology "biased agonism" to explain the receptor mediated effects of antagonist D. They suggested that antagonist D is capable of differentially activating some downstream signalling pathways (via G12) from a neuropeptide receptor whilst simultaneously inhibiting others (via Gq) (discussed in more detail in chapter 6).

Antagonist G has recently completed a phase I clinical trial and will be entering a phase II clinical trial in the near future. The phase I trial was carried out on volunteer cancer patients and evaluated the effects of dose escalation up to 400mg/m^2. Patients received a 6hr infusion of the peptide every three weeks for up to 12 cycles. No dose limiting toxicity was found. Pharmacokinetic analysis of patient plasma showed that the mean plasma concentration of antagonist G at the maximum dose, was 40μM. This concentration is well within the expected range for growth inhibition *in vitro*. A phase II clinical trial is now being planned for post chemotherapy SCLC patients.

In conclusion, the combined research into the mechanism of action of substance P analogues over the last decade, has provided conflicting and confusing evidence which must be clarified if these compounds are to be developed into a successful

therapeutic tool. Understanding both, the exact manner in which these peptides modulate neuropeptide mediated and neuropeptide independent signalling, and pinpointing which of these effects are clinically relevant, will allow the rational design of more potent analogues and aid the clarification of the potential clinical setting of this group of compounds.

1.5 PLAN OF STUDY

It is becoming increasingly evident that neuropeptides are important mitogens for a range of tumour types including lung cancer, breast cancer, prostate cancer, pancreatic cancer and gastric cancers. The presence of autocrine / paracrine neuropeptide growth loops in cancers presents an obvious target for therapeutic intervention and the development of antagonists of these systems is a major step in controlling these diseases.

Analogues of substance P have been developed as broad-spectrum neuropeptide antagonists and one substance P analogue, Antagonist G, is entering a phase II clinical trial as a potential therapy for small cell lung cancer. In order to further the development of substance P analogues as a cancer therapy it is necessary to define their mechanism of action and to increase their therapeutic potential it is necessary to define their clinical placement.

In order to investigate these issues the following work will be carried out.

1) The sensitivity of a panel of tumour cell lines will be assessed to establish against which tumour types substance P analogues may be useful.
2) The importance of neuropeptide receptors in the mechanism of action of substance P analogues will be investigated: The expression levels of neuropeptide receptors in a panel of tumour cell lines will be assessed in order to detect any correlation with sensitivity to substance P analogues.

3) In order to clarify the clinical setting of substance P analogues, small cell lung cancer cell lines from longitudinal follow ups from single patients will be assessed for sensitivity to substance P analogues and responsiveness to neuropeptides. These cell lines and others will be used to assess the relationship between sensitivity or resistance to conventional chemotherapy and responsiveness to growth inhibition by substance P analogues.
4) Mechanistic studies will be carried out to further investigate the agonist effects of substance P analogues. Signal transduction pathways involved in the mechanism of action of substance P analogues will be clarified and the relevance of these pathways in terms of growth inhibition will be investigated.

Sections 1 and 2 will be carried out using the substance P analogue, antagonist G. This analogue was chosen because it is currently undergoing clinical trails as an anti-cancer drug and thus is the most appropriate analogue with which to investigate tumour cell sensitivity. Section 3 will be carried using both antagonist G and related substance P analogue, antagonist D. Finally, section 4 will investigate the mechanism of action of antagonist D. Historically, most of the mechanistic work on this group of compounds has been carried out using antagonist D. To make this work directly comparable to previous research it was decided to concentrate on antagonist D for mechanistic studies in this project.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines

A panel of cancer cell lines was assembled for in vitro experiments. Cell lines included small cell lung cancer lines, non-small cell lung cancer lines, colo-rectal tumour lines, pancreatic tumour lines and ovarian tumour lines (Table 2.1). All SCLC cell lines grew as non-adherent aggregates. All other cell lines grew as adherent monolayers in culture.

Investigations into the mechanism of action of substance P analogues were carried out using non-tumour, fibroblast cell models (Table 2.2). Wild type and transfected Rat-1 fibroblasts were a gift from Dr. E. Rozengurt. Wild type and transfected Balb-3T3 fibroblasts were a gift from Dr. J Battey. All other cell lines used in this project were from ICRF.

For experimental purposes all cell lines were used at the lowest possible passage number and regularly screened for mycoplasma infection. All lines were consistently negative.

2.2 Cell culture

Stocks were maintained in RPMI 1640 (tumour cell lines) or DMEM (non-tumour cell lines)(both from Sigma) medium supplemented with 10% (v/v) foetal bovine serum (Gibco Lifetech) (heat-inactivated at 57^{o}C for 1 h) in a humidified atmosphere of 5% CO_2:95% air at 37^{o}C. Cells were passaged as required. For experimental purposes, cells were either grown in SITA which consists of RPMI 1640 medium

supplemented with, Insulin -Transferrin-Sodium Selenite (SIT) media supplement (Sigma) and 0.25% bovine serum albumin (essentially fatty-acid and globulin free, (Sigma) (Simms et al., 1980) or in quiescent media which consists of 0% FCS (tumour cell lines) or 0.1% FCS (non-tumour cell lines) and 0.25% bovine albumin in either RPMI or DMEM.

Table 2.1 Characteristics of Tumour Cell Lines

Cell Line	Type	Source	Sex	Prior Chemo	Prior Radio	Class
H69	SCLC	PE	M	Yes	Yes	C
H69LX4	SCLC *In vitro* CR	PE	M	Yes (+ *in vitro*)	Yes	C
H510	SCLC	BM	M	yes	-	C
WX330	SCLC	PE	-	no	no	C
GLC4	SCLC	PE	-	no	-	V
GCL4ADR	SCLC *In vitro* CR	PE	-	*In vitro*	-	V
LS274	SCLC	PE	-	no	-	C
LS310	SCLC	PE	-	yes	-	C
GLC14	SCLC	LN	F	no	no	C
GLC16	SCLC	Lung	F	Recurrence of previously treated tumour	yes	C
GLC19	SCLC	Lung	F	yes	yes	C
HT29	Colorectal adeno-carcimoma.	Colon	F	-	-	NA
HCT116	Colorectal	Colon	-	-	-	NA
PEO4	Ovarian	Ascites	-	Yes (resistant)	-	NA
PANC1	Pancreatic	-	-	Yes	-	NA

(Abbreviations: PE – pleural effusion, BM – bone marrow, LN – lymph node, C-classical, V-variant, M-male, F-female, CR – chemotherapy resistant.)

Table 2.2 Characteristics of Non-tumour cell lines

Cell Line	Type	Modifications
Rat-1	Rat Fibroblast	None
Bor15	Rat fibroblast	Stable transfection with bombesin receptors
Balb b1	Mouse fibroblast	Re-clone of Balb 3T3
5ET4	Mouse fibroblast	Balb b1 stable transfection with mouse GRP-R (10^6 receptors /cell)
R288H	Mouse fibroblast	Balb b1 stable transfection with mouse GRP-R with mutated ligand binding site.
CHO-K1	Chinese hamster ovary epithelium.	Sub-clone of CHO cell line.

2.3 Liquid Growth Assay:

SCLC cells were grown in SITA for 3-5 days, then centrifuged at 2,000 rpm for 30s, washed and resuspended in SITA at a density of 5 x 10^4 cells per ml, in the presence or absence of antagonists in triplicate.

Adherent cells were grown in SITA for 3-5 days then plated at a density of 2 x 10^3 cells per ml of SITA, in the presence or absence of antagonists in triplicate.

Cell number was determined using a Coulter Counter (model Z1, Coulter). Prior to counting cell clumps were disaggregated by passing the cell suspension through 19 and 21 gauge needles.

2.4 Determination of intracellular Ca^{2+} concentration:

Aliquots of 4-5 x 10^6 SCLC cells cultured in SITA for 3-5 days, were washed and incubated for 2 h at 37°C in 10 ml fresh SITA medium. Then, 1μM fura-2-tetraacetoxymethylester AME (FURA-2-AM) (Calbiochem) from a stock of 1mM in dimethyl-sulphoxide was added and the cells were incubated for a further 10 minutes. The cell suspension was centrifuged at 2000 rpm for 15 seconds, then the cells were resuspended in 2ml of electrolyte solution (140 mM NaCl, 5 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 16 mM Hepes, 16 mM Tris and a mixture of amino acids at pH 7.2), transferred to a quartz cuvette, and stirred continuously at 37°C.

Adherent cells were grown in DMEM containing 10% FCS until confluent, in 100mm petri dishes then quiesced overnight in DMEM containing 0.1% FCS and 0.25% bovine albumin. Cells were trypsinised to remove them from the dishes then 1mg/ml soya bean trypsin inhibitor was added to the cell suspension. The cells were washed in Hanks balanced salt solution (HBSS without calcium, Sigma) and then incubated with 1μM FURA-2-AM in HBSS (without calcium) for 10 minutes. The cell suspension was centrifuged at 2000 rpm for 15s and the cells resuspended in 2ml HBSS (with calcium), transferred to a cuvette and stirred continuously at 37°C.

Fluorescence was recorded in a Model F2000 fluorescence spectrophotometer (Hitachi). Alternate dual wavelength excitation at 380nm and 410nm allowed ratiometric analysis of bound and unbound FURA-2AM when measured at 505nm

$[Ca^{2+}]_i$ was calculated using the formula:

Rmin = Fmin (λ_1)-Z_1 / Fmin (λ_2)-Z_2
Rmax = Fmax (λ_1)-Z_1 / Fmax (λ_2)-Z_2

R = F(λ_1)-Z_1 / F(λ_2)-Z_2

Concentration C = K (R – Rmin / Rmax – R x Fmin (λ_2) / Fmax (λ_2))

Where;

Fmax (λ_1):	F(λ_1) at saturated Ca concentration.
Fmin (λ_1):	F(λ_1)-at Ca concentration 0.
Fmax (λ_2):	F(λ_2) at saturated Ca concentration.
Fmin (λ_2):	F(λ_2) at Ca concentration 0.
Z_1:	F(λ_1) of sample alone (auto-flourescence).
Z_2:	F(λ_2) of sample alone (autoflourescence)
K:	Constant (dissociation constant of FURA2 = 224nM)

(Further details available in "Measurement of Intracellular Cation Concentration Using a Rapid Scanning Fluorescence Spectrophotometer" Hitachi Scientific Instruments, application notes.)

Fmax was determined by fracturing cell membranes with 0.1% tritonX to release all intracellular calcium. Fmin was determined by chelating all released calcium with 0.25mM EGTA.

2.5 Clonogenic assay

SCLC cells, 3-5 days post-passage, were washed and resuspended in SITA. Cells were then disaggregated into an essentially single cell suspension by two passes through a 19 gauge needle and then through a 20 μm pore size nylon gauze. Viability was judged by trypan blue exclusion on a standard haemocytometer. Cell number was determined using a Coulter Counter. 10^4 viable cells were mixed with SITA containing 0.3% agarose in the presence or absence of antagonists, and layered over a solid base of 0.5% agarose in SITA with antagonists at the same concentration, in 35mm plastic dishes. The cultures were incubated at 37°C for 21 days, then stained with 1mg/ml of the vital stain nitro-blue tetrazolium (NBT, Sigma) overnight at 37°C

Colonies of >120 μm diameter were counted using a microscope with a X4 objective. Colonies from at least 10 different fields were counted for each well.

2.6 Determination of Protein Concentration in Cell Lysates

Samples for analysis of proteins were adjusted to equal protein concentrations using BCA protein assay reagent (Pierce). A standard curve of protein concentration was produced using various dilutions of bovine serum albumin (0.1-0.5 mg/ml) diluted in lysis buffer and protein concentrations of test samples were determined from this curve. Briefly, 200μl of BCA reagent was mixed with 10μl of test or standard sample in a 96 well plate and incubated at 37°C for 30 min. Protein concentration was determined using a 96 well plate reader (MRXII, Dynex technologies) (read at 562nm).

2.7 SDS-PAGE and Western Blotting

Cells were lysed in buffer containing 25mM Hepes (Na salt), 0.3M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5% Triton X-100, 20mM β-glycerophoshate, 0.5mM dithiothreitol and 1mM orthovanadate (pH 7.4). One Complete™ protease inhibitor tablet (Boehringer Manheim) was added per 50ml of lysis buffer. Lysates were cleared by centrifugation at 13000 rpm for 10 minutes at 4°C. Lysates were boiled at 99°C for 5 minutes in sample buffer containing (for 4X stock) 50mM Tris, 10% glycerol, 2% SDS, 0.1% bromophenol blue and 10% mercaptoethanol (pH 7.4).

Samples were loaded onto SDS-polyacrylamide mini-gels (12%) comprised of a stacking gel and a separating gel. Separating gels were prepared from a solution containing H_2O (10ml), 30% acrylamide (8.3ml), 1.5M Tris pH8.8 (6.3ml), 10%SDS

(0.25ml), 10% ammonium persulphate (APS) (0.25ml) and TEMED 0.01ml). Stacking gels were prepared from a solution containing H_20 (6.8ml), 30% acrylamide (1.7ml), 1M Tris pH 6.8 (1.25ml), 10% SDS (0.1ml), 10% APS (0.1ml) and TEMED (0.01ml). Gels were run at 160V for 1hr then transferred onto nitrocellulose membrane for 1hr at 400mA.in buffer containing 210mM glycine, 24.7mM Tris base and 20% methanol.

Nitrocellulose membranes were stained with Ponceau S (Sigma) to confirm equal protein loading then blocked for 1hr at room temperature in PBS containing 5% marvel non-fat dried milk powder and 0.02% tween20. Blocked membranes were incubated for 1-2hrs, with primary antibodies, at various concentrations (table 2.3), diluted in blocking solution then washed several times in PBS containing 0.02% tween20. Membranes were then incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (1:1000) diluted in blocking solution. Following further washes, antibody bound proteins were detected using ECL western blotting detection reagent (Amersham Pharmacia Biotech).

Table2.3. Antibodies used for probing western blots.

Primary Antibody	Source	Working Conc	Supplier
pMAPK (pERK1 & pERK2)	Mouse Monoclonal IgG_1	1:2000	Sigma
Pan-Ras (AB1)	Mouse Monoclonal $IgG_{2b}K$	1:20	Oncogene
H-Ras (C-19)	Rabbit Polyclonal	1:200	Santa Cruz
P21 WAF1 (AB-4)	Mouse Monoclonal IgG_{1K}	1:200	Oncogene
P27 (F-8)	Mouse Monoclonal IgG_1	1:500	Santa Cruz

2.8 MAPK (ERK2) activity assays

Samples were lysed on ice as for SDS-PAGE in 0.5ml lysis buffer. Lysates were cleared by centrifugation at 13000 rpm for 10 min. Cleared lysates were equilibrated for protein concentration (500μg/ml) then vortexed for 2-4hrs at 4^0C with 3μg of agarose conjugated ERK2 pAB (Santa Cruz). Agarose beads were washed three times at 4^oC in a buffer containing 20mM Hepes, 50mM NaCl, 2.5mM $MgCl_2$ and 0.1mM EDTA and once in kinase buffer containing 20mM Hepes, 0.5mM NaF, 7.5mM $MgCl_2$, 0.2mM EGTA, 2mM dithiothreitol, 10mM β-glycerophosphate and 0.5mM Na_3VO_4. One protease inhibitor tablet was added per 50ml of kinase buffer. Each sample was incubated at 30^0C for 20 minutes in 20μl of kinase buffer containing 10μg myelin basic protein, 100μM ATP and 1μCi Redivue [^{33}P]-ATP (500Ci/mmol) (Amersham Pharmacia Biotech). Samples were spotted onto p81 paper and washed three times in 500ml 0.5% phosphoric acid and once in acetone then allowed to dry before counting radioactivity in a Liquid scintillation analyser (1900TR, Packard).

2.9 Measurement of Thymidine Incorporation.

Confluent quiescent cells in 24 well trays were stimulated with various agonists / antagonists in DMEM (0.1 % FCS) for 24hrs at 37°C. During the last 5hrs 250μl of a mixture containing 40μM [6-^{3}H] thymidine (1mCi/ml. 25Ci/mmol) (Amersham Pharmacia Biotech) and 400mM unlabelled thymidine in DMEM was added to each well. Cells were washed twice in ice cold PBS then twice in 5%TCA and finally twice in 70% ethanol. Cells were then solubilised in 250μl base solution containing

2% Na_2 $C0_3$, 0.1N NaOH and 1%SDS which was then transferred to scintillation vials and incorporated radioactivity counted on a liquid scintillation analyser.

2.10 Semi-Quantitative RT-PCR for Neuropeptide Receptors.

RNA was extracted using Tri-Reagent (Sigma) from exponentially growing cells, which had been in SITA medium for 3-5 days and was subsequently treated with DNase1 to remove any DNA contamination. cDNA was produced from this RNA using a 1st strand synthesis kit (Boehringer Manheim).

PCR primers were designed using Gene Jockey software and synthesised by ICRF (table 2.4). All primer sequences were screened against Genbank database to check for any mismatches. None were found.

PCR reactions were standardly optimised for $MgCl_2$, dNTPs and PCR cycling parameters (table 2.5). Taq polymerase was produced by ICRF. By measuring product production over a range of cycles it was ensured that the chosen cycle number for each primer pair, was within the linear phase of product production (Fig 2.1 and table 2.5).

PCR products were run on a 1.5% agarose gel containing 1μg/ml ethidium bromide to enable UV visualisation. Stored images of the gels were analysed by densitometry using Gel Base/ Gel Blot software.

γ-Actin levels were measured as an internal control (Fig 2.2). To compensate for variation in cDNA synthesis efficiency and PCR reagent pippetting accuracy, levels of each product was expressed relative to the levels of γ-actin for the same cDNA batch and for each PCR reaction.

The following PCR protocol was followed for each primer pair:

Step 1: 94°C for 2min 1 cycle

Step 2: a) 92°C for 30sec

b) *various annealing temperatures*

c)72°C for 45 sec *various cycle numbers*

Step 3: 72°C for 5 min 1 cycle.

Table 2.4: Primers for RT-PCR Detection of Neuropeptide Receptors.

Target cDNA	Primer Sequence (F=forward, R=reverse)	Product Size	Annealing Temp (°C)
GRP R	F: ATCTTCTGTACAGTCAAGTCC R: GCTTTCCTCATGGAAGGGATA	374bp	60
V1A R	F: TACCTGCTACGGCTTCATCTGC R: ACACAGTCTTGAAGGAGATGGC	380bp	56
BK2 R	F: CCTGAGTGTCATCACCTTCTGC R: TTGATGACACGGCAGAGG	141bp	62
GASTRIN R	F: CCTATCTCCTTCATTCACTTGC R: AGTGTGCTGATGGTGGTGTAGC	220bp	62
γ-ACTIN	F: ATGGCATCGTCACCAACTGG R: ATGACAATGCCAGTGGTGGC	240bp	57

All primers were stored at –20°C in DEPC treated H_2O

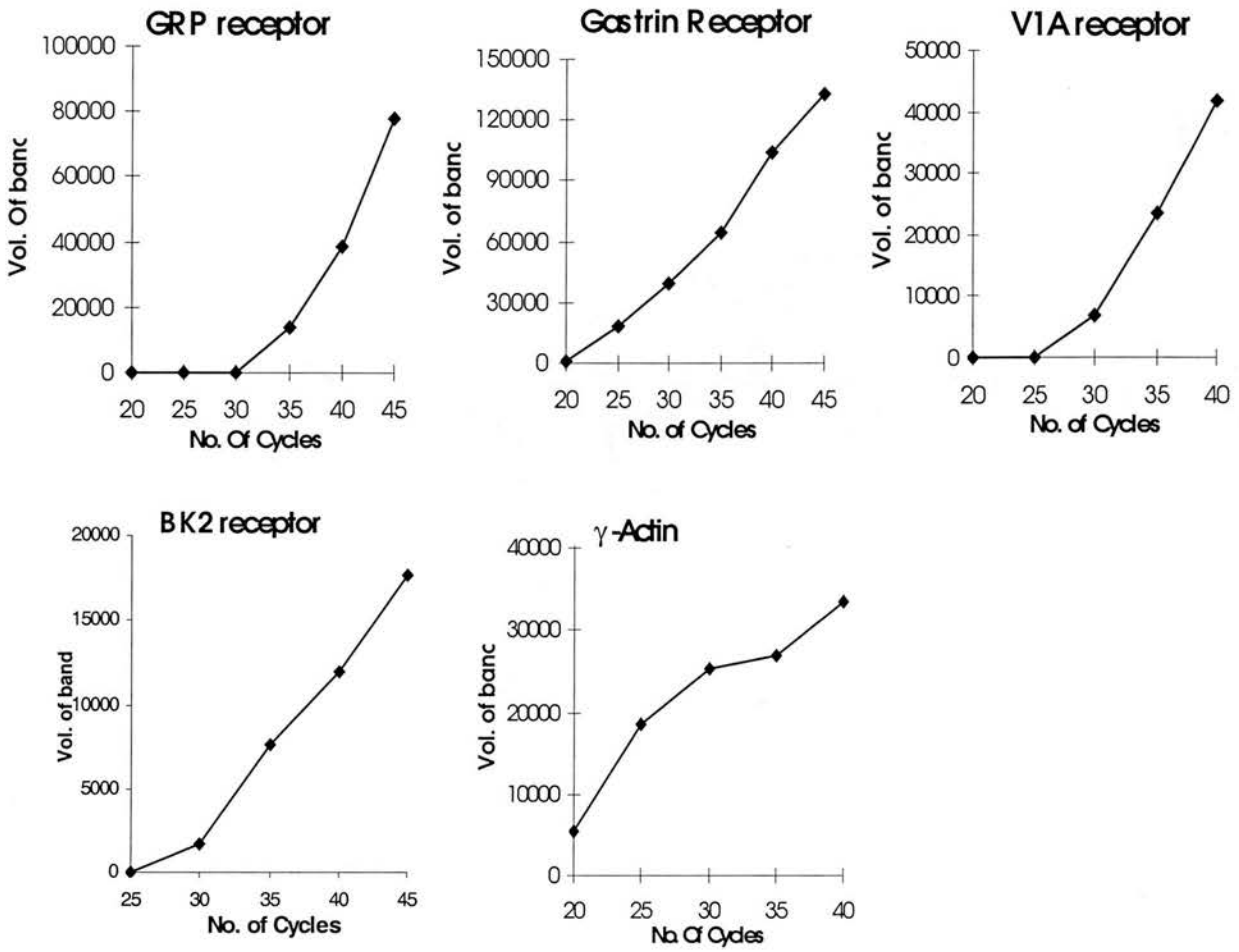


Fig 2.1: PCR Cycle Optimisation for semi-quantitative RT-PCR PCR cycles were optimised for each primer pair to ensure that product production was in a linear phase. Product production was measured by densitometry between 20 and 45 reaction cycles. Each primer pair was optimised in a cell line which was expected to have a mid-range expression level of a particular receptor. Table (2.5) shows the optimal number of cycles for each set of primers.

Table 2.5: Optimisation of PCR cycles

cDNA Detected	RNA source	Optimal number of PCR cycles
GRP-R	HT29	40
Gastrin Receptor	H510	40
V1A-R	H510	35
BK-2-R	H69	40
γ-Actin	H510	35

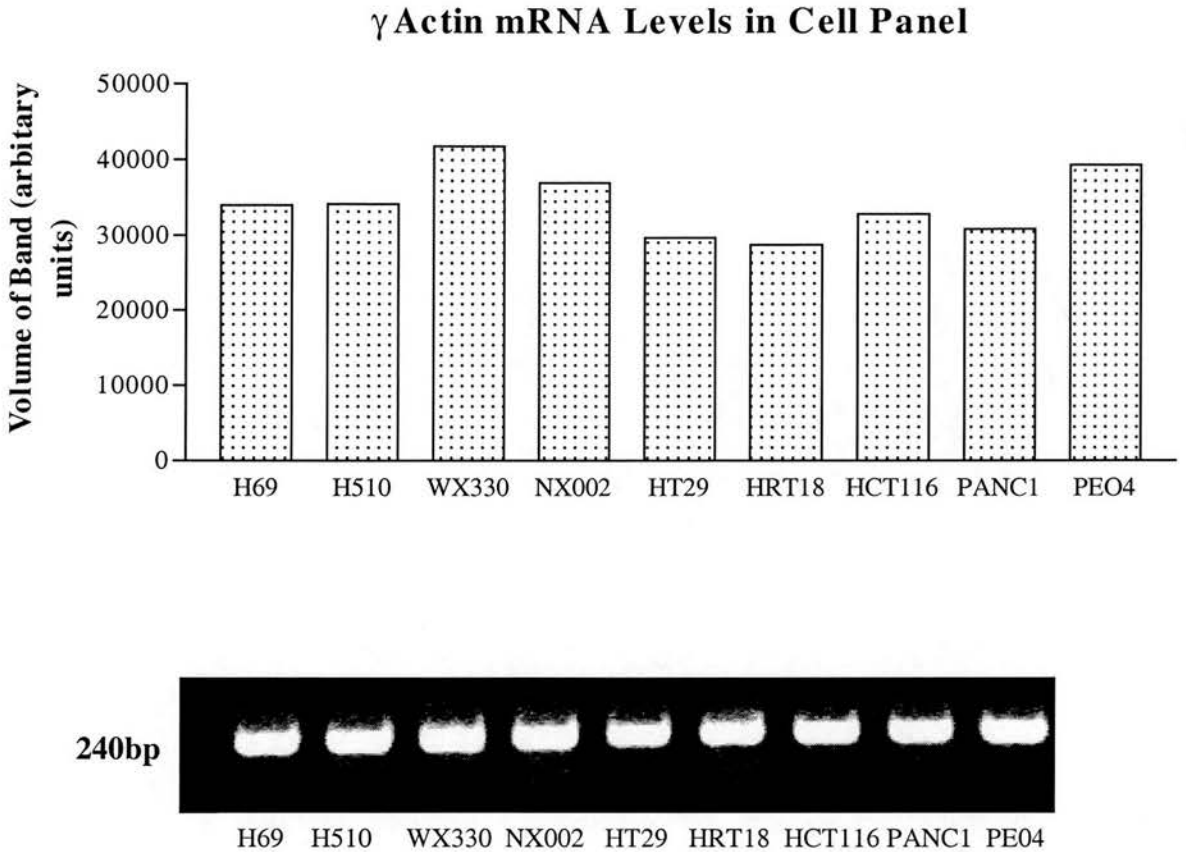


Fig2.2. γ Actin mRNA levels across tumour cell panel. γ actin mRNA levels were similar across all nine cell lines. Chart shows desitometrical analysis of a representative RT-PCR gel (shown below). The mean γ-actin level in arbitrary units, was 34250 (+/- 4396).

2.11 Detection of Apoptosis

2.11.1 Morphological detection.

Cells were quiesced in media containing 0% (SCLC cells) or 0.1%FCS (all other cells) in the presence or absence of test agents for 24hrs. The cells were harvested, and aliquots were cytocentrifuged onto glass slides and stained with May-Grunwald-Giemsa stain. Slides were examined by light microscopy and cells displaying apoptotic morphology (cell shrinkage, chromatin condensation) were counted from >400 cells from random fields.

2.11.1 Annexin V binding

Cells were quiesced in media containing 0% or 0.1%FCS in the presence or absence of test agents for 24hrs. Cells were harvested and washed twice in cold PBS then re-suspended in 250µl of annexin V (Bonder Medsystems) diluted 1:250 in binding buffer (5mM $CaCl_2$ in Hanks balanced salt solution). Cells were incubated in the dark at 4^0C for 10min then annexin V positive cells were detected by flow cytometry (Coulter Profile II).

2.11.2 Analysis of caspase activation

CPP32 (caspase 3) activity, as an indicator of apoptosis, was measured using an ApoAlert™ kit (Clontech). SCLC cells were grown in SITA for 3 days prior to experimentation. $1x10^6$ cells were treated with antagonist G or a positive control for 24hrs at 37°C. Cells were lysed using the provided lysis buffer. All samples were incubated with DEVD –AFC, a CPP32 substrate. Upon activation of CPP32, this substrate is cleaved to form a fluorescent complex which was detected at 505nm using a fluorescence spectrophotometer (Model 2000 –Hitachi). Parallel samples were incubated with a caspase inhibitor (DEVD-CHO) to ensure that the increased fluorescence at 505nm was due to caspase activity. Further samples were analysed without incubation with DEVD-AFC to determine background fluorescence emissions.

2.12 Transformation of Bacteria and Extraction of Plasmid DNA.

The V1A receptor cDNA was a gift from Professor. M Threbonnier and was provided in a pcDNA3 plasmid. The GRP receptor cDNA was a gift from Dr. J Battey and was provided in a modified pcDII plasmid.

Plasmid DNA was sub-cloned into DH5α™ competent bacterial cells (Life Technologies) and following overnight growth of the bacterial culture, media containing appropriate antibiotic for selection of plasmid expression, the plasmid DNA was extracted using Wizard plus SV Miniprep / Maxiprep kits (Promega). To check for efficient transformation, some extracted plasmid DNA was digested using appropriate restriction enzymes to release the insert, and ran on a 1% agarose gel containing ethidium bromide to enable UV visualisation.

2.13 Stable Transfection of Rat-1 Fibroblasts.

Cells were transfected using Lipofectamine PLUS™ reagent (Life Technologies). $1x10^6$ Rat-1 fibroblasts were plated in 100mm petri dishes in 10%FCS DMEM until 70% confluent (overnight). The following day, 10μg of cDNA for either the GRPR or the V1AR was pre-complexed with PLUS reagent in serum free DMEM. Lipofectamine reagent was diluted in serum free DMEM and then combined with the pre-complexed cDNA for 15min at room temperature. The media on the cells was replaced with 4ml of serum free DMEM and the DNA-PLUS-Lipofectamine complexes were then added gently to the petri dishes and incubated for 3hrs at 37°C. After this period the media was topped up to normal volume and serum concentration. The following day the cells were trypsinised and distributed into 5 x 100mm petri dishes, containing 10ml of 10%FCS DMEM, and incubated overnight

at 37°C. 24 hrs later, 800μg/ml geneticin (G418) (Promega) was added and the media changed every three days until isolated colonies were visible. The cell suspension was diluted in 10% FCS DMEM containing 800μg/ml G418 to a density of 1 cell/200μl and 200μl was added to each well of a 96 well plate. Individual wells were trypsinised following clonal growth and 24 clones from each transfection were expanded. The clones were screened for the presence of transfected receptors by testing their ability to mobilise intracellular calcium in response to the receptor ligands and also by their ability to bind radiolabelled ligand.

2.14 Radio-Ligand Binding Assay using [^{125}I]GRP.

Rat-1 fibroblasts were transfected with cDNA encoding the Gastrin Releasing peptide receptor (above) and screened for their ability to bind radiolabelled GRP. Cells were grown to confluence in 6 well plates and quiesced overnight in DMEM containing 0.1%FCS. The following day, the cells were washed twice in serum free DMEM then incubated at 37°C in 750μl of binding medium (30mM HEPES, 5mM $MgCl_2$ (pH8)) containing [^{125}I]GRP (Amersham) at a final concentration of 1nM. Non- specific binding was measured by incubating the cells with the above binding medium in the presence of both 1nM radiolabelled GRP and 1μM unlabelled GRP. Binding was measured after 30 minutes by washing the cells with ice-cold phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), then solubilising the cells with 500μl NaOH containing 0.1% sodium dodecyl sulphate (SDS) and 2% Na_2CO_3. Samples were transferred to scintillation vials and radioactivity was counted in a β scintillation counter. Total binding was calculated by subtracting the non-specific binding value from the test samples.

2.15 Detection of Active Ras using c-Raf-1:GST Pull-outs.

Active Ras was measured by its ability to bind to the Ras binding domain of Raf-1 and detected by western blotting. Plasmid encoding the 42kDa fusion protein clone (Ras binding domain of human c-Raf1 - amino acids 1-149) was a gift from Dr. J Downward (ICRF) and were provided in BL-21 bacteria.

Bacteria were grown overnight in a shaking incubator at 37°C in liquid broth (LB) containing 100μg/ml ampicillin and the following day this culture was used to inoculate a 500ml flask of LB/Amp. The culture was grown to an OD of 0.35-0.6 (measured at 600nm) and then induced with 1mM Isopropylthio-β-D- galactoside (IPTG) for 2hrs at 37°C. The bacteria were centrifuged at 4000rpm for 15min at 4°C and the pellet was resuspended in 5ml of lysis buffer containing 100mM NaCl, 50mM HEPES (pH 7.5) 1% Triton X-100, 1mM EDTA (pH8) 1mM EGTA (pH 8), 1mM Na_3VO_4, 20mM Na , 12mM $MgCl_2$ and one protease inhibitor tablet per 50ml. The suspension was sonicated in a water bath. The lysate was cleared by centrifugation at 13000rpm for 10min at 4°C and incubated on a wheel for 1hr at 4° with 1ml of glutathione agarose (Sigma, 1:1 slurry in PBS). The beads were washed 4 times in buffer A containing 20mM HEPES (pH 7.5), 120mM NaCl, 10% Glycerol, 0.5% triton X-100, 2mM EDTA and 1 protease inhibitor tablet per 50ml and then stored as a 1:1 slurry in buffer A.

Cell lysates were prepared from stimulated or non stimulated cells grown to confluency in 100mm petri-dishes using 500μl of the above lysis buffer. 60μl of the above fusion protein preparation was added to cleared lysates and incubated at 4°C on a rotating wheel for 30min. The beads were then washed three times with cold PBS containing 5mM $MgCl_2$ and 0.1% triton X-100. Following the final wash, 40μl

of 1X sample buffer was added and samples boiled for 5 min at 99^{0}C before running on SDS-PAGE (12%).

Nitrocellulose membranes were probed with either anti-Pan-Ras (AB-1, mouse monoclonal, diluted 1:200. Oncogene research products) or anti-R-Ras (C-19, rabbit polyclonal, diluted 1:20. Santa Cruz).

2.16 HPLC Analysis of Antagonist G and its Metabolites

High performance liquid chromatography was carried out on a liquid chromatograph consisting of a Model 500 solvent delivery system, a Model 680 controller, a Wisp autosampler (all from Waters), a Model5100A Coulochem electrochemical detector with a pre-column Model 5020 guard cell (GC) and a twin electrode (D1 and D2) Model 5011 high sensitivity analytical cell (ESA inc). The stationary phase was μ-Bondpack C18 packed in a 30cm x3.9mm I.D. stainless steel column (Waters) and the mobile phase consisted of 0.15% TFA in 10mM ammonium acetate (pH2.75)-acetonitrile: 60:40 (v/v).

Samples of antagonist G for HPLC analysis were prepared in 1ml volumes in various media then frozen at –20^{0}C until analysed. Prepared samples were kindly analysed by Alex McLellan (ICRF).

2.17 Statistical Analysis and Curve Fitting.

Statistical analysis and curve fitting were carried out using Prism software (GraphPad Prism version 3.00 for windows, GraphPad software, Santiago USA). Statistical tests used were paired, two-tailed T-tests and Spearman-rank correlation tests, as indicated.

RESULTS: CHAPTER 3

3.1 Investigation into the Sensitivity of a Panel of Tumour Cell Lines to Antagonist G

Antagonist G is entering a phase II clinical trial for the treatment of small cell lung cancer. Autocrine loops of neuropeptide growth factors are central in maintaining the unregulated growth of small cell lung cancer cells. Thus, the use of broad spectrum neuropeptide antagonists as a therapy for SCLC is an exciting development in cancer therapy.

It is well known that neuropeptides can stimulate the growth of many other types of cancers including pancreatic cancer, colo-rectal cancer, prostate cancer, ovarian cancer, breast cancer and non-small cell lung cancer. The aim of this work was to determine whether cell lines from various tumour types are sensitive to antagonist G and to investigate the range of sensitivities within a panel of 11 cancer cell lines.

The nature of the growth inhibition will be discussed. It has been previously reported that broad-spectrum neuropeptide antagonists can induce apoptosis and this will be confirmed using the H69 SCLC cell line.

Finally, HPLC analysis of the rates of metabolism of antagonist G will be investigated. This work was carried out in order to determine the stability of antagonist G in SITA media and also to examine whether differential metabolism rates of sensitive and resistant cells is a factor determining the potential of antagonist G to cause growth inhibition. The presence of antagonist G in cells will be examined over a time course to

determine how long the drug is associated with the cells in the tissue culture environment.

3.1.1 Determination of Sensitivity to Antagonist G in Liquid Culture

A panel of 11 tumour cell lines was assembled representing a spectrum of tumour types (Table 3.1). The sensitivity of each cell line to antagonist G was determined in liquid growth assays. IC_{50} values (the concentration of drug required to reduce cell growth by 50% of the control growth level) for growth inhibition were calculated using a non-linear regression, curve fitting application in Prism data analysis software.

Table 3.1: Panel of tumour cell lines (see materials and methods for further details).

CELL LINE	TUMOUR TYPE
H69	SCLC
H510	SCLC
WX330	SCLC
GLC4	SCLC
CORL51	SCLC
NX002	NSCLC
HT29	Colo-rectal
HRT18	Colo-rectal
HCT116	Colo-rectal
PEO4	Ovarian
PANC1	Pancreatic

IC_{50} values were calculated during the exponential growth phase of each cell line. This was between day 6 and day 10 in each case.

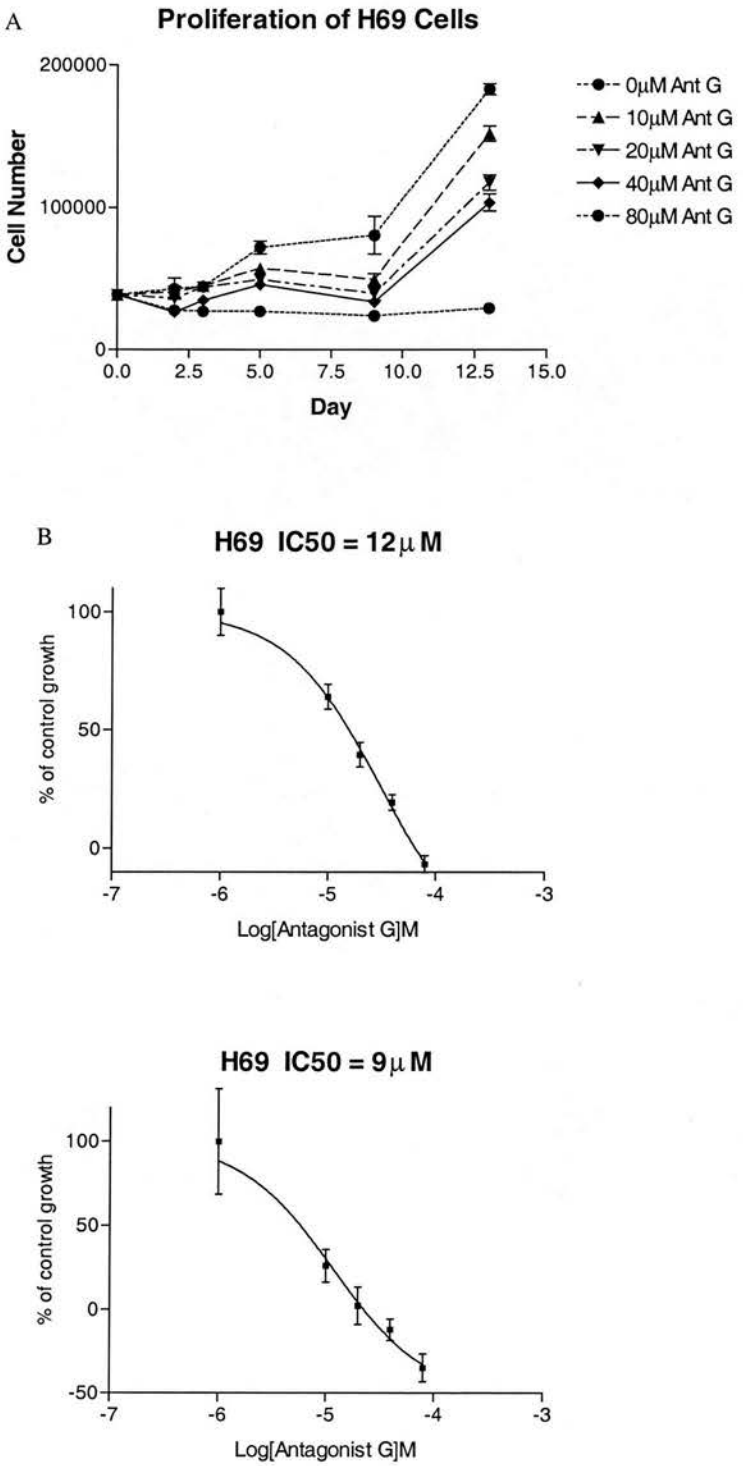


Fig 3.1 A-Proliferation of SCLC cell line H69 in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.

B- IC_{50} determinations for two independent experiments. Day 8 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the H69 cell line =10.5μM (+/-2.1μM)

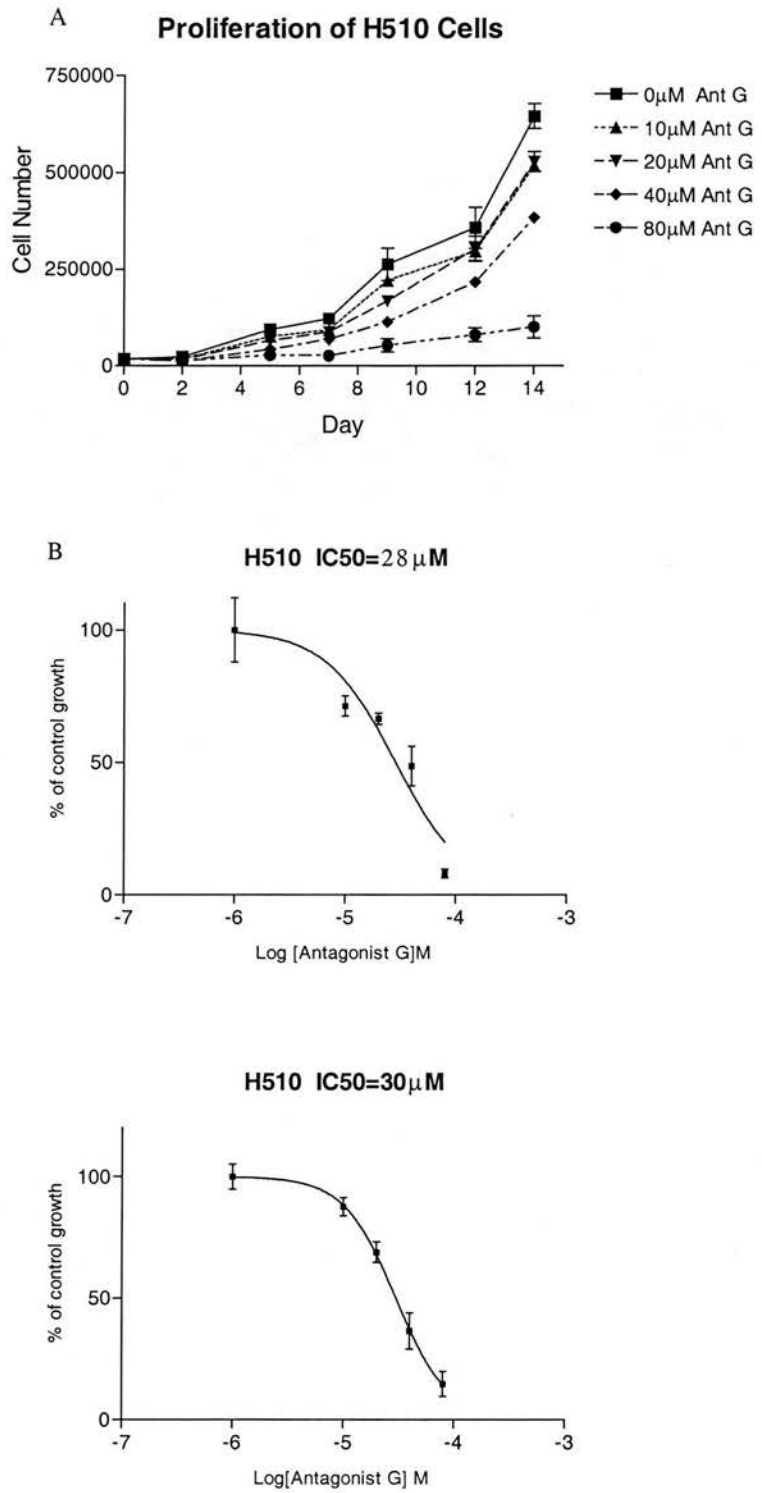


Fig 3.2 A-Proliferation of SCLC cell line H510 in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.

B- IC_{50} determinations for two independent experiments. Day 9 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the H510 cell line =29μM (+/-1.4μM)

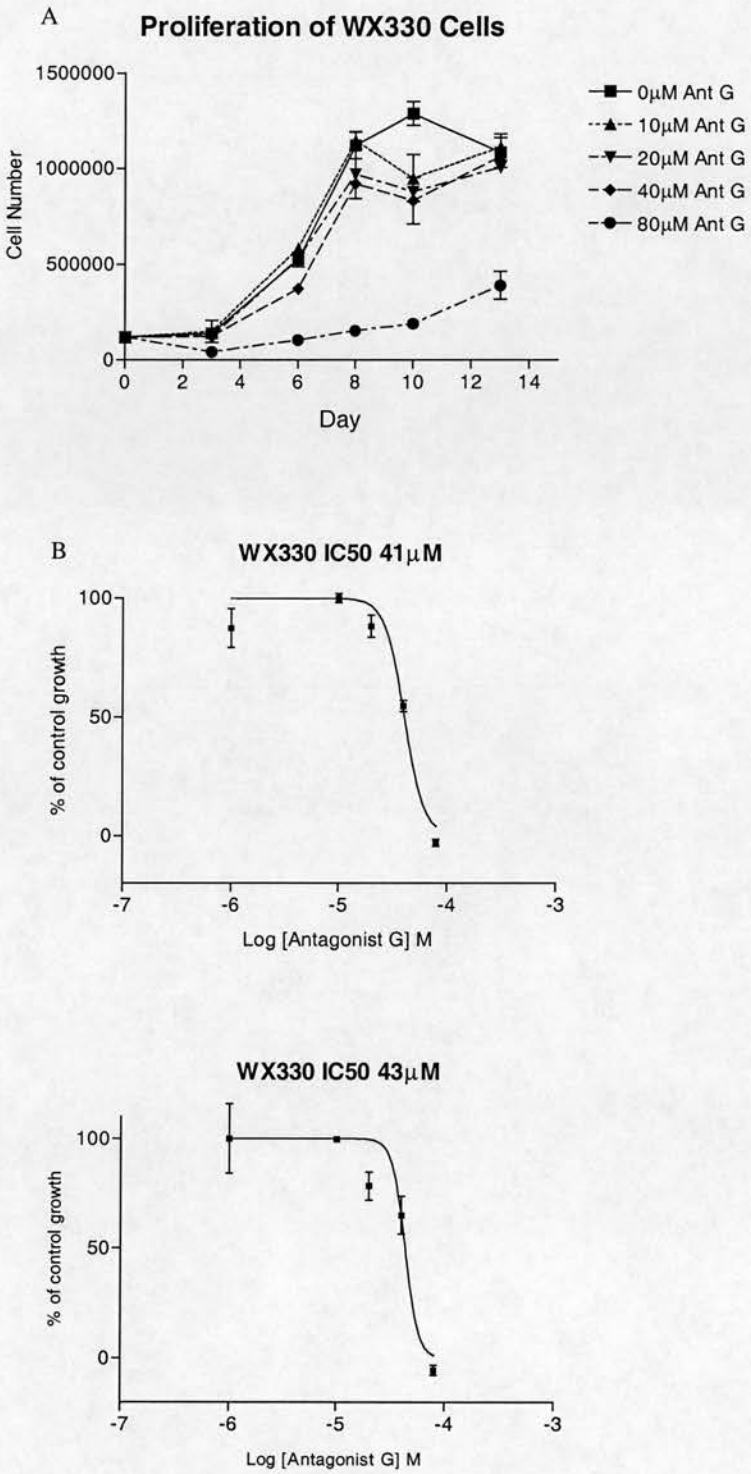


Fig 3.3 A-Proliferation of SCLC cell line WX330 in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.

B- IC_{50} determinations for two independent experiments. Day 6 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the WX330 cell line =42μM (+/-1.4μM)

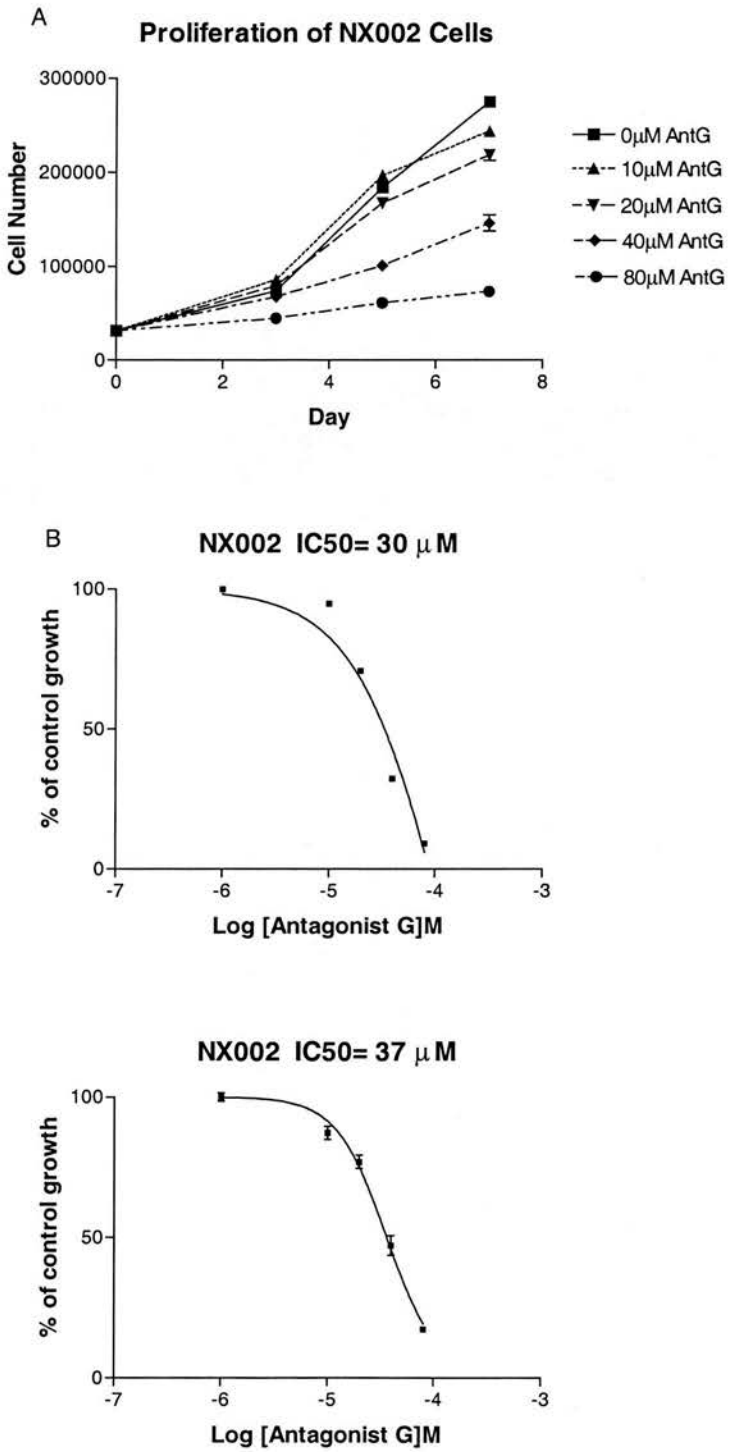


Fig 3.4 A-Proliferation of NSCLC cell line NX002 in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.

B- IC_{50} determinations for two independent experiments. Day 7 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the NX002 cell line =33.5μM (+/- 4.9μM)

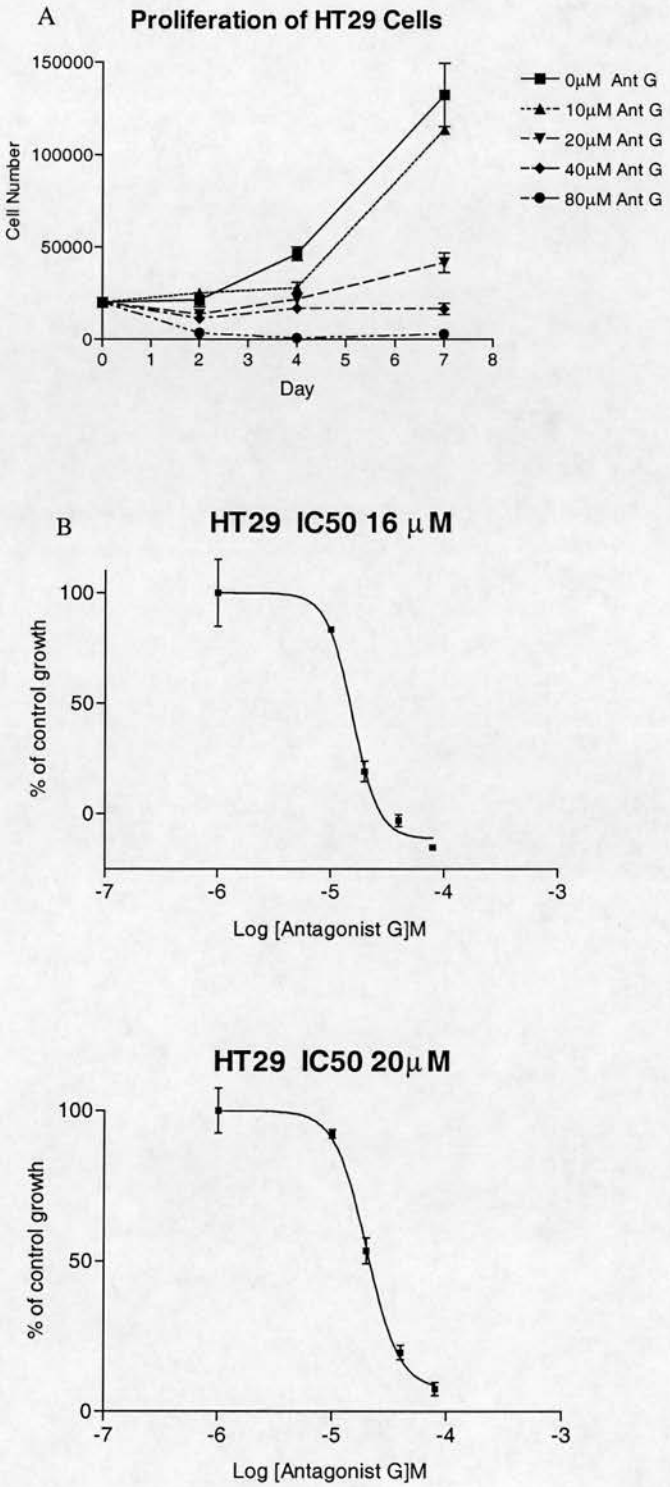


Fig 3.5 A-Proliferation of Colo-rectal carcinoma cell line, HT29, in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.
B- IC_{50} determinations for two independent experiments. Day 7 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the HT29 cell line =18μM (+/-2.8μM)

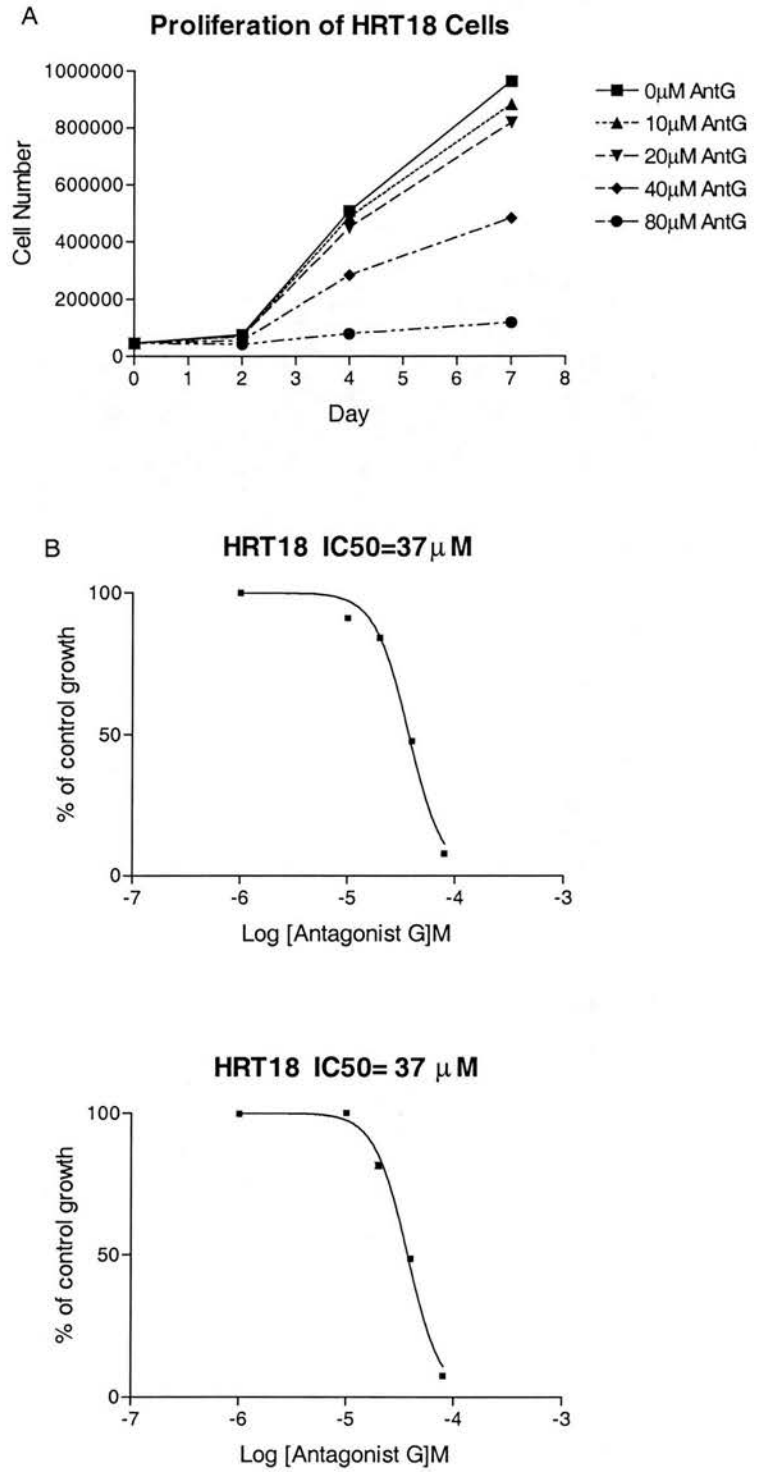
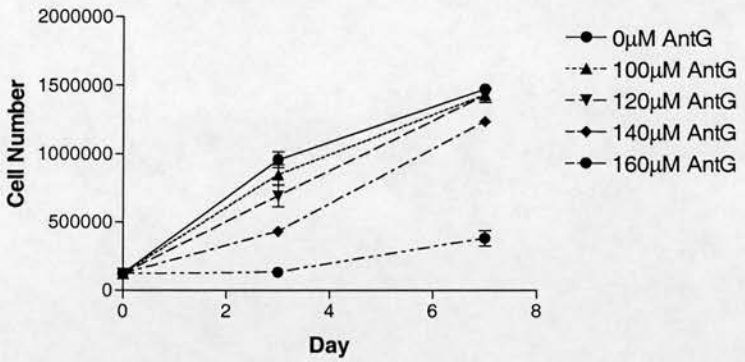


Fig 3.6 A-Proliferation of Colo-rectal carcinoma cell line, HRT18, in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.
B- IC_{50} determinations for two independent experiments. Day 7 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the HRT18 cell line =37μM

A



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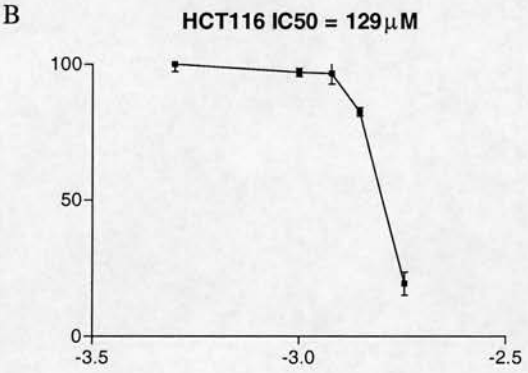


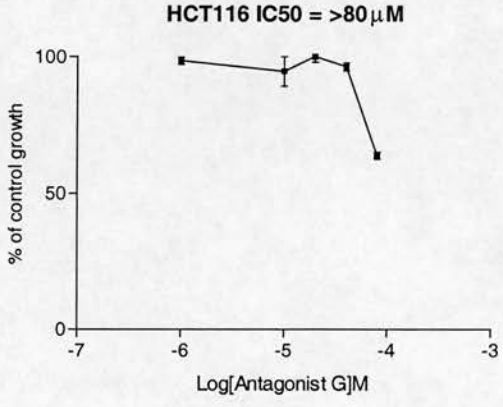


Fig 3.7 A-Proliferation of Colo-rectal carcinoma cell line, HCT116, in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.
B- IC_{50} determinations for two independent experiments. Day 7 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

IC_{50} for the HCT116 cell line ~129μM

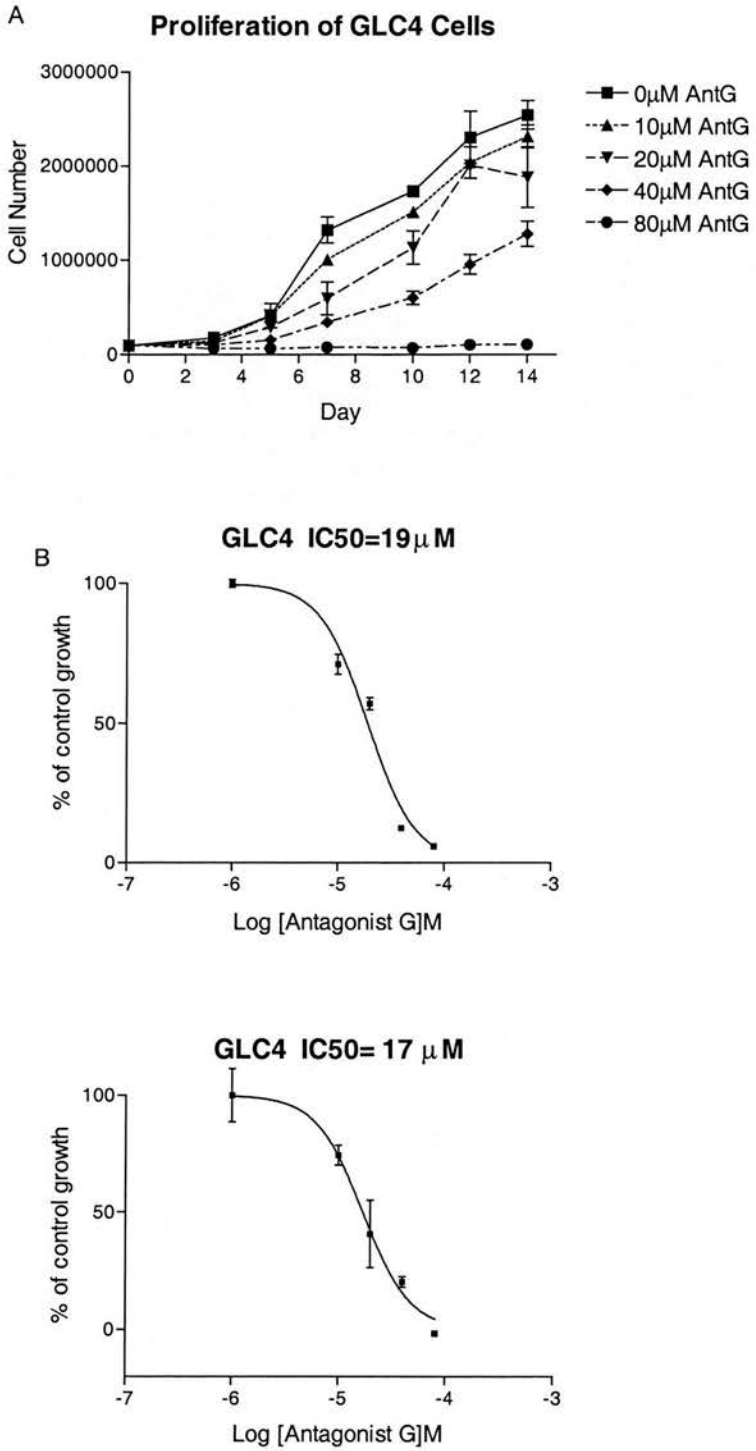


Fig 3.8 A-Proliferation of SCLC cell line, GLC4, in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.

B- IC_{50} determinations for two independent experiments. Day 10 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the GLC4 cell line =18μM (+/- 1.4μM)

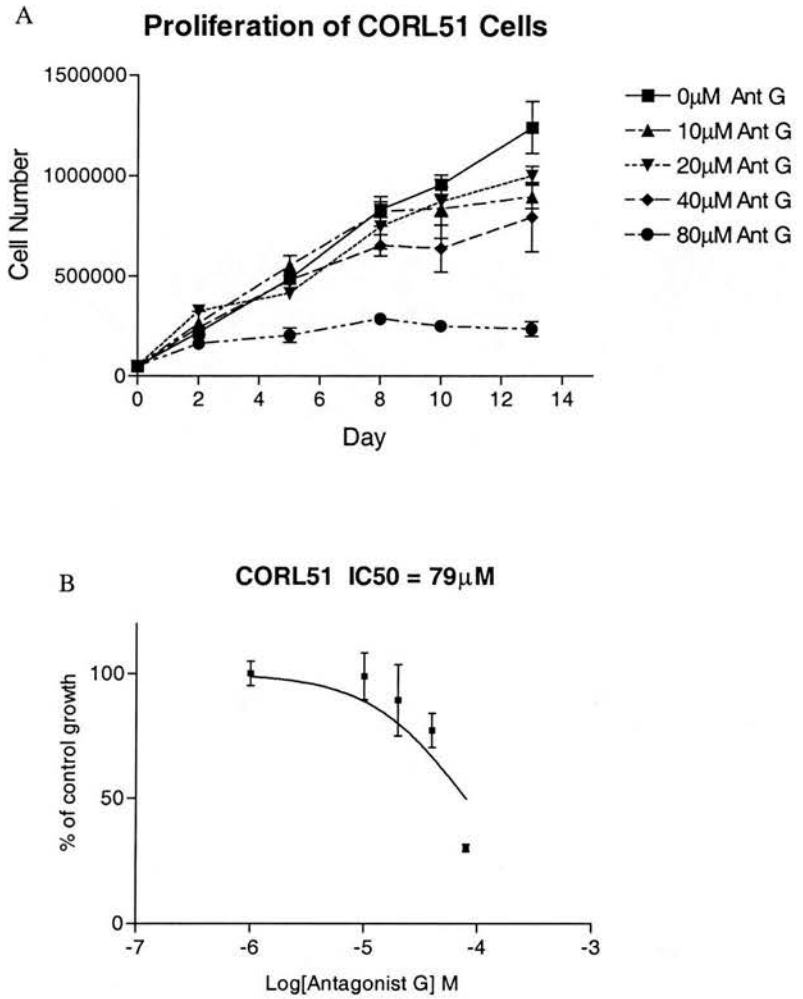


Fig 3.9 A-Proliferation of SCLC cell line, CORL51, in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Data points represent the mean of triplicate samples.
B- IC_{50} determination. Day 8 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

IC_{50} for the CORL51 cell line =79μM

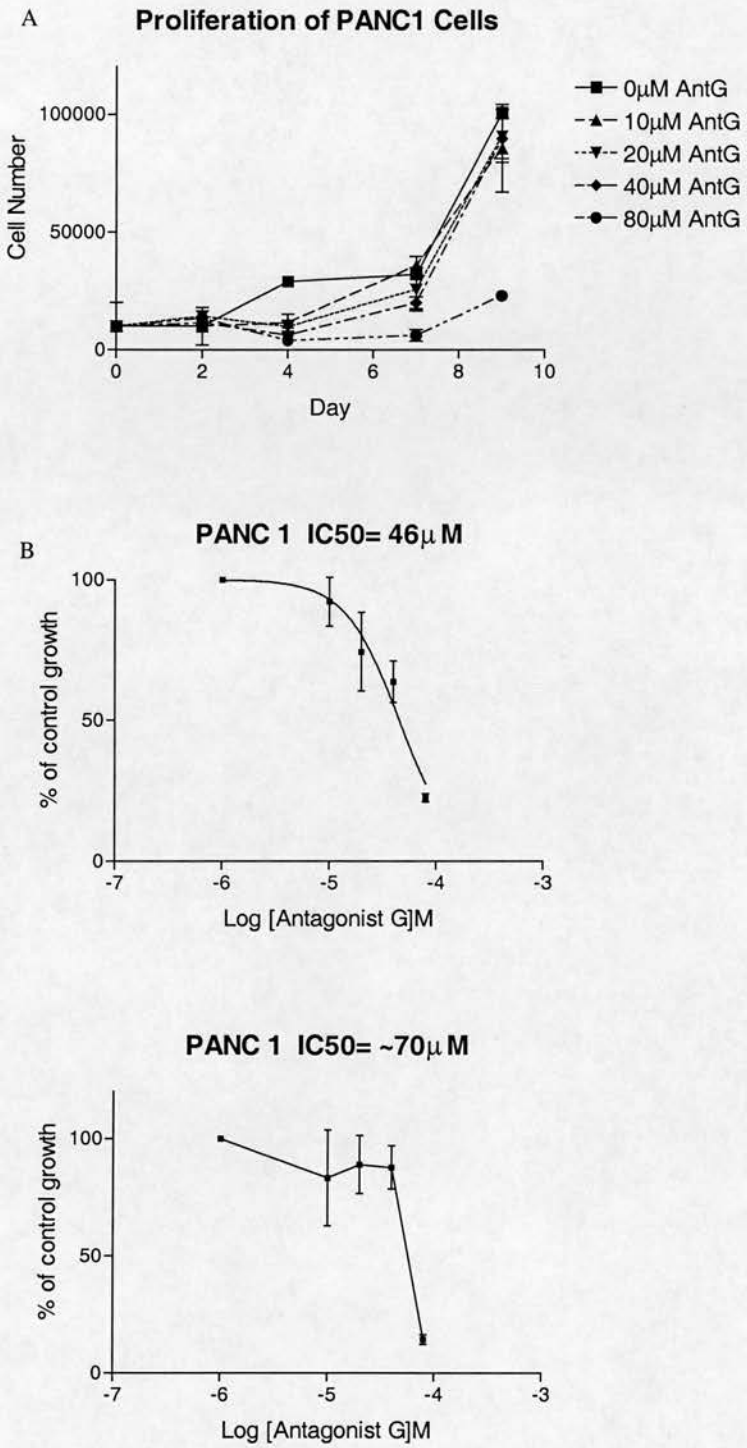


Fig 3.10 A-Proliferation of pancreatic carcinoma cell line, PANC1, in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Graph is representative of at least two independent experiments. Data points represent the mean of triplicate samples.
B- IC_{50} determinations for two independent experiments. Day 9 cell counts were used to plot curves. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

Mean IC_{50} for the PANC1 cell line = ~58μM

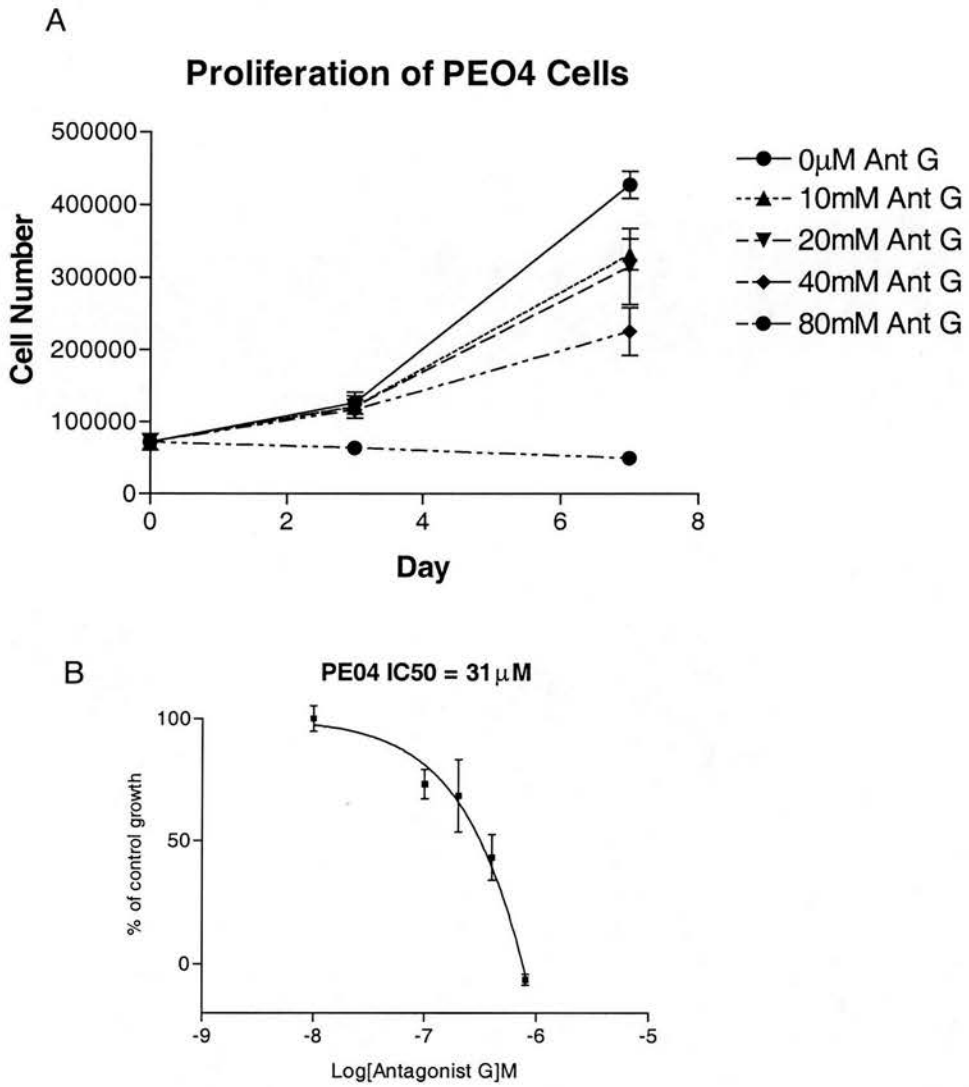


Fig 3.11 **A**-Proliferation of ovarian carcinoma cell line, PEO4, in the presence or absence of various concentrations of Antagonist G. Cell number was determined using a coulter counter at the intervals indicated. Data points represent the mean of triplicate samples.
B- IC_{50} determination. Day 7 cell counts were used to plot curve. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

IC_{50} for the PEO4 cell line =31μM

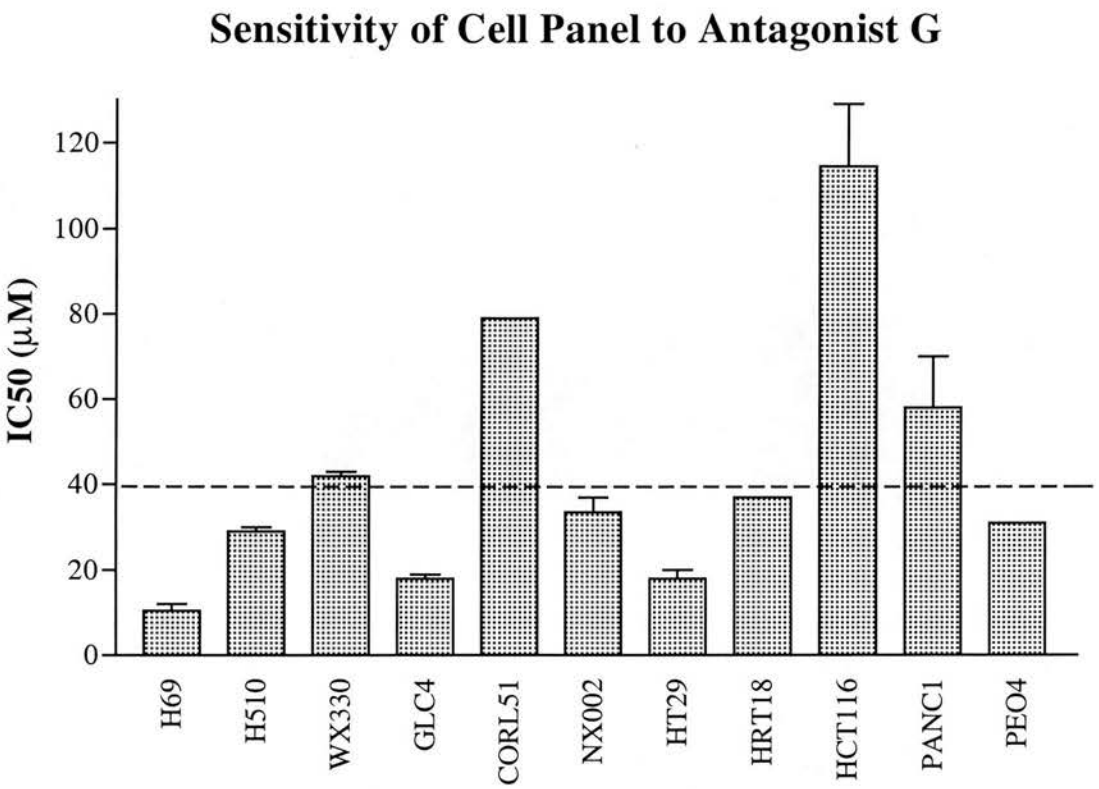


Fig 3.12 Sensitivity of 11 tumour cell lines to growth inhibition by antagonist G.
The above figure summarises IC_{50} values from figures (3.1-3.11). The cell panel includes small cell lung cancer cell lines (H69, H510, WX330, GLC4 and CORL51), a non-small cell lung cancer cell line (NX002), colo-rectal carcinoma cell lines (HT29, HRT18 and HCT116), a pancreatic carcinoma cell line (PANC1) and an ovarian cell line (PEO4). The IC_{50} value for the cell line HCT116 is an estimation as high concentrations of antagonist G (80μM) did not produce a sufficient growth inhibition to calculate an accurate IC_{50} value. IC_{50} values were determined from two independent experiments, except for CORL51 and PEO4, which are the results of single determinations.
The dotted line represents mean plasma levels of Antagonist G achievable in patients, with no dose limiting toxicity.

Within the cell panel of 11 tumour cell lines, the IC_{50} values ranged from 10μM to >100μM. The mean IC_{50} value was 42.7μM.

The sensitivity of the cell panel to growth inhibition by antagonist G, was not confined to tumour type. The three most sensitive cell lines were the H69 SCLC cell line (IC_{50} - 10.5μM), the GLC4 SCLC cell line (IC50-18μM) and the HT29 colo-rectal cell line (IC50 18μM). 4/6 lung cancer cell lines had IC_{50} values below the mean IC_{50} value.

The most resistant SCLC cell line was CORL51 with an IC^{50} value of 79μM, however, this cell line grew in extremely tight aggregates and this may have affected the cell number determinations and subsequent IC_{50} calculations. The colo-rectal cell line, HCT116 was the most resistant with an estimated IC_{50} of ~115μM. Both the ovarian carcinoma and the non-small cell carcinoma were sensitive to growth inhibition by antagonist G, with IC_{50} values of 31μM and 31.5μM respectively.

During the phase 1 clinical trial of Antagonist G, plasma levels of up to 40μM were achievable with no dose limiting toxicity. Interestingly, 75% of all cancer cell lines tested had IC_{50} values near or below this concentration.

Information as to the nature of the growth inhibition by antagonist G can not be gained from the above liquid growth assays. Although, in the majority of cases, the final cell number of treated samples was not below the initial plating density, this does not necessarily mean that antagonist G acts as a cytostatic drug. Cytotoxicity, resulting in cell death, may also be occurring but the ratio of mitosis versus apoptosis/necrosis may mask any observable cytotoxic effect. Further experiments are required to determine the nature of growth inhibition by antagonist G. These experiments will be discussed next.

3.2 Detection of Apoptosis Induced by Antagonist G

Three methods of detection of apoptotic cells were employed to confirm previous findings which suggested that substance P analogues could induce apoptosis in SCLC cells (Reeve and Bleehen, 1994). The H69 cell line was chosen to investigate antagonist G induced apoptosis.

3.2.1 Annexin V Binding in H69 Cells

During the early stages of apoptosis, plasma membrane alterations occur which cause the membrane phospholipid, phosphatidyl-serine, to translocate from the inner to the outer leaflet of the membrane. Annexin V is a calcium dependent phospholipid binding protein which can be flourescently conjugated and used as a probe to detect phosphatidyl-serine by flow cytometry.

The incubation of H69 cells with annexin V was performed at 4^0C as there were concerns that antagonist G may be able to insert into membranes and expose phosphatidyl-serine in a non-specific manner. By reducing incubation temperature it was unlikely for this non-specific movement of membrane phospholipids to occur.

It was found that antagonist G dose dependently increased annexin V binding to H69 cells. Antagonist G, at physiologically relevant concentrations, increased basal annexin V binding by up to 50% of control. Etoposide (25μg/ml) was used as a positive control and increased annexin V binding by approximately 100% of control. (Fig 3.13). Phosphatidyl-serine can also be exposed on necrotic cells so in order to confirm the presence of apoptosis, alternative methods of detection were also employed.

It was unknown whether antagonist G could induce apoptosis in a non-specific manner by, for example, disrupting membrane integrity, or whether its pro-apoptotic effects were specific to a certain type of cell. To investigate this question we attempted to induce apoptosis with Antagonist G in Chinese hamster ovary (CHO) cells, which are non-cancerous, non-neuroendocrine cells. It was found that antagonist G did not significantly increase annexin V binding in CHO cells whereas cyclohexamide, a non-specific cytotoxic drug, increased annexin V binding by over 150% of control (Fig3.16).

3.2.2 Caspase 3 Activation in H69 Cells

CPP32, or caspase 3, is an ICE family cysteine protease, which is activated in the early stages of apoptosis. The ICE proteases initiate cellular breakdown by specific structural, regulatory and DNA repair proteins and their activation is a marker of apoptosis. Activation of caspase 3 was investigated in response to antagonist G, in the H69 cell line, using the ApoAlert^{TM} caspase 3 activation kit. The assay measures the activation state of caspase 3 in treated and untreated cell lysates by detecting the cleavage of a caspase 3 substrate Asp-Glu-Val-Asp (DEVD) -pNA. Upon cleavage of the substrate, pNA is released and can be detected colourimetrically. Background readings are determined by incubating an induced sample with the caspase inhibitor DEVD-flouromethylketone (fmk) and subtracting these values from each test sample.

It was found that antagonist G induced caspase 3 activation in a dose dependant fashion and at 30μM caspase activation was increased by 50% of control levels. Etoposide (25μg/ml) was again used as a positive control. This concentration produces sub-maximal pro-apoptotic effects thus making any additive effects with antagonist G treatment apparent. Interestingly it was found that incubation of H69 cells with 25μg/ml etoposide together with 30μM antagonist G had an additive effect when compared with either treatment alone (Fig3.14).

3.2.3 Morphological Detection of Apoptosis

Finally, it was decided to confirm the above findings by morphological examination of H69 cells treated with antagonist G. Cytocenrifuged cells were stained with May-Grunwuld-Giemsa stain and examined by light microscopy. It was found that antagonist G induced a dose dependant increase in the presence of the morphological

characteristics of apoptosis (cell shrinkage and nuclear condensation). Maximal concentration of cyclohexamide was used as a positive control. (Fig3.15)

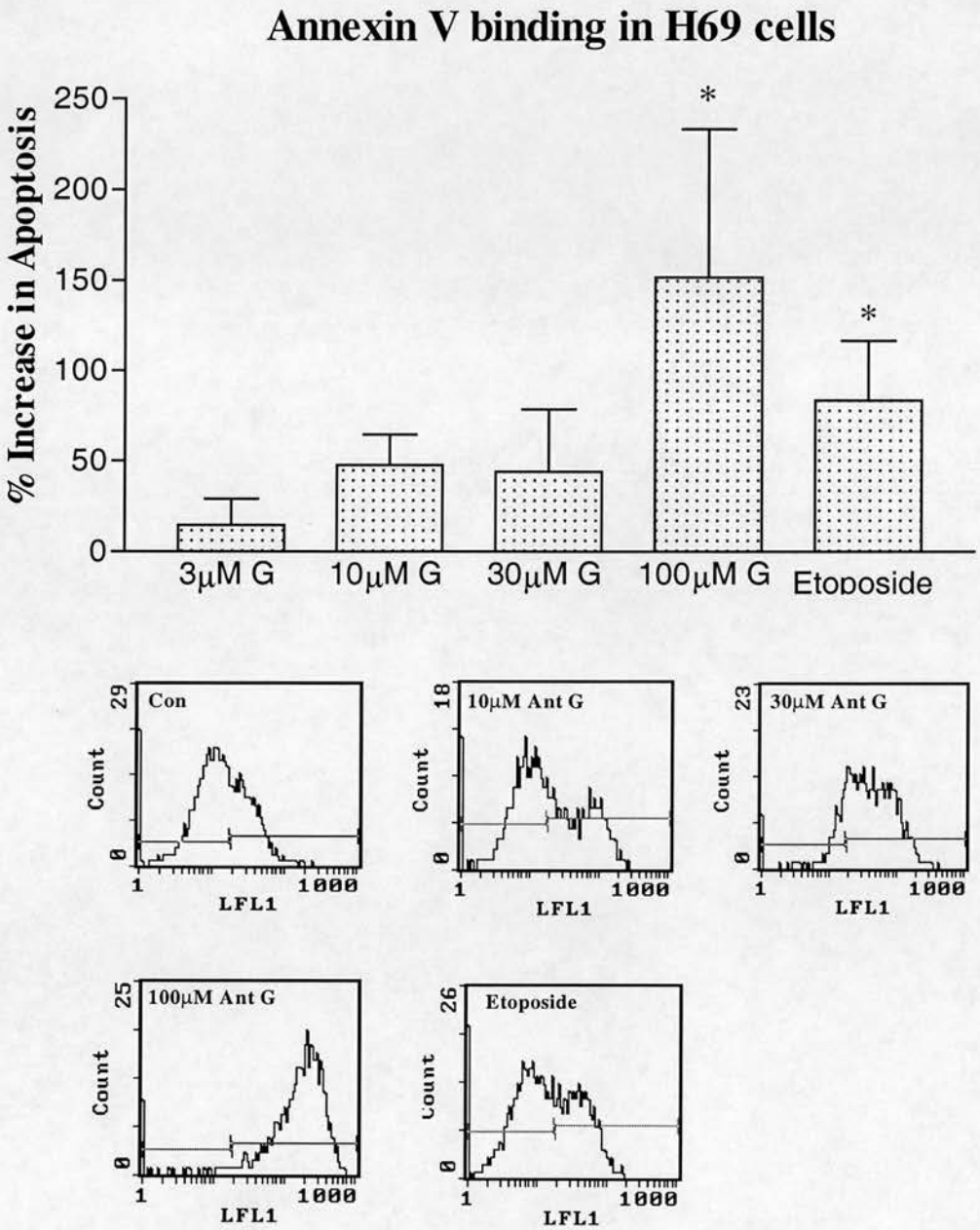


Fig 3.13: Annexin V binding in H69 SCLC cell line (top). Cells were quiesced overnight then treated with drugs for a further 24 hrs. Antagonist G was used at the concentrations indicated. Etoposide was used at 25μg/ml. Annexin V binding was assayed as described in materials and methods. Data represents the mean of three independent experiments performed in triplicate. (* Significantly different from control $P<0.05$)

Flow cytometry histograms of annexin V binding in H69 cells (bottom). Antagonist G produces a dose dependent shift in FL1 detection, which represents increased annexin V binding.

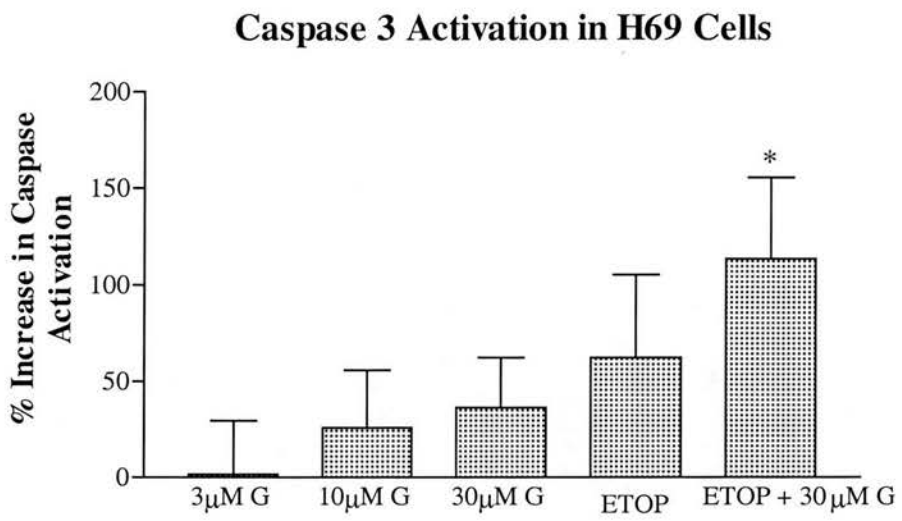


Fig 3.14) Caspase 3 activation in H69 SCLC cell line. $5x10^5$ cells were quiesced overnight then treated with drugs for a further 24 hrs. Antagonist G was used at the concentrations indicated. Etoposide was used at 25μg/ml. Caspase 3 activation was assayed as described in materials and methods. Data represents the mean of three independent experiments performed in triplicate. (* = Significantly different from control P<0.05)

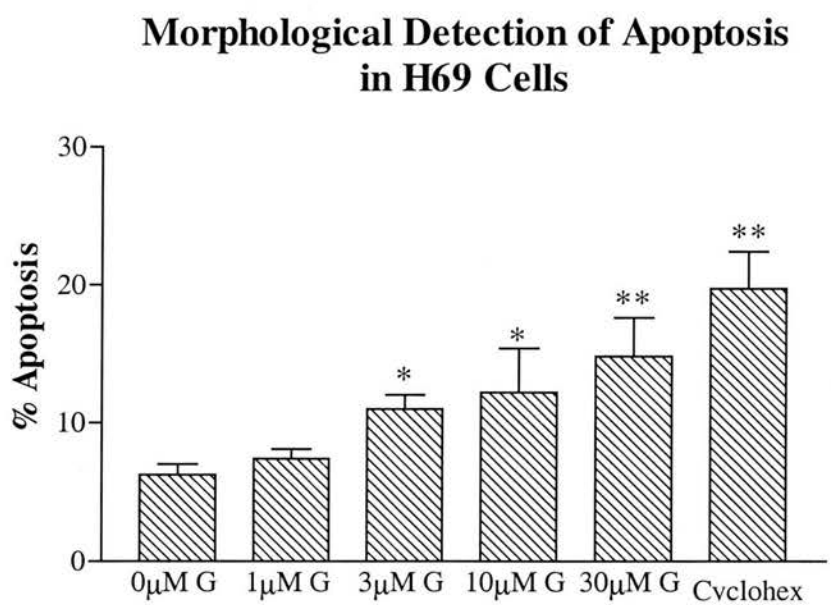


Fig 3.15) Morphological Detection of Apoptosis in H69 SCLC cell line. Cells were quiesced overnight then treated with drugs for a further 24 hrs. Antagonist G was used at the concentrations indicated. Cyclohexamide was used at 100μM. Apoptosis was detected on cytocentrifuged samples as described in materials and methods. Data represents the mean of three independent experiments performed in triplicate. (* = Significantly different from control *P<0.05, ** P<0.01)

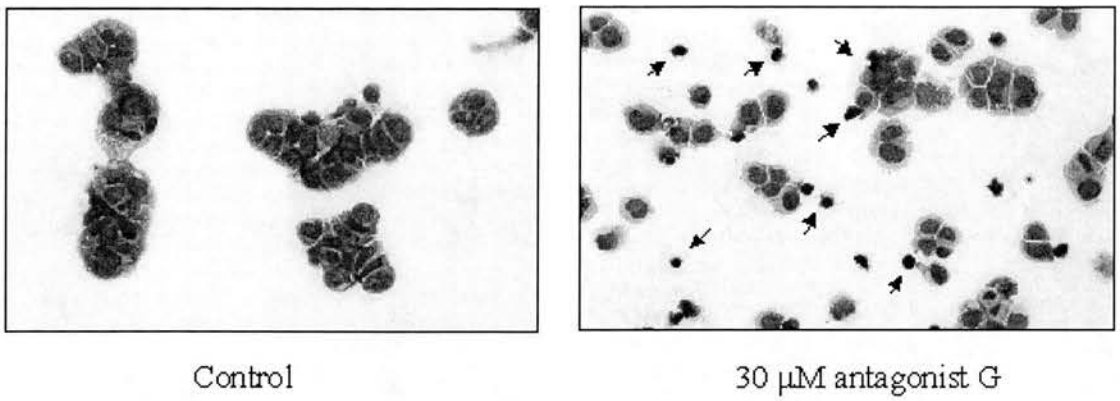


Fig 3.16. Morphological detection of apoptosis in H69 SCLC cells. H69 cells were treated for 24hrs with 30μM Antagonist G. Samples were cytocentrifuged onto glass slides and then stained with May-Grunwald-Giemsa stain as described in materials and methods. Apoptotic cells (arrows) show cell shrinkage, condensed nuclei and reduced clumping when compared to control cells.

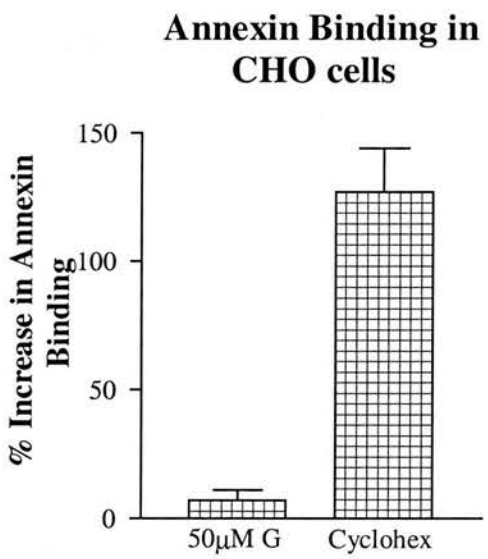


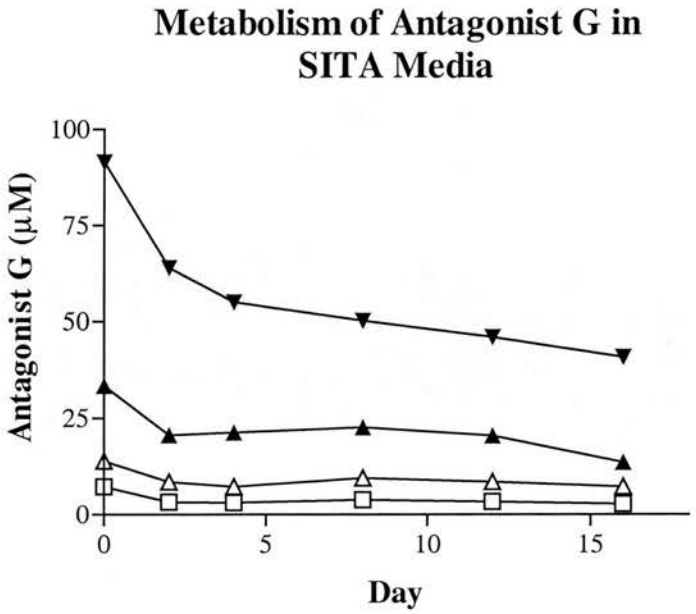
Fig 3.17 Annexin V binding in CHO cells. Cells were quiesced overnight then treated with drugs for a further 24 hrs. Antagonist G was used at 50μM. Cyclohexamide was used at 100μM. Annexin V binding was detected using flow cytometry as described in materials and methods. Data represents the mean of three independent experiments performed in triplicate.

3.3 Investigation into the Metabolism of Antagonist G in Tissue Culture.

The growth inhibition demonstrated by antagonist G was investigated in liquid growth assays (SITA media) as discussed earlier. It was unclear as to the stability of antagonist G in SITA over the time course of these experiments. In order to address this question, various concentrations of antagonist G were incubated in media at 37^0C and the levels of both intact antagonist G and the three main metabolites of antagonist G were determined by HPLC. It was found that over an initial 4 day period, levels of intact antagonist G were reduced by an average of 40% and then remained stable for the remainder of the 16 day time course. This reduction of intact antagonist G was accompanied by an increase of metabolites 2 and 3 over an initial 7 day period, however, the increases in metabolites were very small and did not account for the reduced presence of antagonist G. Levels of metabolite 1 did not alter throughout the 16 day period (Fig3.18)

The sensitivity of individual cell lines to substance P analogues varies from 10μM-100μM as discussed earlier in this chapter. To determine whether the differences in sensitivity was due to the difference in the cell lines ability to metabolise antagonist G, the ability of the HT29 cell line (IC_{50}-21.5μM) and the PANC1 cell line (IC_{50}-57μM) to metabolise antagonist G were compared. These cell lines were chosen as they represented two different sensitivities and also because they were adherent cell lines and therefore easier to wash off non-associated drug. Both cell lines were incubated with 40μM antagonist G and levels of intact drug in the media were determined by HPLC on days 4 and 7. There were no significant differences in media levels of antagonist G between the two cell lines on either day (Fig 3.19).

It is unclear whether the association of antagonist G with cells was a transient event or whether the drug remains associated with cells over a longer time course. To determine the length of time antagonist G could remain associated with cells, the HT29 and PANC1 cell lines were incubated with the drug for either 24hrs, 4 days or continually for 7 days. HPLC analysis of cell associated antagonist G was performed on day 7 in each case. It was found in both cell lines that after both 24hr and 4 day exposures to antagonist G, significant levels of intact drug was detectable on day 7. However, these levels were lower than levels found in cells continually exposed to the drug (Fig3.20).



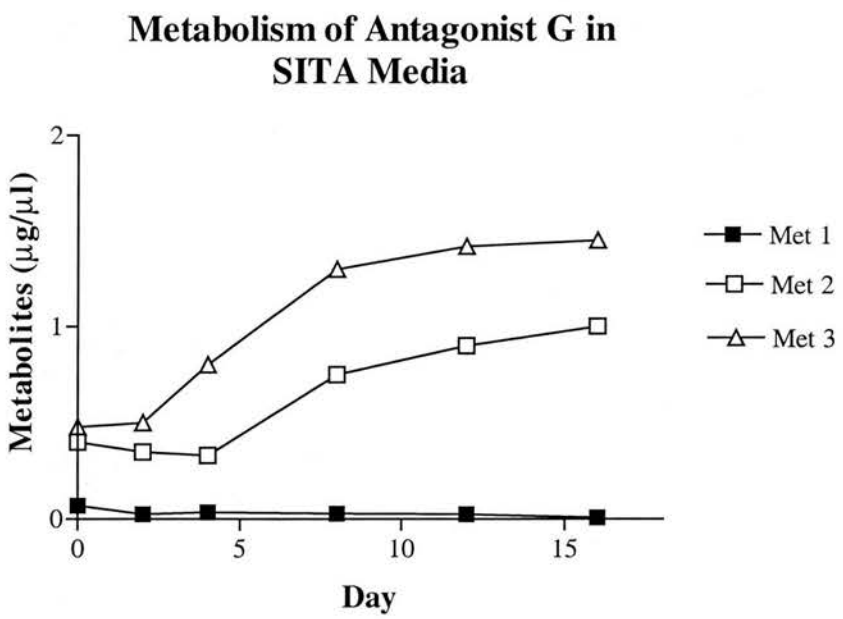


Fig 3.18 A: HPLC analysis of Antagonist G in cell free SITA media. Four initial concentrations of Antagonist G (90μM, 30μM, 15μM and 7.5μM) in SITA were incubated for 0-16 days at 37°C. Aliquots were frozen at the indicated time points for HPLC analysis of antagonist G content as described in materials and methods. Data points represent the means of duplicate samples.

B: HPLC analysis of metabolites 1,2 and 3 in cell free SITA media. 40μM antagonist G in SITA was incubated as above. At the indicated time points, aliquots were frozen and subsequently analysed by HPLC for the presence of metabolites 1,2 and 3 as described in materials and methods.

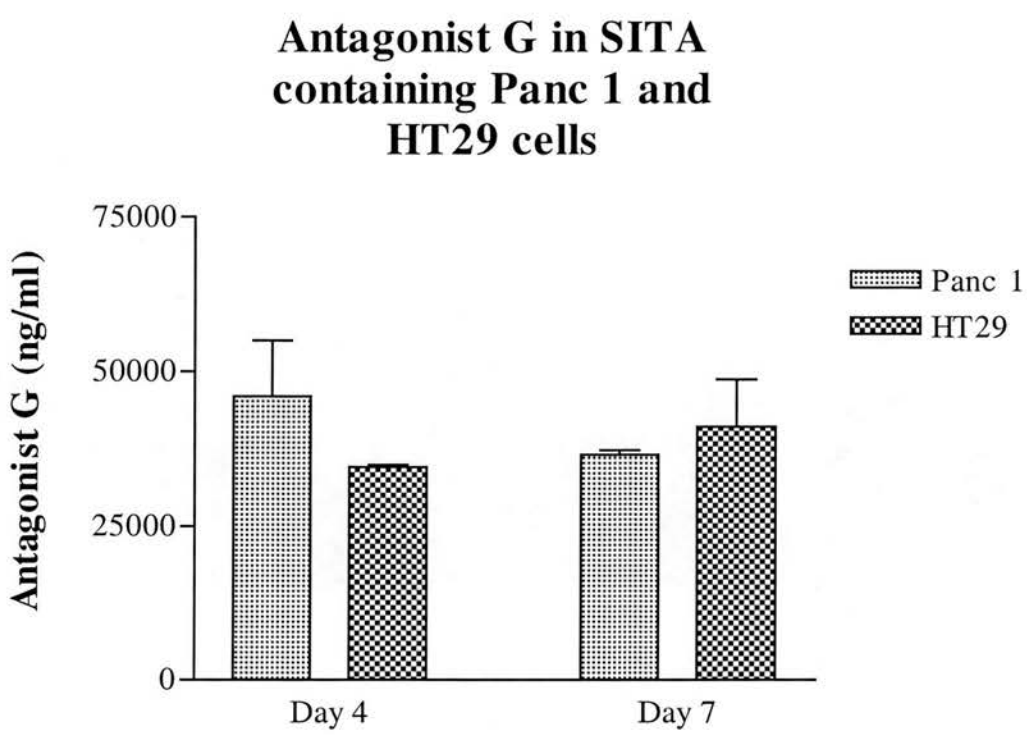
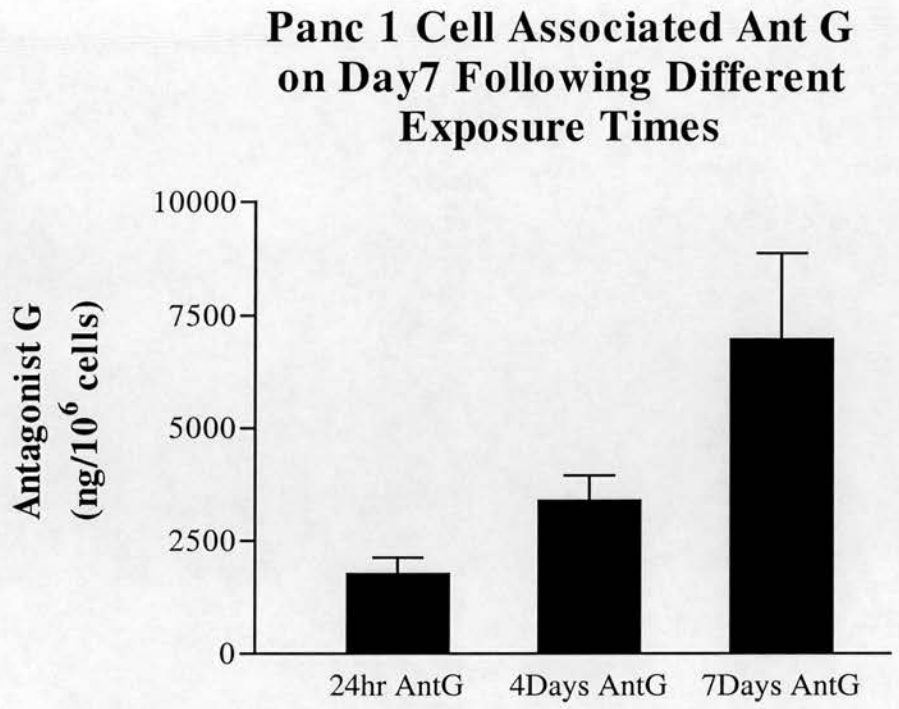


Fig 3.19 HPLC analysis of antagonist G in media containing HT29 or PANC1 Cells. HT29 cells (sensitive to Antagonist G) and Panc1 cells (resistant to antagonist G) were incubated with 40μM antagonist G in SITA on day 0. On day 4 and day 7, media was removed then levels of antagonist G present in the media were determined by HPLC. Data represents the mean and SD of triplicate samples.



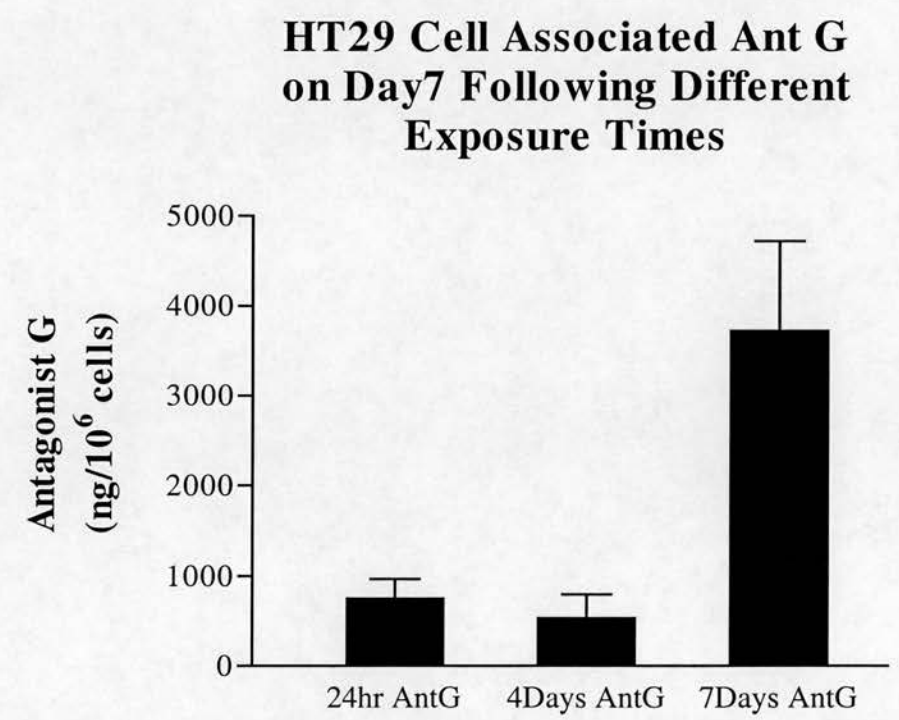


Fig 3.20 HPLC analysis of cell associated antagonist G on day 7 following either a 24hr, 4 day or 7 day exposure to antagonist G. 40μM antagonist G in SITA was added to PANC1 cells and HT29 cells on day 0. Following either 24hr, 4 days or 7 days, samples were washed twice in PBS and fresh media was added. The drug free cells were left to continue growing until day 7 when all cells were harvested. Cell samples were washed twice in PBS and then antagonist G content was determined by HPLC as described in materials and methods. Parallel samples were counted to determine cell number. Data represents the mean of triplicate samples.

3.4 Discussion of Results

As discussed, the sensitivity of the tumour cell lines, to antagonist G, differed across the panel with a range of >1 log order (10μM - >100μM). These differences strongly suggest that antagonist G inhibits cell growth by some specific mechanism. If antagonist G acts as a non-specific cytotoxic or cytostatic compound then the observed differences in sensitivity between cell lines would be unlikely.

IC_{50} values for 72% of all cell lines tested were near to or below 40μM, which is approximately the maximum concentration of antagonist G that can be obtained in patient plasma, without dose limiting toxicity. Tumour cells may well show different sensitivity to antagonist G *in vivo*, however, it is still encouraging that significant growth inhibition can be observed at physiologically relevant concentrations *in vitro*.

Antagonist G is in clinical trial as a potential therapy for small cell lung cancer. Based on these results of this screen, it seems that antagonist G could be used therapeutically against several other tumour types in addition to SCLC. 4/5 SCLC, 2/3 colo-rectal carcinoma 1/1 non-SCLC and 1/1 ovarian carcinoma cell lines had IC_{50} values which were in the range of physiologically obtainable plasma concentrations.

Growth inhibition assays do not provide information as to the nature of the growth inhibition. Antagonist G could be acting as a cytostatic drug which does not cause cell death but prevents cells from undergoing mitosis. Alternatively antagonist G may be a cytotoxic drug which causes one of two mechanisms of cell death, namely necrosis or apoptosis. Previous studies have shown that substance P analogues can induce apoptosis and the pro-apoptotic effects of antagonist G were confirmed using three methods of detection; annexin V binding, caspase 3 activation and observation of morphological changes which are characteristic of apoptosis. All methods provided

evidence that antagonist G induces apoptosis in H69 SCLC cells. Using both annexin V binding and caspase 3 activation assays, antagonist G dose dependently increased the frequency of apoptosis and at 30μM, antagonist G induced up to 50% increase of basal rates. Using morphological detection of apoptosis, the same concentration of antagonist G increased a basal rate of 7% apoptosis to 14% apoptosis. These antagonist G induced, pro-apoptotic effects are likely to cause marked changes in the overall growth rate of tumours. It would be interesting to measure antagonist G induced apoptosis in tumour samples from xenografts in mice in order to confirm this phenomenon *in vivo*.

In order to confirm that the apoptotic effects of antagonist G were of a cell specific nature, the non-cancerous, non-neuroendocrine Chinese hamster ovary cells (CHO-K1) were incubated with high concentration antagonist G (50μM). Only a small (7%) increase in apoptosis was seen following antagonist G treatment whereas, in contrast, there was a 127% increase in apoptosis seen following incubation with the cytotoxic drug cyclohexamide (Fig 3.17). This result strongly suggests that the pro-apoptotic effects of antagonist G are of a specific nature. In addition, unpublished observations from our laboratory show that antagonist G and D do not induce apoptosis in human neutrophils. If these drugs activate a non-specific apoptotic pathway then it would be expected that all cell lines would be sensitive to their pro-apoptotic effects. The lack of responsiveness of CHO cells and neutrophils to antagonist G is encouraging as many currently used, cytotoxic cancer therapies induce cell death in an non-selective manner which results in damage to non-cancer cells.

Using the caspase 3 activation assay, it was observed that the co-incubation of H69 cells with 30μM antagonist G plus a maximal dose of the cytotoxic drug, etoposide, produced an additive apoptotic effect which was greater than the effect of either drug alone. This

is an interesting observation because etoposide is currently a drug of choice for the treatment of SCLC and this experiment suggests that combined treatment with antagonist G may increase its effectiveness. Further *in vitro* studies have since been carried out in our lab, and have shown that antagonist G has synergistic effects on growth inhibition and apoptosis, when used along-side several cytotoxic agents (in press MacKinnon et al, Brit Jnl Cancer, Sept 2000). These findings have important implications for the clinical scheduling of antagonist G.

Studies have been carried out to investigate the *in vivo* metabolism of antagonist G. Metabolism studies in mice bearing the H69 SCLC xenograft showed that metabolism is restricted almost exclusively to the C-terminal of the peptide producing 4 major products (Fig 3.21). The major pathway of metabolism was peptidase mediated catabolism and the minor pathway was the oxidation of the C-terminal methionine residue. Metabolism was initiated by deamidation to produce metabolite 1 followed by exopeptidase removal of the C-terminal methionine to yield metabolite 2. Both metabolite 1 and 2 retain growth inhibitory activity *in vitro*. Further exopeptidase metabolism at both the C and N terminals produced metabolites 4 and 4b respectively. Oxidation of antagonist G produced metabolite 3 (before deamidation) and metabolite 3b (after deamidation) (Cummings et al., 1995).

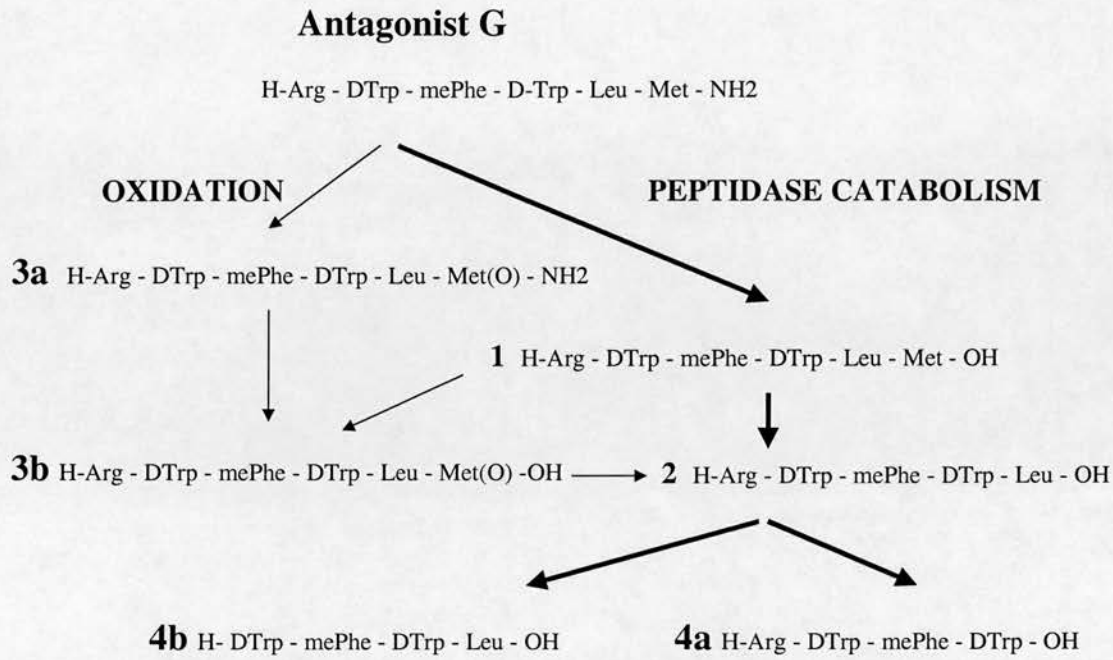


Fig 3.21 *In vivo* metabolism of Antagonist G. Data derived from liver and H69 xenograft homogenates (Cummings et al., 1995).

All of the tissue culture studies in this chapter were carried out in SITA media. The stability of antagonist G in this media was unknown so HPLC analysis of the stability of antagonist G in cell free, SITA tissue culture media was carried out. Over a 16 day period the levels of both intact antagonist G and its metabolites were monitored. Interestingly, an average of 40% of the starting concentrations of antagonist G were lost over the first 4 days and then levels were stable for the remaining time period. Part of this reduction in antagonist G levels can be explained by metabolism of the compound as there was a concurrent increase in levels of metabolite 2 and 3. However, the increased presence of these metabolites does not account for the total reduction in the intact parent peptide. It may well be the case that the unaccountable portion of the intact antagonist G was stuck to the plastic surface of the tissue culture vessels and was therefore not detected when the media samples were analysed. Cummings et al

(Cummings et al., 1995), performed a similar study to analyse the stability of antagonist G in HITES medium (hydrocortisone, insulin, transferrin, estradiol and sodium selenite) and found that the levels of intact peptide were not reduced over a 7 day period. There is a possibility that the presence of the globular protein albumin, in SITA media could effect the analysis of antagonist G levels and may account for the findings of this study. This work has only been carried out in a single study and further work will be necessary to enable final conclusions to be drawn.

In an attempt to discover whether differences in sensitivity to antagonist G were due to different rates of metabolism of the drug, HPLC analysis of media and cell associated antagonist G was carried out for two cell lines; the relatively sensitive HT29 colorectal cell line and the more resistant PANC1 pancreatic carcinoma cell line. There was very little difference between the levels of antagonist G present in the media of either cell line and there were no significant changes in the media levels between day 4 and day 7 in either cell line. This suggests that the rates of metabolism of antagonist G by the sensitive HT29 cell line and the resistant PANC1 cell line, are similar and that variations in metabolism rates do not account for the differences in sensitivity between these two cell lines. As in the above study, this experiment has only been performed once and will need to be repeated before final conclusions can be made.

A study was performed to asses the association of antagonist G with HT29 and PANC1 cells, following short exposures of 24hrs and 4 days in comparison with continuous exposure. Cell associated antagonist G was measured following 7 days of cell growth in each case. In both cell lines it was found that a significant level of intact peptide was still associated with cells on day 7, following a period of 6 days in drug free media (24hr exposure) and 3 days in drug free media (4 day exposure). The levels of

antagonist G following these shorter exposures were lower than levels found after continuous exposure, however, it is possible that antagonist G present following shorter exposures may still exert a growth inhibitory effect. Further experiments are required to determine the growth inhibitory potential of these various exposure times. These experiments will have important implications in the scheduling of antagonist G treatment for clinical use. Results of the phase 1 clinical trial have shown that antagonist G is rapidly cleared from patient plasma but these preliminary results suggest that continuous infusion of the drug may not be necessary as the drug can remain associated with tumour cells for some time after administration.

In this section it has been demonstrated that some cancer cell lines are sensitive to growth inhibition by antagonist G and other cancer cell lines are not. What cellular factors confer sensitivity to antagonist G? This question shall be addressed in the following chapter.

Chapter 4

Sensitivity to Growth Inhibition by Antagonist G Correlates with High Expression of Neuropeptide Receptors.

Substance P analogues are able to antagonise the mitogenic effects of neuropeptide growth factors. They are capable of blocking calcium mobilisation and mitogenesis induced by bombesin, bradykinin, cholecystokinin, neurotensin, gastrin, galanin, endothelin and substance P (Woll and Rozengurt, 1988; Woll and Rozengurt, 1990; Sethi and Rozengurt, 1991; Sethi and Rozengurt, 1992). These antagonists can also inhibit the binding of neuropeptides bombesin and vasopressin to their receptors (Seckl et al., 1995). It is likely that substance P analogues can recognise and bind to some part of either the neuropeptide receptor or to a protein which can regulate receptor function such as a G protein. However, it is unclear whether or not their ability to inhibit cell growth in the absence of exogenous neuropeptides, involves neuropeptide receptors, or some other mechanism.

In order to address this question a panel of tumour cell lines, representing a spectrum of sensitivity to Antagonist G, were tested for expression of four neuropeptide receptors; vasopressin (V1A) receptor, gastrin releasing peptide (GRP) receptor, bradykinin receptor (BK2) receptor and gastrin receptor. Relative levels of these receptors were determined by semi-quantitative RT-PCR. This technique was utilised because of its specific nature and its ability to detect receptors expressed at very low density. Antibodies against these receptors are not commercially available so detection by western blotting was not possible. Radio-ligand binding was not used to avoid excessive use of radioactivity and to keep costs minimal. The aim of

this work was to attempt to compare sensitivity to antagonist G, with the expression levels of these receptors.

Detection of mRNA by RT-PCR gives an accurate indication of transcription of a particular gene but gives no solid information regarding the expression levels of functional proteins, although a tight correlation between transcription and protein levels would be expected. Regardless, it was decided to confirm the presence of functional receptors by measuring the ability of the cells to mobilise intracellular calcium in response to neuropeptide stimulation. This was carried out for all of the SCLC cell lines in the panel.

4.1 Detection of Neuropeptide Receptors in a Panel of Tumour Cell Lines

Using semi-quantitative RT-PCR, the levels of GRPR, V1AR, BK2R and Gastrin R were determined in 9 tumour cell lines (Table 4.1).

Table 4.1 Panel of human tumour cell lines for detection of neuropeptide receptors

Cell Line	Tumour type	IC50 (μM)
H69	SCLC	10.5
H510	SCLC	29
WX330	SCLC	42
NX002	NSCLC	31.5
HT29	Colo-Rectal	18
HCT116	Colo-Rectal	>100
HRT18	Colo-Rectal	37
PANC1	Pancreatic	58
PEO4	Ovarian	31

Receptors for vasopressin (V1AR) were detected in 4/4 lung cancer cell lines (3 SCLC and 1 NSCLC). The WX330 SCLC cell line was the highest expresser of V1AR with approximately three times more mRNA detected than in the other three lung cancer lines. None of the three colo-rectal cancer cell lines expressed any detectable V1AR and the pancreatic and ovarian cancer lines expressed very low levels (Fig 4.1).

Receptors for gastrin releasing peptide (GRPR) were detected in 3/4 lung cancer cell lines (2 SCLC and 1 NSCLC). Both H69 (SCLC) and NX002 (NSCLC) expressed high levels of GRPR. 3/3 colo-rectal cell lines expressed GRPR. The HT29 cell line was the highest expresser, whereas the HCT116 cell line showed a very low level of GRPR expression. The pancreatic cell line PANC1 had a very low expression level. The PEO4 ovarian cancer cell line was the highest expresser of GRPR in the cell panel. (Fig 4.2)

Receptors for bradykinin (BK2R) were detected in all cells of the cell panel. WX330, PANC1 and PEO4 had relatively low receptor levels in comparison to the rest of the panel. Other studies have found an almost ubiquitous expression of bradykinin receptors in human lung cancers (Bunn et al.,1992a,b). Interestingly the colo-rectal cell lines were high BK2R expressers. (Fig 4.3) There is no published data implicating bradykinin as a growth factor for such cell lines and further work to determine the role of bradykinin in colo-rectal cancer would be interesting.

Gastrin receptors were detected in 4/4 lung lines (3 SCLC and 1 NSCLC) and to a small degree in the ovarian line PEO4. The highest expresser was the SCLC cell line H510. Interestingly, the colo-rectal cell lines did not express gastrin receptors as may have been expected, bearing in mind the site of origin of these tumours (Fig

4.4). This finding is confirmed in a study by Reubi et al who found that the gastrin receptor was rarely expressed in colo-rectal cancer cell lines (Reubi et al., 1997). The gastrin receptor is more commonly found in other forms of gastric cancer (discussed in chapter 1).

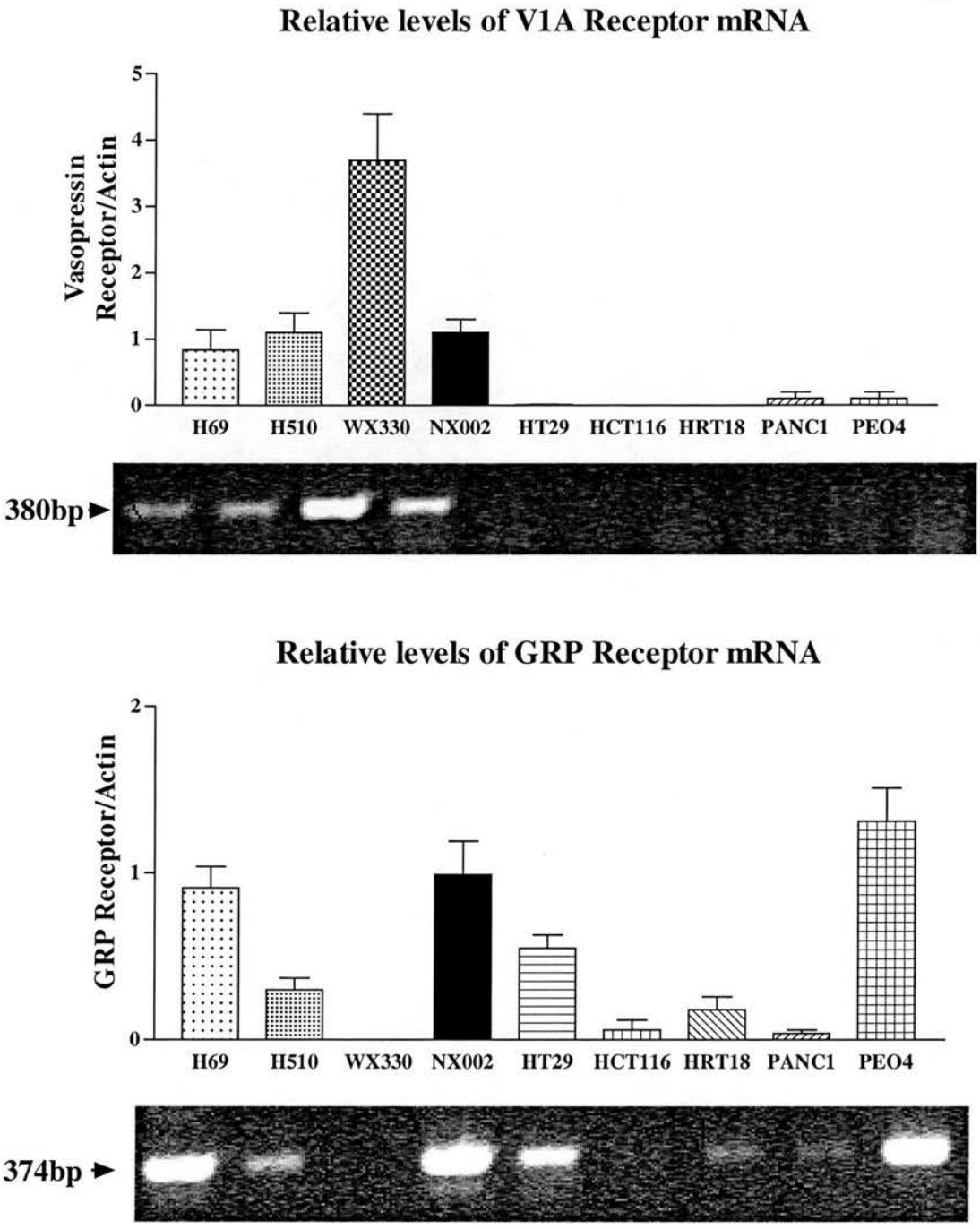
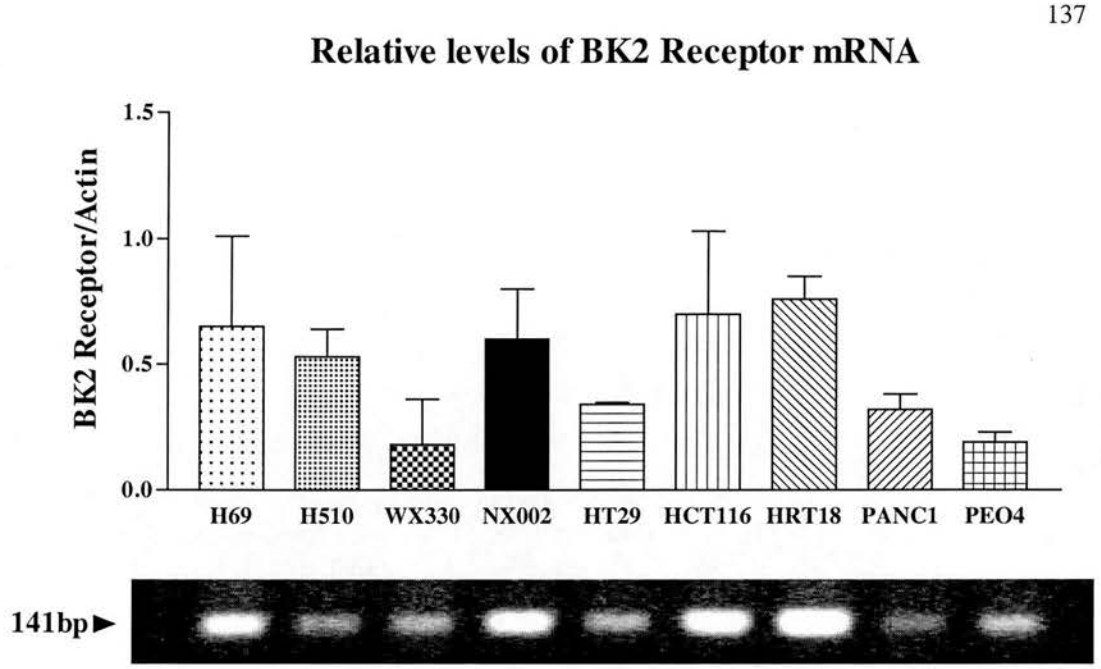


Fig 4.1 V1AR expression in tumour cell panel.
Fig 4.2 GRPR expression in tumour cell panel.

Receptors were detected by RT-PCR and gels were analysed by densitometry. Results of this analysis are expressed as a ratio of levels of γ-actin for the same cDNA batch. Data represents the mean of 3-6 separate determinations from at least three different cDNA synthesis reactions. Below the charts are representative PCR gels for the respective receptors.



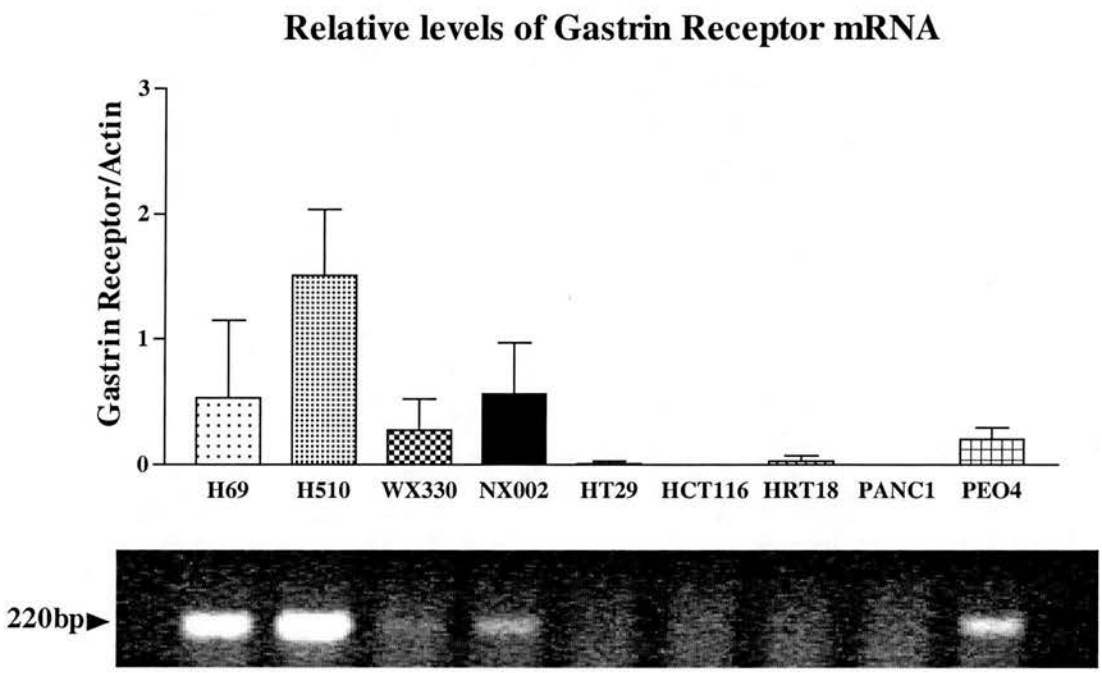


Fig 4.3 BK2R expression in tumour cell panel.
Fig 4.4 Gastrin R expression in tumour cell panel.

Receptors were detected by RT-PCR and gels were analysed by densitometry. Results of this analysis are expressed as a ratio of levels of γ-actin for the same cDNA batch. Data represents the mean of 3-6 separate determinations from at least three different cDNA synthesis reactions. Below the charts are representative PCR gels for the respective receptor

4.2 Mobilisation of Intracellular Calcium in Response to Neuropeptide Ligand Stimulation to Confirm the Presence of Functional Receptors.

The presence of mRNA for a particular neuropeptide receptor does not necessarily mean that a functional receptor is present on the surface of the cell. In order to confirm the presence of functional receptors, high concentrations (100nM) of neuropeptide receptor ligands were tested for their ability to induce the mobilisation of intracellular calcium in the three SCLC cell lines of the cell panel. The four neuropeptide receptors analysed by RT-PCR (bombesin/GRP, vasopressin, bradykinin and gastrin) were tested, in addition to galanin and neurotensin.

It was found that the ability of the cell lines to mobilise intracellular calcium in response to stimulation with neuropeptide ligands, corresponded with high expression of mRNA for the respective receptor. Cell lines expressing very low mRNA levels failed to respond to ligand stimulation. None of the three cell lines mobilised calcium in response to neurotensin and only the H510 cells line showed any response to galanin.

In this study it was found, in three SCLC cell lines, that neuropeptide receptor activation results in mobilisation of intracellular calcium. This phenomenon was adopted as an indication of the presence of functional receptor protein, however, it is not clear as to the significance of calcium mobilisation in terms of a mitogenic response. The colo-rectal carcinoma cell line HT29 has been shown in this study and in others to express high levels of GRP receptors. Stimulation of this cell line with GRP results in a proliferative response but not in the mobilisation of intracellular calcium. This may suggest that, in some cell lines at least, the mobilisation of calcium is not critical for mitogenisis via neuropeptide receptors and that these cell

lines utilise a different downstream pathway in response to neuropeptide receptor activation.

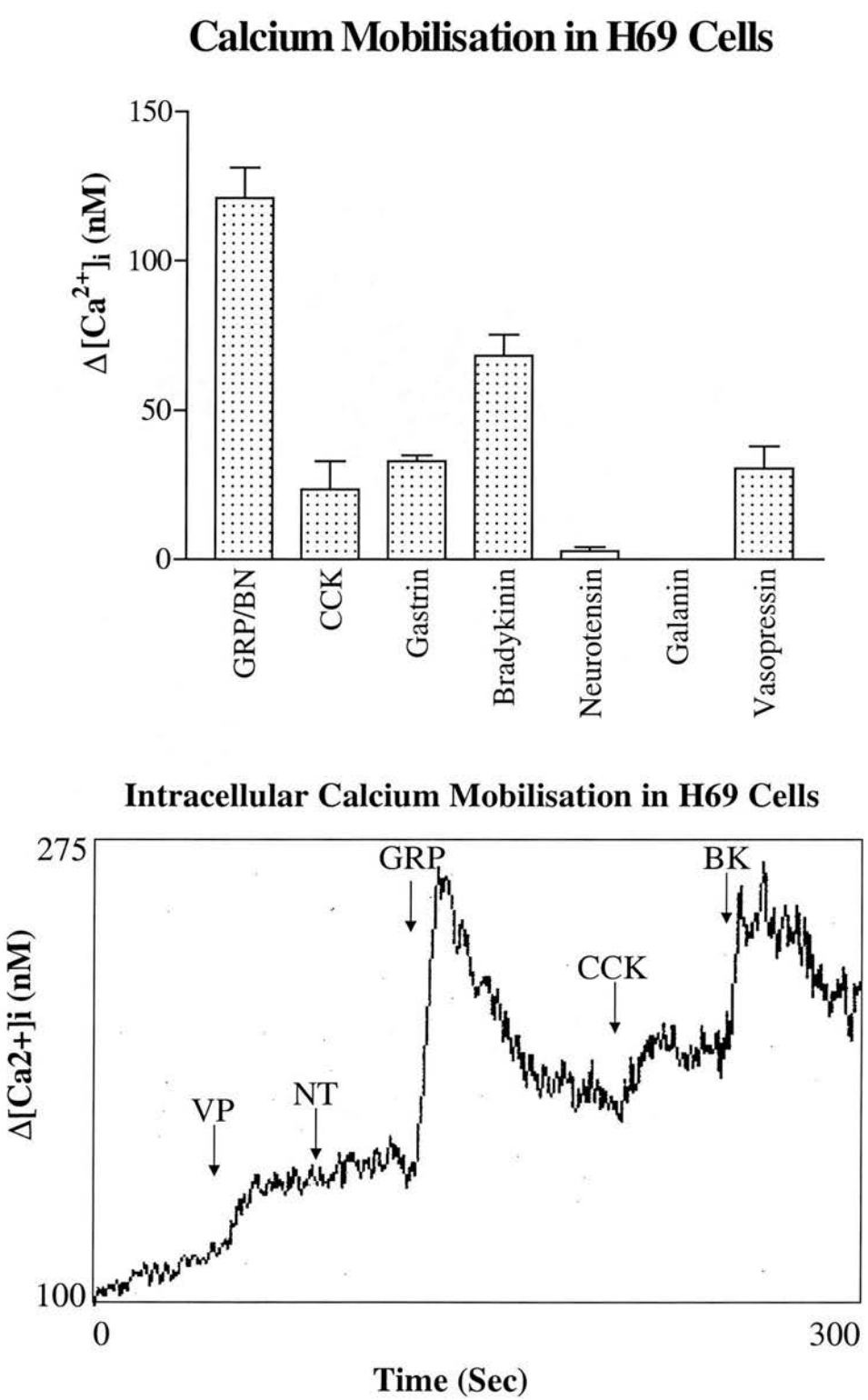


Fig 4.5. Calcium mobilisation in H69 SCLC cell line. Intracellular calcium mobilisation was measured as described in materials and methods. Neuropeptides were used at 100nM and were used in a random order for each determination. Graph represents the mean increase in intracellular calcium concentration for 4 independent experiments. The trace below is representative of 4 independent experiments and shows real time increases in calcium concentration upon exposure to 100nM of the indicated neuropeptides.

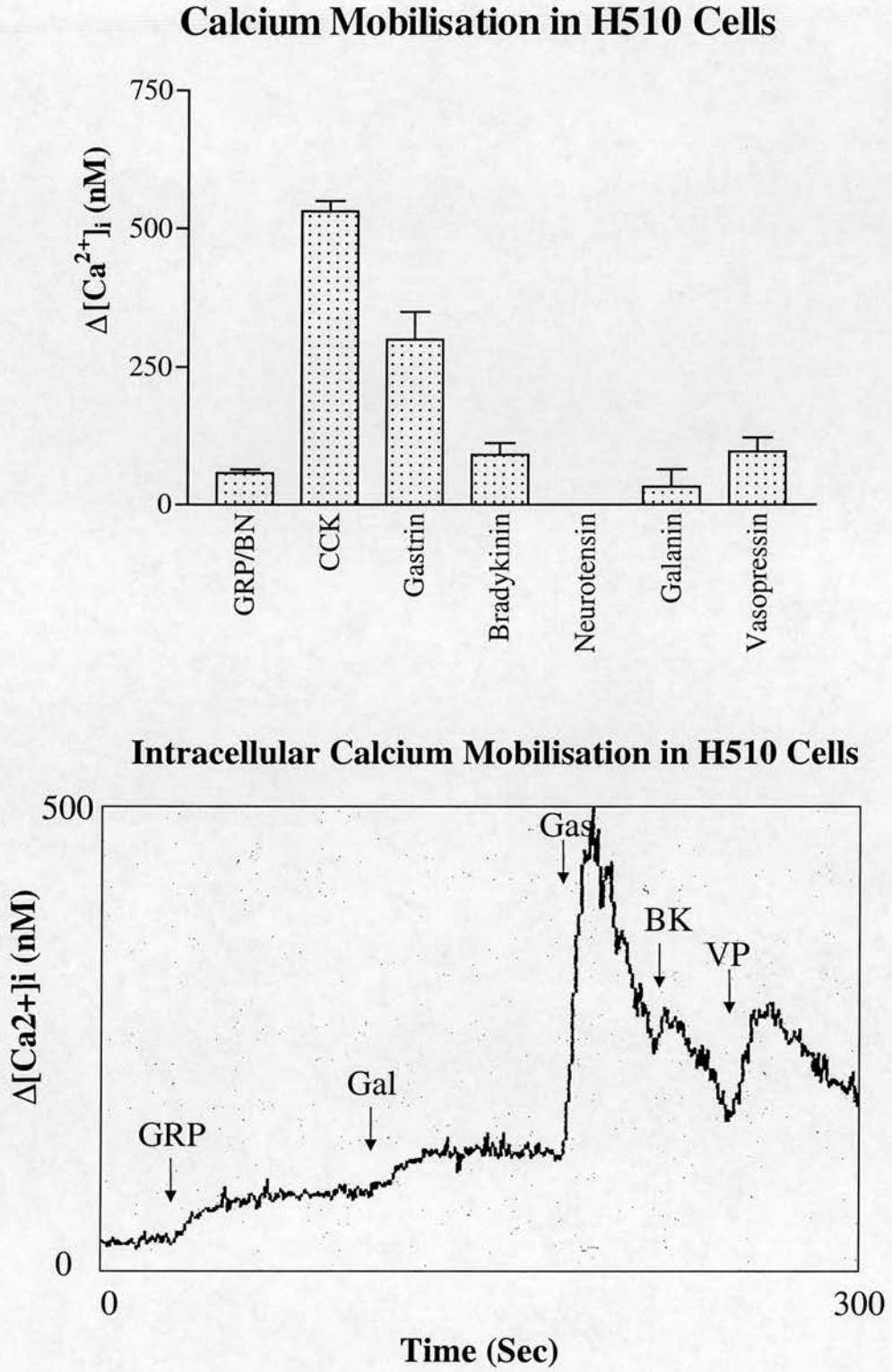


Fig 4.6. Calcium mobilisation in H510 SCLC cell line. Intracellular calcium mobilisation was measured as described in materials and methods. Neuropeptides were used at 100nM and were used in a random order for each determination. Graph represents the mean increase in intracellular calcium concentration for 4 independent experiments. The trace below is representative of 4 independent experiments and shows real time increases in calcium concentration upon exposure to 100nM of the indicated neuropeptides.

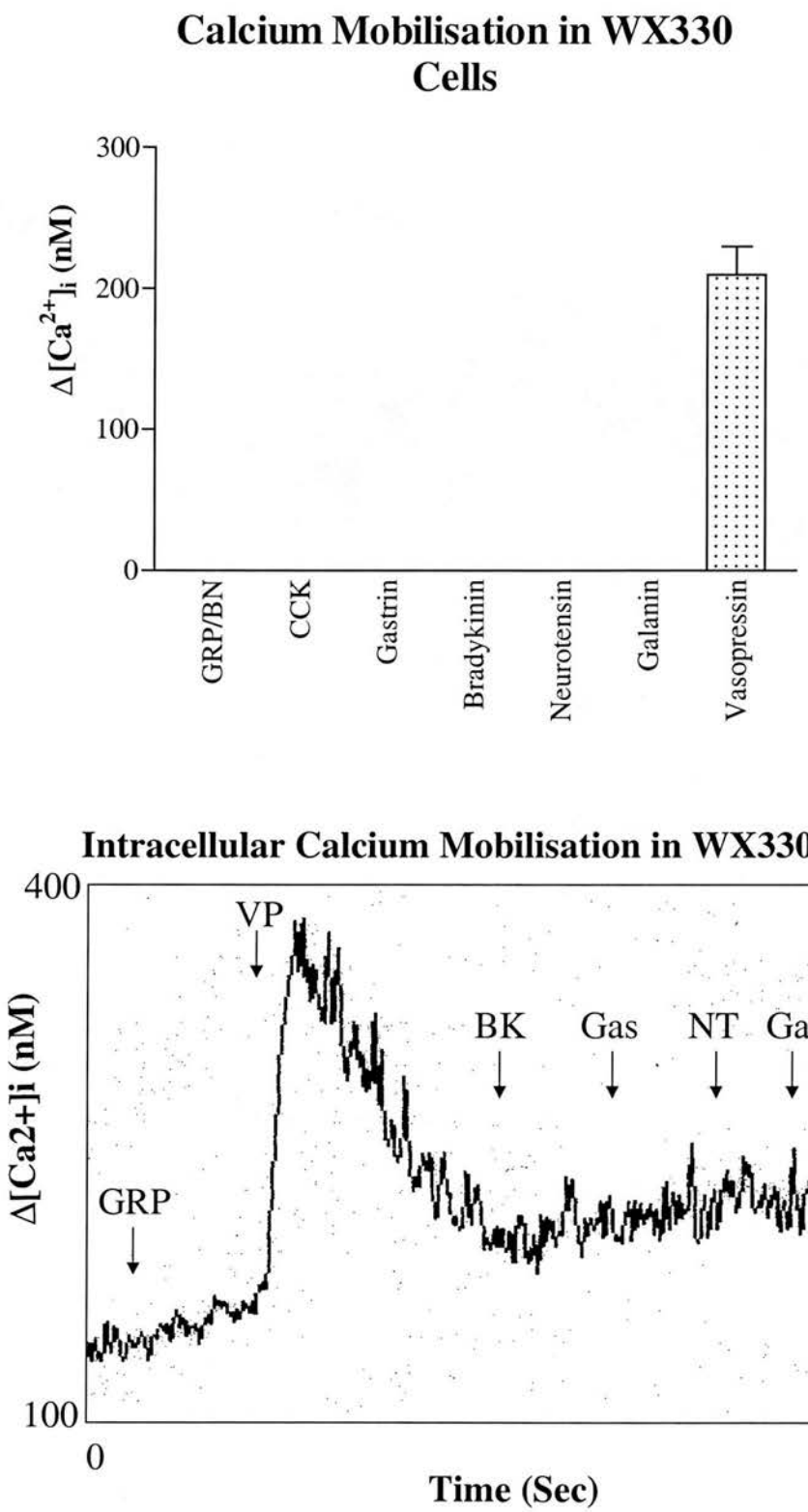


Fig 4.7. Calcium mobilisation in WX330 SCLC cell line. Intracellular calcium mobilisation was measured as described in materials and methods. Neuropeptides were used at 100nM and were used in a random order for each determination. Graph represents the mean increase in intracellular calcium concentration for 4 independent experiments. The trace below is representative of 4 independent experiments and shows real time increases in calcium concentration upon exposure to 100nM of the indicated neuropeptides.

4.3 Comparison of Neuropeptide Receptor Levels and Sensitivity to Antagonist G

In order to determine whether the presence of a single class of neuropeptide receptor confers sensitivity to antagonist G, the IC50 values of the tumour cells were compared with the relative levels of GRPR, V1AR, BK2R or Gastrin R (Fig 4.8).

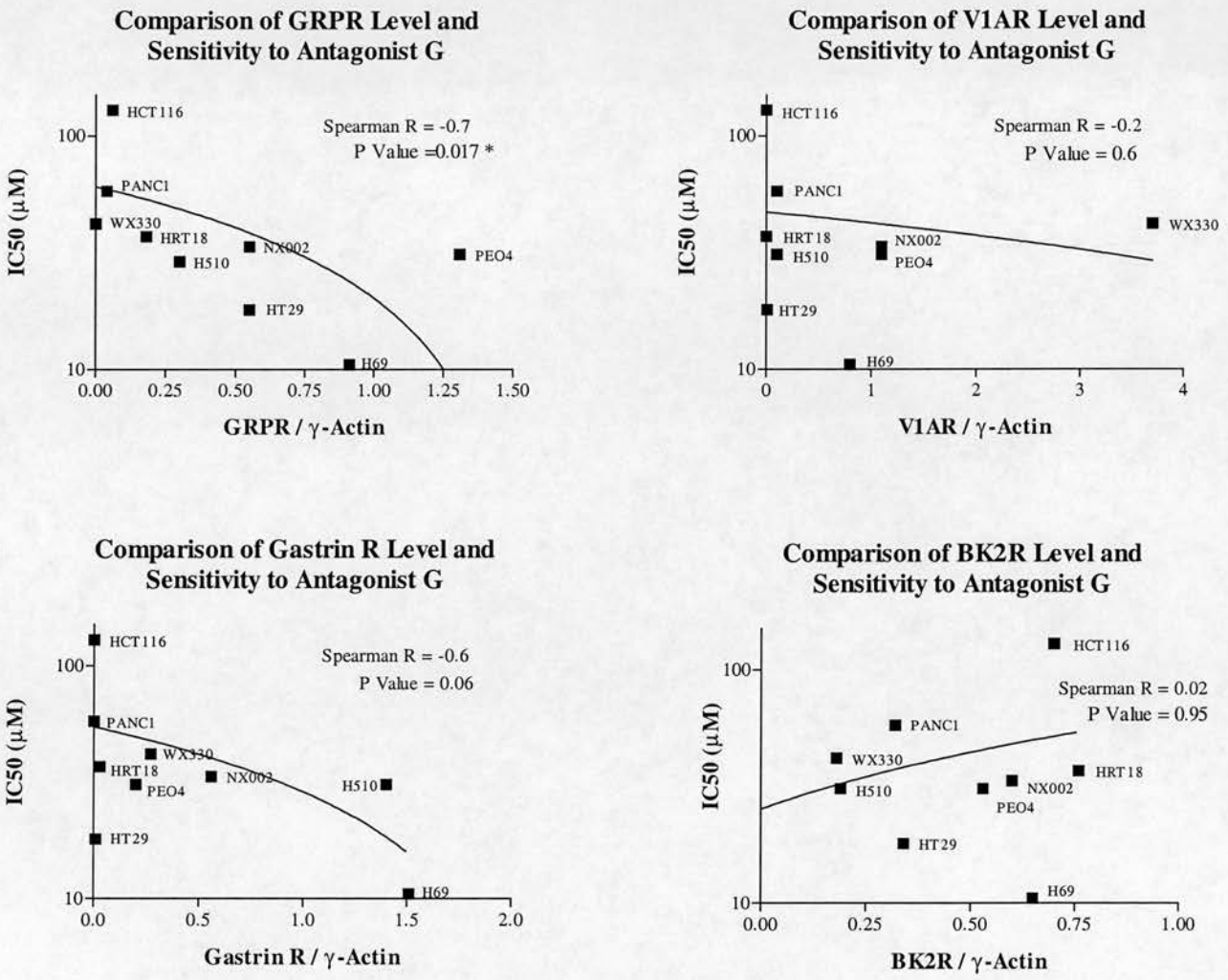


Fig 4.8 Comparison of IC50 value with expression of neuropeptide receptors. The four graphs show relative levels of GRPR, V1AR, BK2R or gastrin R plotted against the IC50 value for the corresponding cell line. Correlation between receptor level and IC_{50} value was analysed by Spearman- rank analysis using Prism software. Spearman R values are shown; when R= -1 a perfect negative correlation can be assumed. The Spearman R value for the GRPR/ IC_{50} correlation is significant with a P value of <0.05.

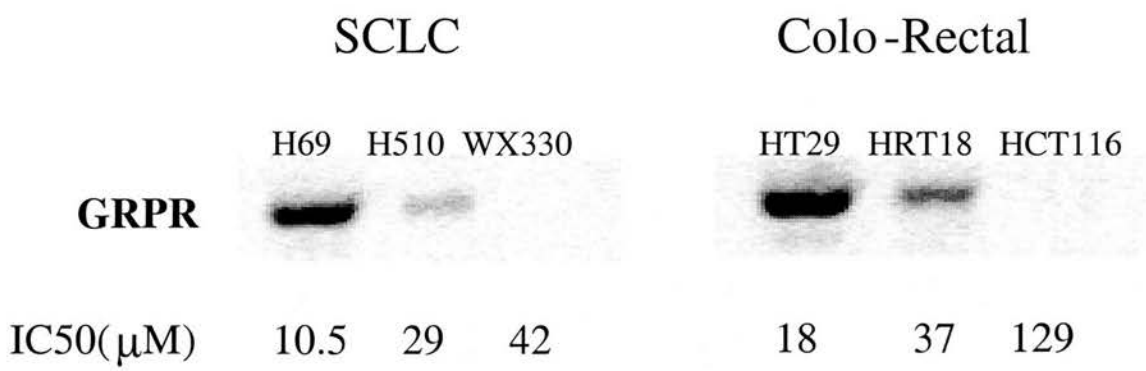


Fig 4.9 Representative PCR gels (inverted images) showing GRPR levels in three SCLC cell lines and three colo-rectal cancer cell lines. In both groups, high GRPR expression correlates with low IC50 values for growth inhibition by antagonist G. Conversely, the absence of GRPR expression correlates with increased resistance to growth inhibition by antagonist G. Cell lines with mid-range expression of GRPR exhibit mid range sensitivity to antagonist G.

The above comparisons of IC_{50} value and neuropeptide receptor levels highlight the importance of neuropeptide receptor expression in dictating the sensitivity of tumour cell lines to growth inhibition by substance P analogues (Figs 4.8 and 4.9). Analysis of this correlation by a Spearman-rank test shows that increased sensitivity to antagonist G significantly correlates with increased GRP receptor expression. It is worth noting that, although not statistically significant, there is also a correlation between gastrin and vasopressin receptor expression and sensitivity to antagonist G. To further explore these issues, the substance P analogue sensitivity of Rat-1 fibroblasts, which do not express GRP receptors, were compared to the sensitivity of Rat-1 fibroblasts stably transfected with the Bombesin/GRP receptor. These results will be discussed in the following section.

4.4 Comparison of Sensitivity to Antagonist G in Rat-1 Fibroblasts with and without transfected Bombesin Receptor.

The apparent importance of neuropeptide receptor status in order for cell lines to be sensitive to antagonist G was further tested in Rat1 fibroblasts. The BOR15 cell line is a Rat-1 fibroblast clone, stably transfected with the bombesin receptor (amphibian homologue of the gastrin releasing peptide receptor). This transfected cell line has been shown to respond normally to bombesin stimulation (Charlesworth et al., 1996)in terms of signal transduction and stimulation of DNA synthesis. The ability of bombesin to stimulate calcium mobilisation and DNA synthesis in BOR15 cells was confirmed prior to experimentation with this cell line (Figs 4.10 and 4.11). The sensitivity to antagonist G, of the bombesin receptor bearing BOR15 cells were compared with the bombesin receptor negative parent Rat-1 cells. IC50 values for growth inhibition by antagonist G were determined as described in materials and methods. It was found that the parent Rat-1 cells were resistant to antagonist G with an IC50 value of 135μM whereas the BOR15 cells showed increased sensitivity with an IC50 value of 44μM on day 7 (Fig 4.14). Rat 1 cell growth was inhibited only by 80μM antagonist G however some growth occurred at this high concentration. In contrast, BOR15 cell growth was completely inhibited by 80μM AntG and growth was also reduced by 40μM AntG. (figs 4.12 and 4.13). This work shows that the presence of the bombesin receptor leads to increased sensitivity to antagonist G.

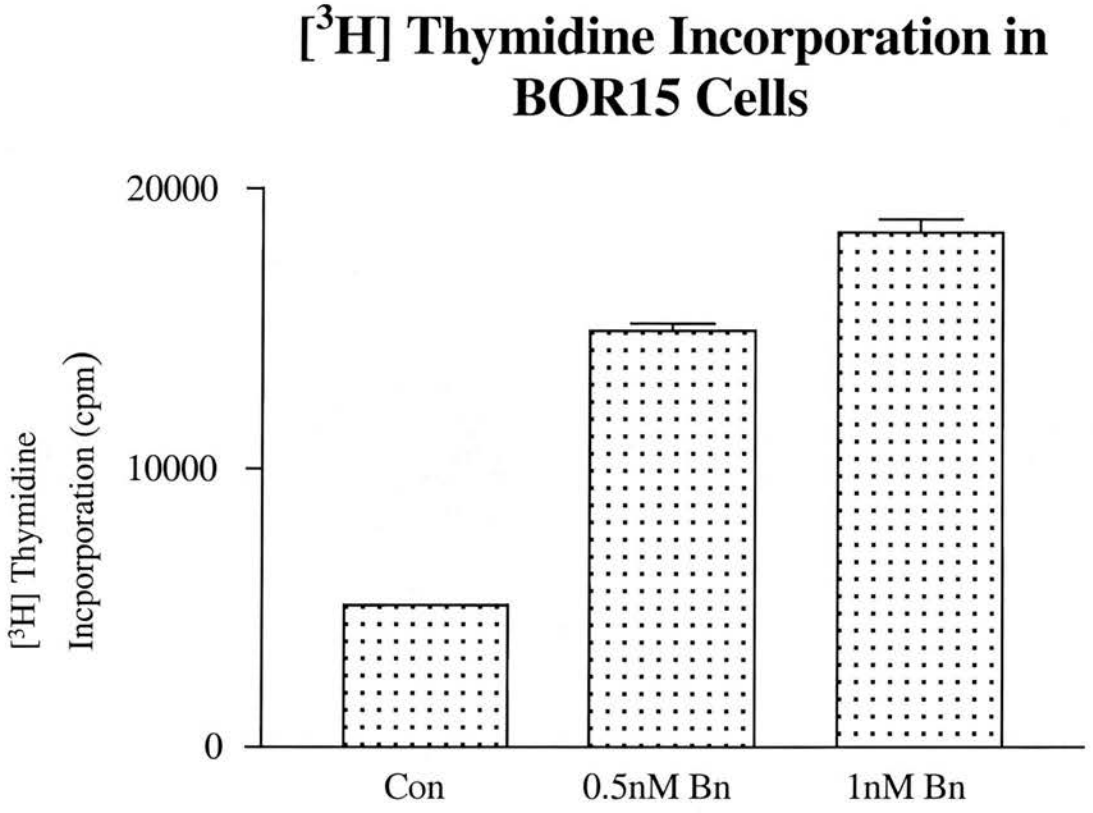


Fig 4.10 Incorporation of [^{3}H]Thymidine in BOR15 cells. Measurement of [^{3}H] thymidine incorporation was performed as described in materials and methods. BOR15 cells were treated with the indicated concentrations of bombesin for 18hrs.

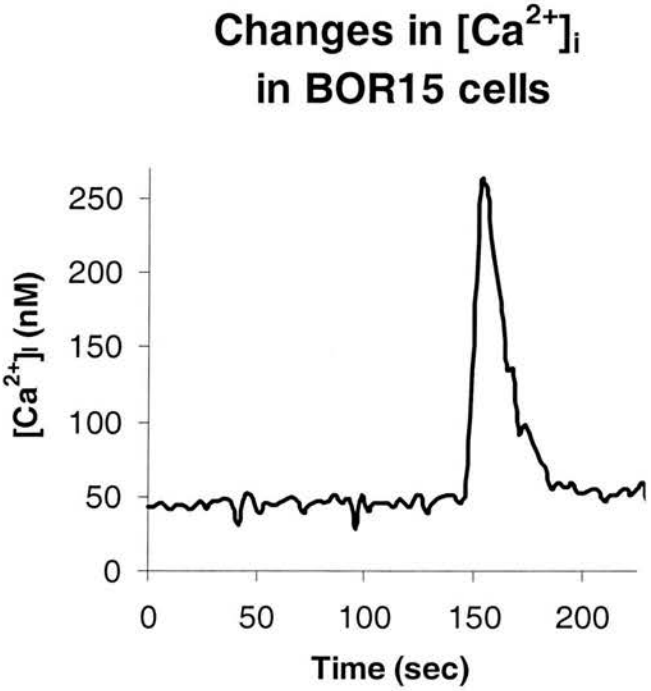


Fig 4.11 Calcium mobilisation in BOR15 cells. Measurement of intracellular calcium mobilisation was carried out as described in materials and methods. BOR15 cells were stimulated with 100nM bombesin.

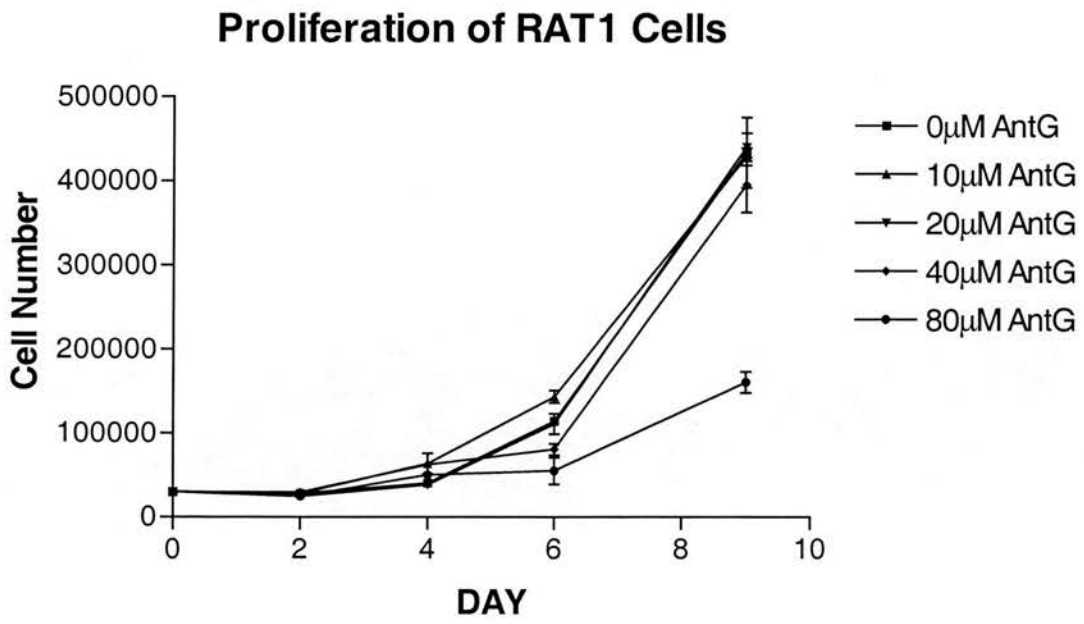


Fig4.12. Proliferation of RAT1 cells in liquid culture in the presence or absence of antagonist G.
Rat1 fibroblasts were incubated in the presence of various concentrations of antagonist G. Cell number was determined using a coulter counter at the time points indicated.
Graphs are representative of two independent experiments. Data points are means of three samples counted in triplicate.

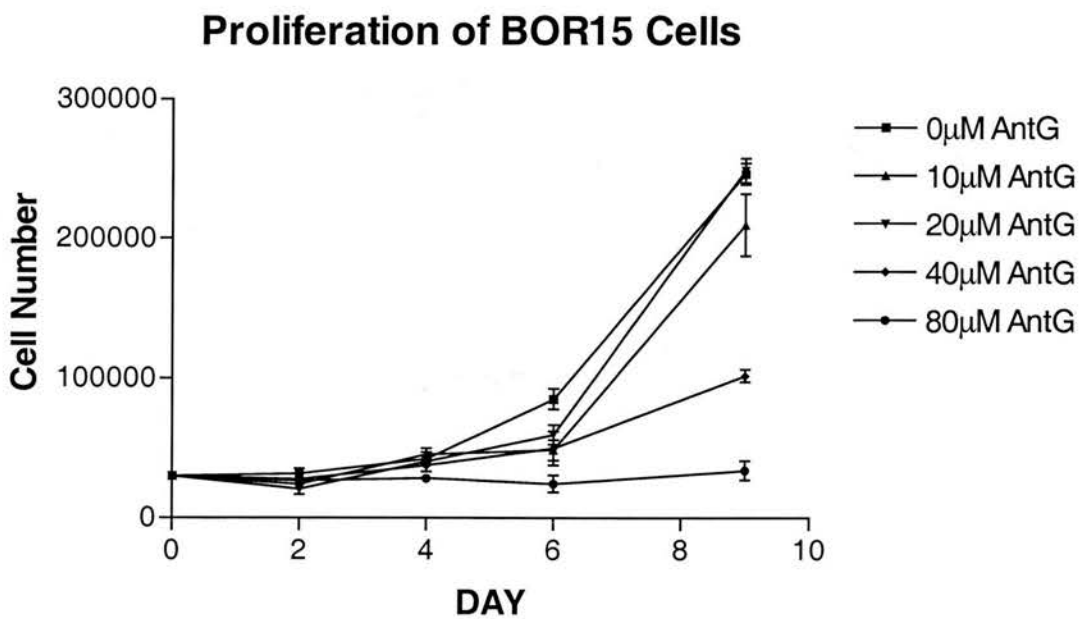


Fig4.13 Proliferation of BOR15 cells in liquid culture in the presence or absence of antagonist G.
BOR15 cells were incubated in the presence of various concentrations of antagonist G. Cell number was determined using a coulter counter at the time points indicated.
Graph is representative of two independent experiments. Data points are means of three samples counted in triplicate

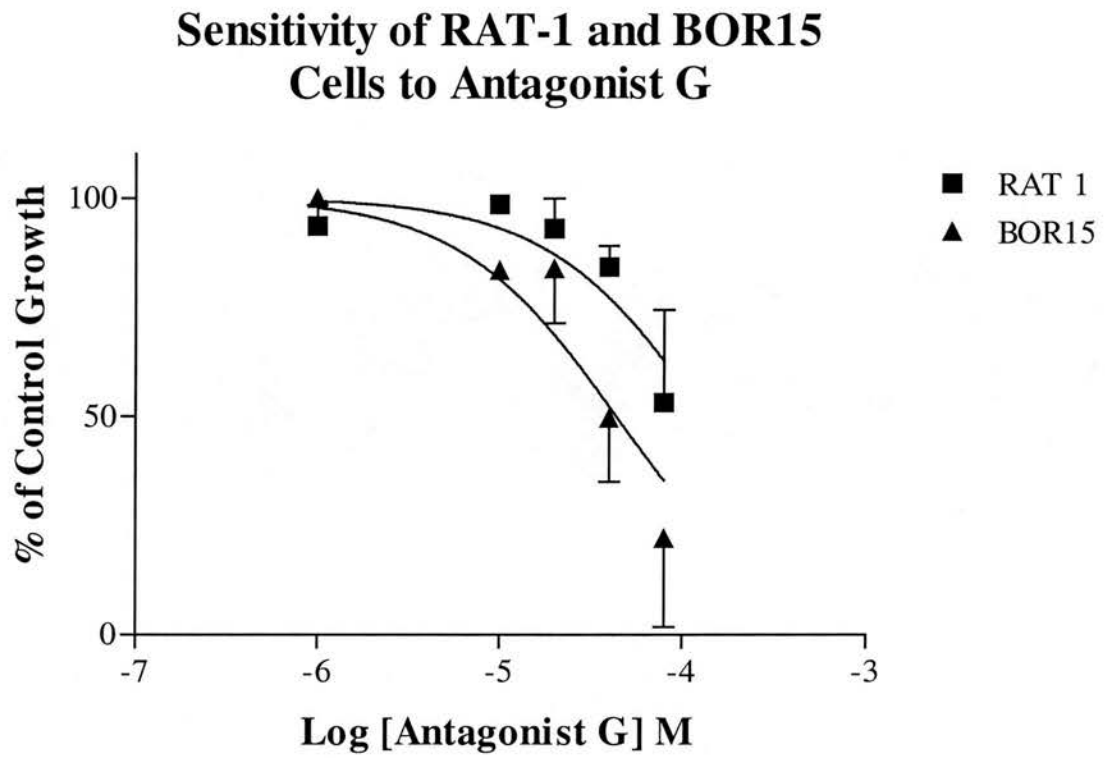


Fig 4.14 IC50 determinations for growth inhibition by antagonist G in Rat-1 and BOR15 cells.
IC50 values were calculated during the exponential growth phase of each cell line (Day7).
Data represents the means of two independent experiments, performed in triplicate.
The IC50 value for **Rat-1** cells is **139μM**.
The IC50 value for **BOR15** cells is **44μM**

4.5 Stable Transfection of Rat-1 Fibroblasts with V1AR and GRPR.

It was apparent that the presence of the bombesin receptor, in Rat-1 fibroblasts, conferred sensitivity to growth inhibition to antagonist G. It was decided to attempt to produce Rat-1 derived cell lines which stably expressed the vasopressin (V1A) or the GRP receptor. This was carried out in order to determine whether the presence of V1AR could increase sensitivity to antagonist G and to confirm the results obtained with the BOR15 cell line, in a Rat-1 derived cell line bearing the GRPR, rather than the bombesin receptor.

Rat-1 fibroblasts were transfected with cDNA encoding the V1AR as described in materials and methods. The resulting cultures were grown in the presence of 800μg/ml G418 for 1 week and then dishes were trypsinised and cell suspensions transferred to a 96 well plate at a density of 1cell/well in the presence of 800μg/ml G418. Two weeks later, the resulting clones were split then transferred to 100mm petri dishes and screened for the ability of 100nM vasopressin to induce mobilisation of intracellular calcium. Of 24 clones originally chosen for screening only two showed any response to vasopressin (Fig 4.15). These cell lines were expanded and stocks frozen in liquid nitrogen.

The same procedure was carried out in order to create a cell line bearing the GRPR. Resulting clones were screened for the ability of 100nM GRP to induce the mobilisation of intracellular calcium. Of 24 clones originally selected 4/24 cells responded to GRP stimulation. Calcium mobilisation in clone 14 is shown in figure 4.16. These four cell lines were expanded and stocks frozen in liquid nitrogen. During this period the four GRP responsive cell lines were screened for their ability to bind radiolabelled [I^{125}]-GRP as described in materials and methods. All four cell

lines were positive for [I^{125}]-GRP binding (Fig 4.15). The GRP6 clone was the most efficient at binding [I^{125}]-GRP, so this cell line was chosen for further examination. Calcium mobilisation in response to increasing concentrations of GRP was carried out. The EC50 for calcium mobilisation by GRP in the GRP6 cell line was 2.1nM (fig 4.17) which is similar to the EC50 for calcium mobilisation by bombesin in the BOR15 cell line.

All vasopressin or GRP responsive cell lines were maintained in 400μg/ml G418 while they were being further characterised. Unfortunately during this period the cells repeatedly failed to respond to stimulation with GRP or VP in terms of mobilisation of calcium. It was presumed that, over time, the cell lines had lost the cDNA for their respective receptors. This can occur for various reasons. The presence of the cDNA or the receptor protein may confer a negative selective pressure on the cell population. In this situation the cell line quickly evolves into a population which does not express the protein in question. Secondly, it is possible for inserted cDNA to fragment and the cells may reject the sequence coding for the receptor but retain the sequence coding for resistance to G418. This may explain why the above cell lines were still able to survive in high concentrations of G418 without expressing the GRP or V1A receptors.

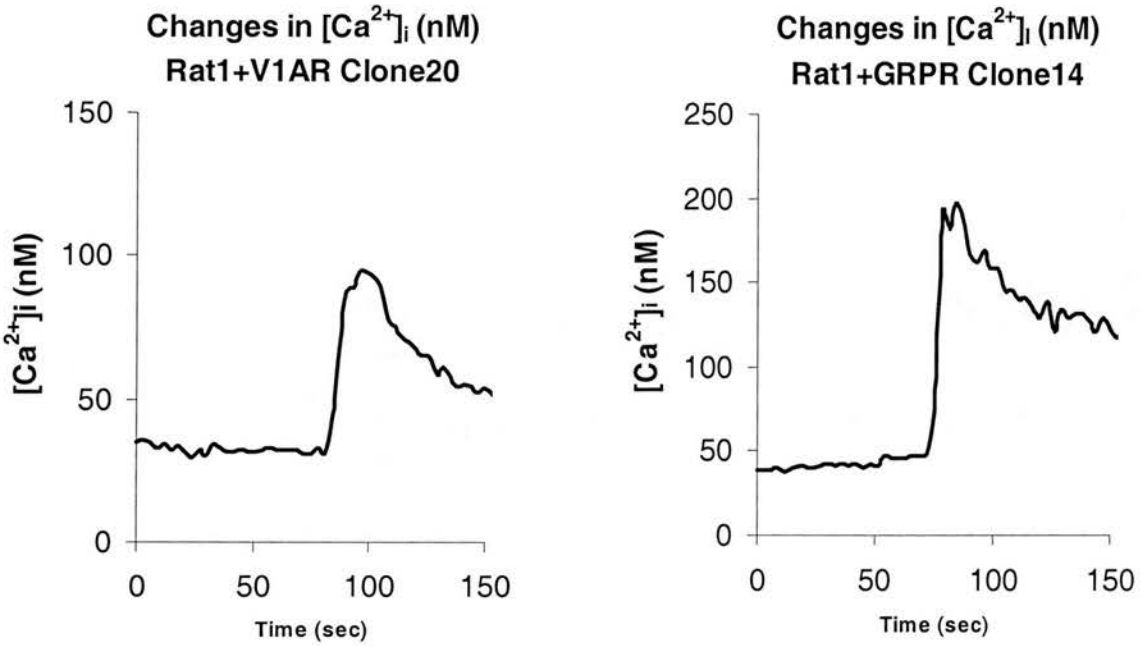


Fig 4.15 Calcium mobilisation in Rat-1 fibroblasts transfected with the V1AR or the GRPR. Calcium mobilisation was determined as described in materials and methods. Traces are examples of the effects of stimulation with 100nM vasopressin (1st trace) or gastrin releasing peptide (2nd trace) in Rat-1 fibroblasts stably transfected with V1AR or GRPR, respectively.

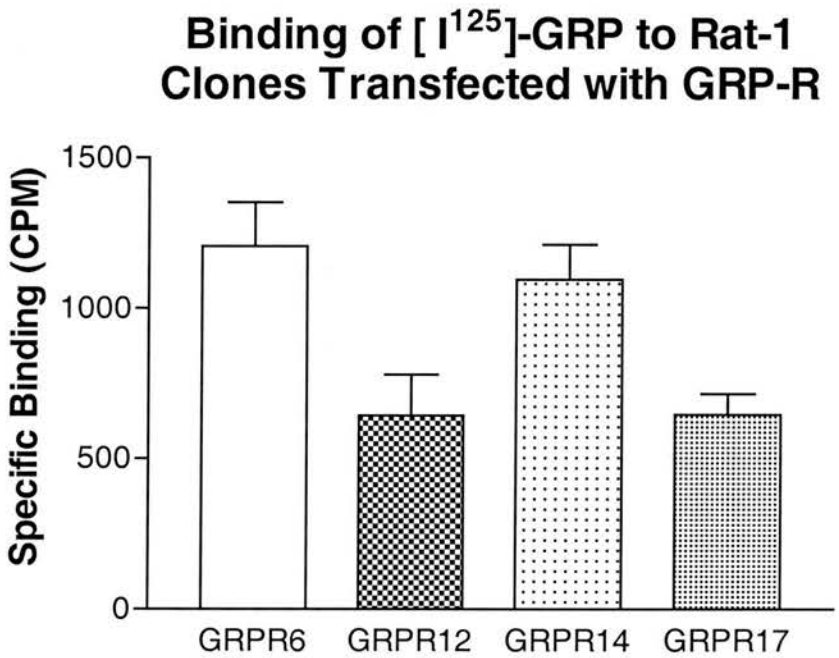


Fig 4.16 Specific binding of [I^{125}]-GRP to four Rat-1 derived cell lines, stably transfected with GRPR. Determination of [I^{125}]-GRP binding was performed in four GRP responsive Rat-1 clones, as described in materials and methods. All cell lines tested were capable of binding [I^{125}]-GRP. Results represent a single experiment, performed in triplicate.

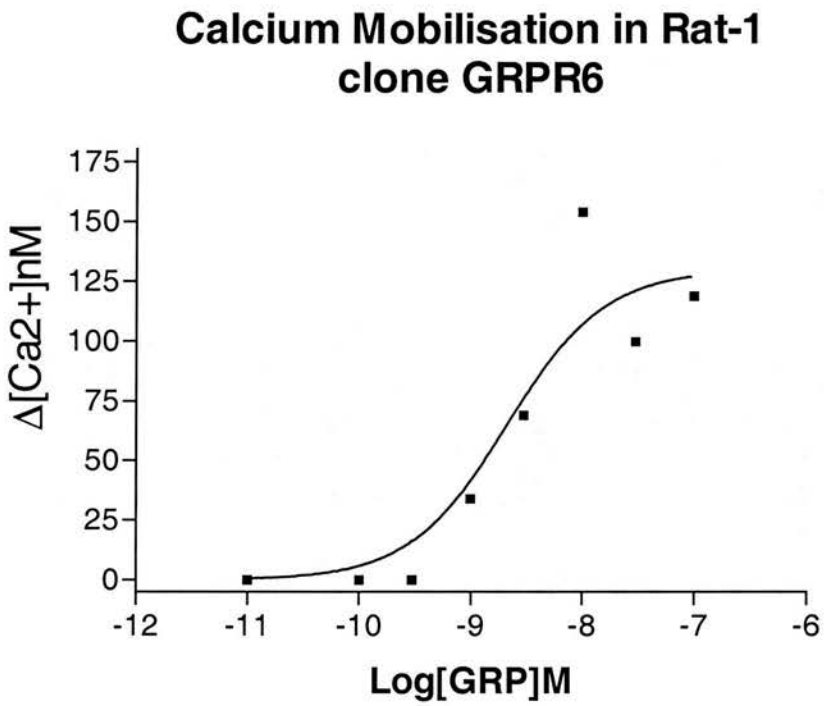


Fig 4.17 Calcium mobilisation in Rat-1 clone GRP6. Calcium mobilisation was determined as described in materials and methods. GRP6 cells (Rat-1 fibroblasts stably transfected with the GRPR) were exposed to increasing concentrations of gastrin releasing peptide. EC50 for mobilisation of calcium by GRP6 cells is 2.1nM.
Data represents single determinations.

4.6 Discussion of Results.

These experiments have shown that cell lines with high expression of receptors for GRP, vasopressin or gastrin are more likely to be sensitive to growth inhibition by antagonist G. Within the SCLC lines of the cell panel, detection of mRNA for a particular receptor correlated well with the ability to mobilise intracellular calcium in response to ligand stimulation. This showed that the receptors detected by RT-PCR were functional.

Bradykinin receptors were expressed by all cell lines tested and expression levels did not relate to sensitivity to antagonist G. The only statistically significant correlation was between high expression of the GRPR and increased sensitivity to antagonist G. GRP and GRPR expression are the hallmark of the neuroendocrine phenotype, thus it is likely that cell lines with high expression of GRPR represent a more neuroendocrine-like phenotype than other cell lines.

Unfortunately, due to the nature of semi-quantitative RT-PCR, only levels of a single receptor type can be compared across the cell panel and therefore the levels of one receptor can not be accurately compared with another receptor type. Ideally, levels of total neuropeptide receptors should be compared across the cell panel but this would require either real-time quantitative RT-PCR or binding studies to determine numbers of each receptor type. Neither of these techniques were available or practical during this project.

It was apparent that neuropeptide receptor expression is a pre-requisite for cells that are sensitive to growth inhibition by antagonist G and to further test this hypothesis, sensitivity to antagonist G was compared between non-neuropeptide receptor expressing Rat-1 fibroblasts and Rat-1 fibroblasts stably transfected with the

bombesin receptor (BOR15 cells). The BOR15 fibroblasts showed increased sensitivity to growth inhibition by antagonist G with a shift in IC50 from 135μM (wild type) to 44μM (BOR15). This work shows that in Rat-1 cells the presence of the bombesin receptor confers increased sensitivity to antagonist G and supports the hypothesis that cell lines which express bombesin/GRP receptors are susceptible to the growth inhibitory effects of antagonist G.

It is still unclear as to whether the presence of other neuropeptide receptors such as the vasopressin receptor, confers increased sensitivity to antagonist G. It was attempted to create a Rat-1 fibroblast derived cell line expressing the V1A receptor in order to investigate this question. The resulting cell lines initially expressed the V1AR but soon became unresponsive to vasopressin stimulation and this experiment was abandoned. It would be interesting to re-attempt these transfections, perhaps using different techniques to enable higher initial transfection efficiency and quicker, more robust screening methods such as FACS analysis.

This work has important implications for the therapeutic use of substance P analogues. Screening tumour biopsy samples for GRPR or other neuropeptide receptor expression may provide insight into the likelihood of patients to respond to treatment with substance P analogues.

Chapter 5

An Investigation into the Effects of Pre-exposure to Chemotherapeutic Agents on Sensitivity to Substance P Analogues.

Small cell lung cancer is initially sensitive to chemotherapy and radiotherapy but it almost invariably relapses and is resistant to further treatment. The two year survival rate for SCLC patients is less than 5%. Little is known about the responsiveness of SCLC cells to neuropeptide antagonists when the tumour progresses from chemotherapy sensitive to chemotherapy resistant. It was of interest to discover whether tumour cells which were resistant to conventional chemotherapy, which often are cross-resistant to a variety of treatment, showed any differences in their sensitivity to substance P analogues.

A useful panel of three cell lines have been developed which have been established from one patient during longitudinal follow-up (Berendsen et al., 1988). During this period the tumour changed from sensitive to resistant to chemotherapy and the *in vitro* sensitivity to chemotherapeutic agents of the cells reflected the clinical resistance to treatment (de Vries et al., 1989). These cell lines (GLC14, 16 and 19) represent an *in vitro* model in which to study the *in vivo* development of drug resistance in SCLC. Another pair of cell lines LS274 and LS310 were established from a similar clinical follow-up of a single patient, prior to chemotherapy and following the onset of chemotherapy resistance (provided by J.Plumb, CRC).

In addition to the above panels of cell lines with *in vivo* derived resistance to chemotherapy, two other pairs of cell lines (SCLC cell lines GLC4 and H69) were used in which chemotherapy resistance was derived *in vitro* to create the cell lines

GLC4(ADR) and H69(LX4) respectively (Reeve et al., 1989; Zijlstra et al., 1987). Together these cell lines provide a model for analysis of the effects of pre-exposure to chemotherapy, on the sensitivity of the cell lines to substance P analogue induced growth inhibition.

To assess the sensitivity of the above panels of cell lines to antagonist D and G induced growth inhibition, growth was measured using two assays; measurement of cell proliferation in liquid culture media (SITA) and measurement of colony formation in semi-solid media (SITA / agarose).

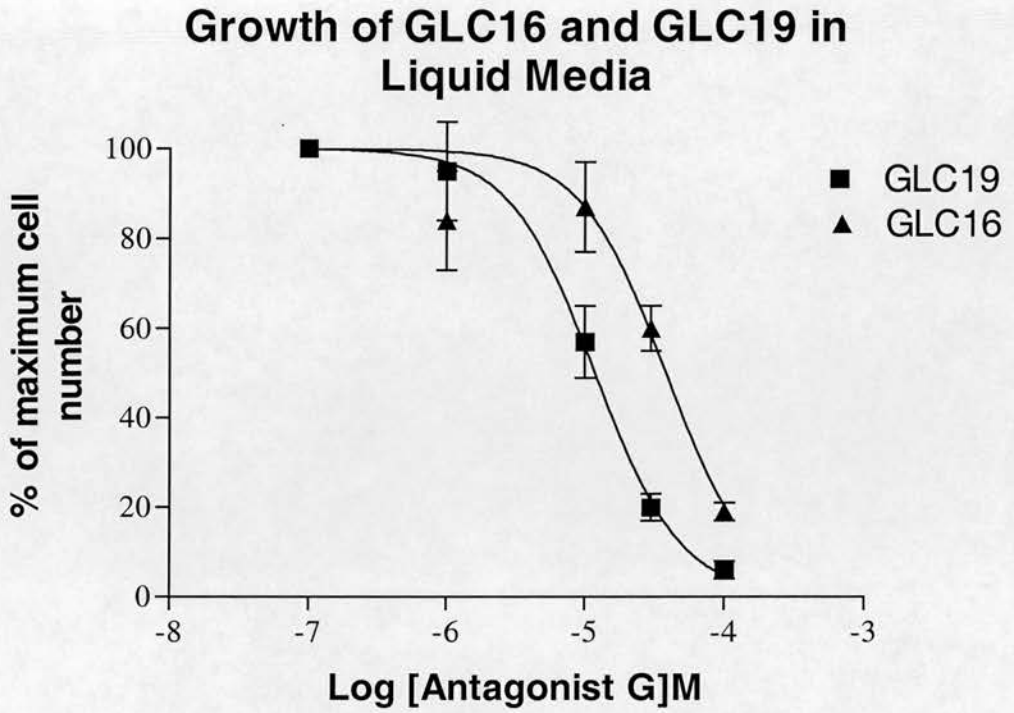
5.1 Sensitivity of GLC16 and GLC19 SCLC cells to Antagonist G and Antagonist D.

The GLC14 cell line was derived before treatment, from a supraclavicular lymph node. After chemotherapy, the patient was in complete remission. After four months she relapsed, further chemotherapy resulted in a partial response and the GLC16 cell line was established from a recurrence in the lung. After further therapy, the lung appeared free from tumour however, three months later the tumour recurred in the lung, from which the GLC19 cell line was derived, this was resistant to further treatment and the patient died two months later.

Ideally this work should have been carried out using the GLC14 and 19 cell lines to compare before and after chemotherapy, however stocks of the GLC14 cell line were suspected to be contaminated with mycoplasma so the GLC16 and 19 cell lines were compared as an alternative.

In the presence of 0-80μM antagonist D or G, proliferation of cells in liquid SITA media were measured using a coulter counter. Cell number was determined on day 7. The GLC19 cell line showed increased sensitivity to both antagonist D and antagonist G when compared to the sensitivity of the GLC16 cell line (Fig 5.1). The IC50 values for growth inhibition by antagonist G for GLC16 and 19 were 38μM and 11μM respectively. The IC50 values for growth inhibition by antagonist D for the GLC16 and 19 were 39μM and 7.5μM respectively.

In addition to measuring growth inhibition in liquid culture, prevention of cell growth was measured in semi-solid media containing 0-100μM antagonists G or D. This assay measures the ability of single cells to form visible colonies which were counted 7 days after seeding. Again the GLC19 cell line showed increased sensitivity to both antagonist D and G with IC50 values for colony inhibition of 2μM and 2.6μM respectively. In comparison the IC50 values for colony inhibition in GLC16 cells, by antagonists D and G, were 18μM and 33μM respectively (Fig 5.2)



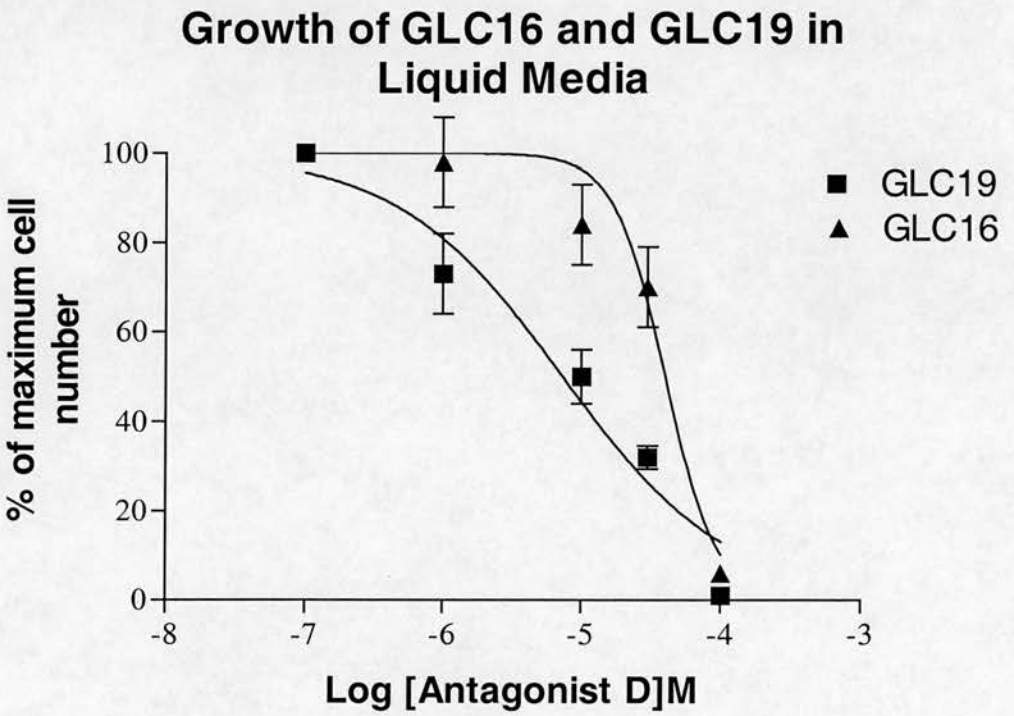


Fig 5.1 Proliferation of SCLC cell lines GLC16 and GLC19 in Liquid Culture.
Cells were grown in SITA media in the presence or absence of the indicated concentrations of antagonist G (top) and antagonist D (bottom). Cell number was determined using a coulter counter following 7 days of growth. Graphs represent the mean of two independent experiments performed in triplicate. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

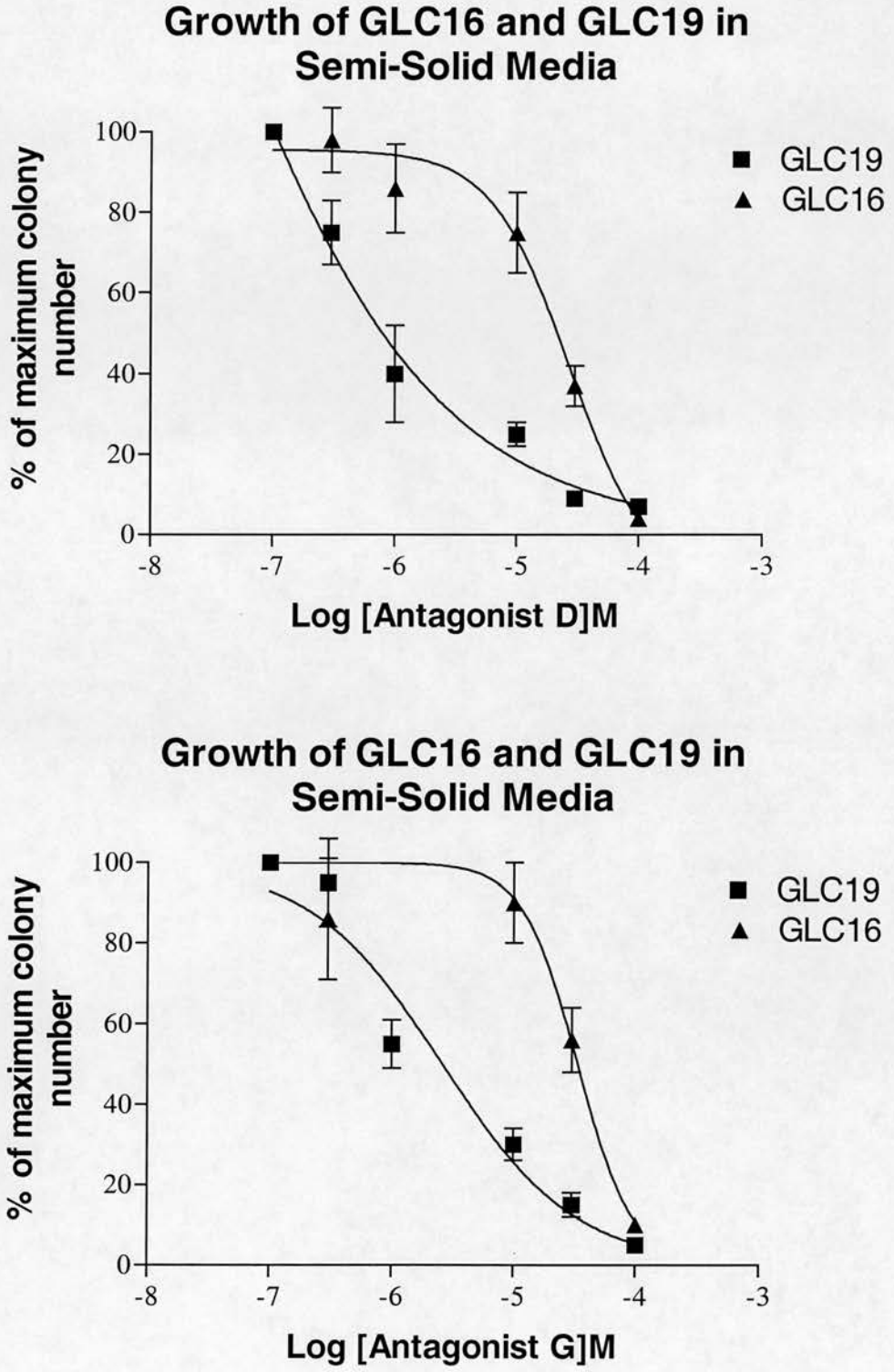


Fig 5.2 Proliferation of SCLC cell lines GLC16 and GLC19 in Semi-Solid Media.
Cells were grown in SITA/agarose media in the presence or absence of the indicated concentrations of antagonist D (top) and antagonist G (bottom). Colony number was determined following 7 days of growth. Graphs represent the mean of two independent experiments performed in triplicate. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

5.2. Sensitivity of LS274 and LS310 SCLC cells to antagonist G and D.

The LS274 and LS310 SCLC cell lines were established from a single SCLC patient, before and after chemotherapy respectively.

As in the previous panel of cell lines, sensitivity to growth inhibition by substance P analogues was determined using both liquid growth assays and colony assays in semi-solid media.

In contrast to the GLC16 and GLC19 cell lines, when the LS274 and LS310 cells were exposed to antagonist G in liquid culture their growth was inhibited to a similar degree. The IC50 values for growth inhibition by antagonist G in liquid culture for the LS274 and LS310 cell were 19μM and 18μM respectively. The dose response curves for these two cell lines were superimposable. Growth inhibition by antagonist D also occurred to a similar degree in these two cell lines. The IC50 values for growth inhibition by antagonist D in liquid culture for the LS274 and LS310 cell were 7.5μM and 7μM respectively (Fig 5.3). When the inhibition of colony formation was measured in the LS274 and LS310 cell lines, again both cell lines were similarly sensitive. The IC50 values for colony inhibition by antagonist G were 7μM for the LS274 cells and 4μM for the LS310 cells. For antagonist D mediated colony inhibition, the IC50 values were 4.2μM for the LS274 cell line and 2μM for the LS310 cell line (Fig 5.4).

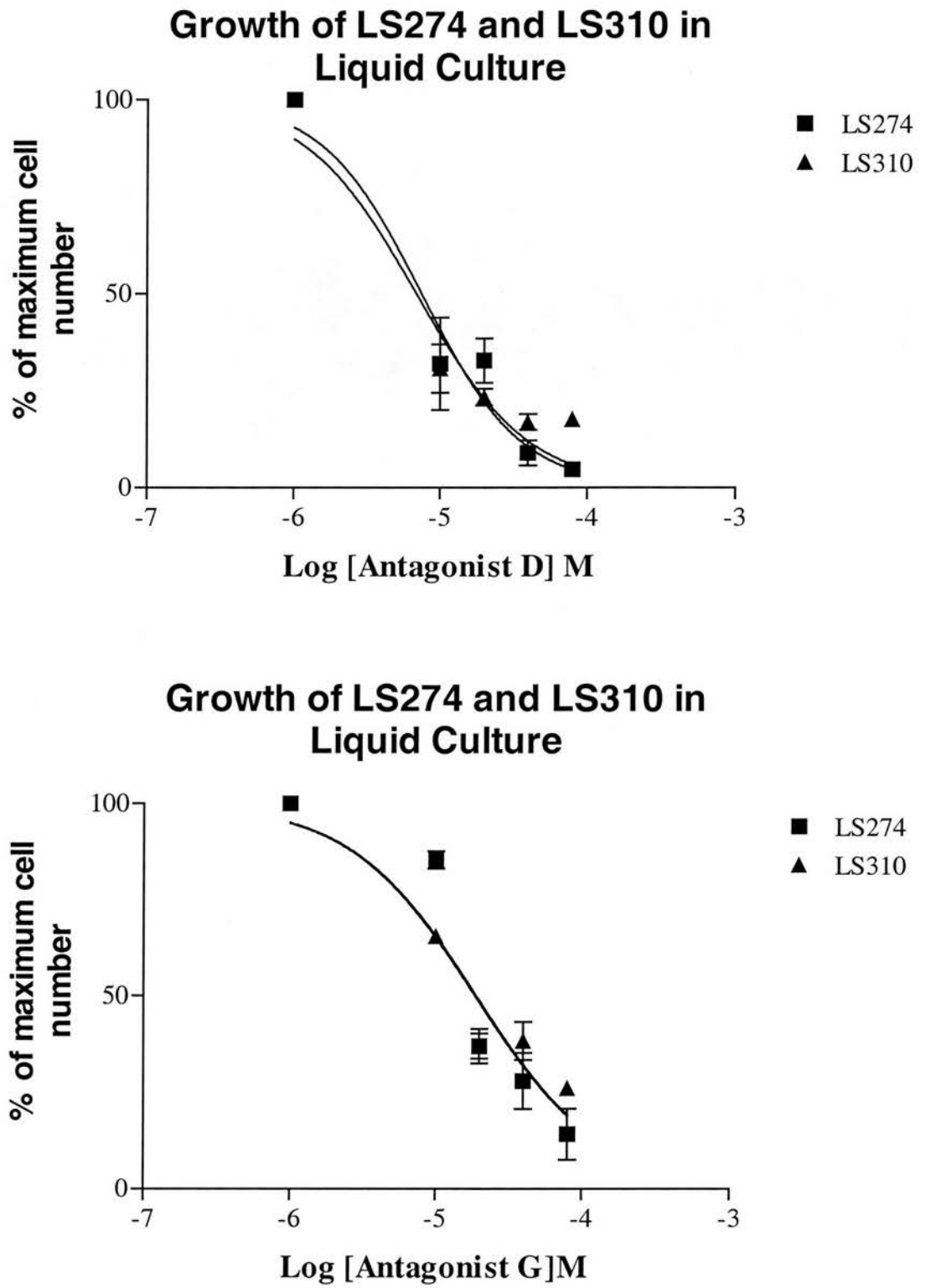


Fig 5.3 Proliferation of SCLC cell lines LS274 and LS310 in Liquid Culture.
Cells were grown in SITA media in the presence or absence of the indicated concentrations of antagonist D (top) and antagonist G (bottom). Cell number was determined using a coulter counter following 7 days of growth. Graphs represent the mean of two independent experiments performed in triplicate. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

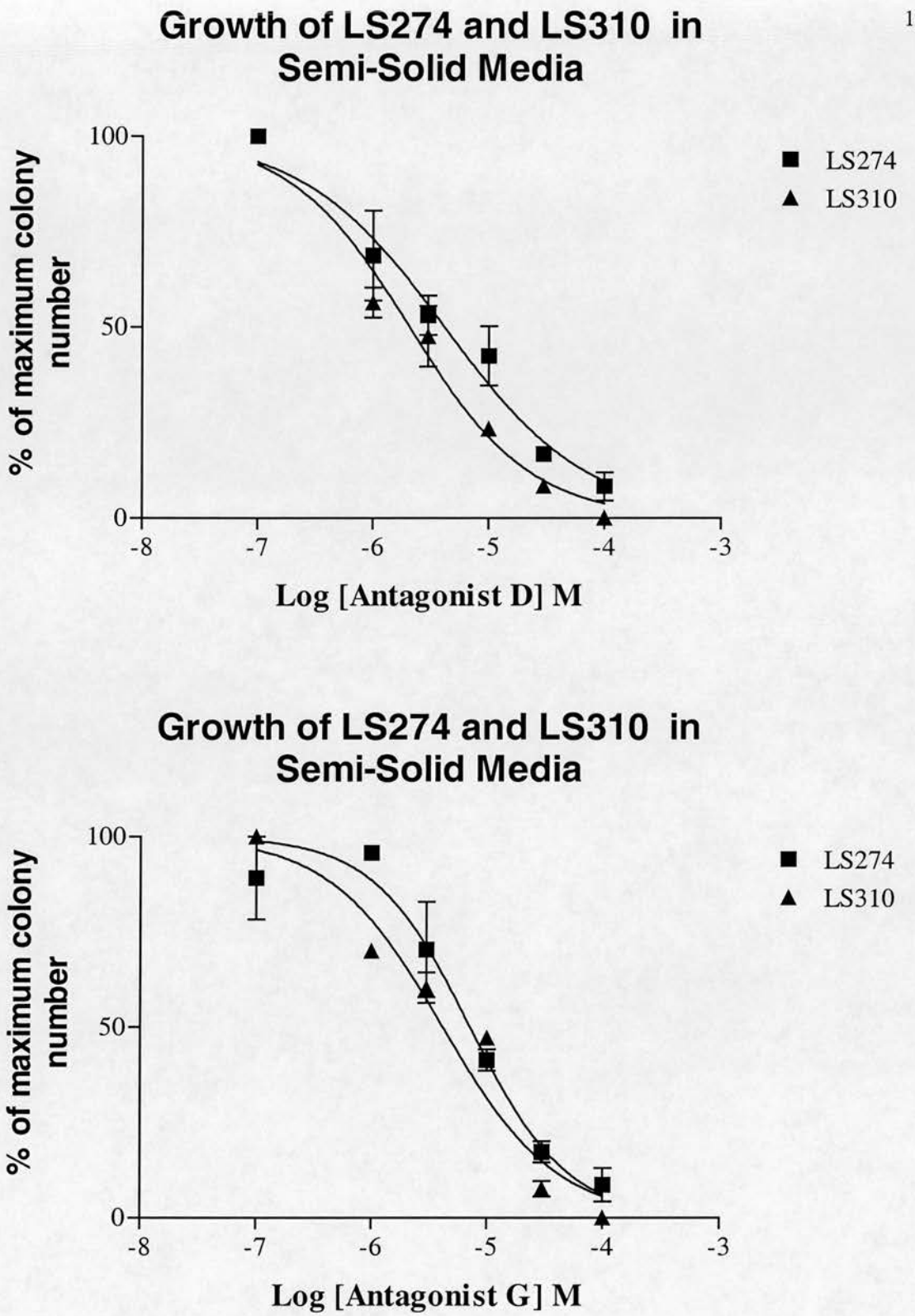


Fig 5.4 Proliferation of LS274 and LS310 SCLC cell lines in semi-solid media.
Cells were grown in SITA/agarose media in the presence of the indicated concentrations of antagonist D (top) and antagonist G (bottom). Colony number was determined following 7 days of growth. Graphs represent the mean of two independent experiments performed in triplicate. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

5.3 Sensitivity of the SCLC Cell Lines GLC4 and GLC4ADR to Growth Inhibition by Antagonist G

The GLC 4 cell line was derived from a pleural effusion of a patient with SCLC. It was characterised as a variant SCLC cell line (Carney et al., 1985). The GLC4ADR cell line was derived by culturing GLC4 cells in 18nM adriamycin until a resistant cell line was obtained. The resulting cell line was multi-drug resistant with a 40-fold increase in IC50 for adriamycin induced growth inhibition (Zijlstra et al., 1987).

The sensitivity of the GLC4 and GLC4ADR cell lines to antagonist G were compared by liquid growth assay. The IC50 values for the two cell lines were not significantly different although the GLC4ADR cell line was marginally more resistant with an IC50 value of 26μM compared to an IC50 value of 18μM for the parent cell line (Fig 5.5).

5.4 Sensitivity of the SCLC Cell Lines H69 and H69LX4 to Growth Inhibition by Antagonist G

The H69 cell line and its multidrug resistant partner, H69LX4 were provided by P.Twentyman (MRC). The H69LX4 cell line has been show to express the mutidrug resistance glycoprotein p180 (Reeve et al., 1989).

The sensitivity of the H69 and H69LX4 cells to antagonist G, were compared by liquid growth assay. The IC50 value for growth inhibition for the H69 cells was 6μM and the IC50 for the H69LX4 cells was 16μM although this difference was not significant.

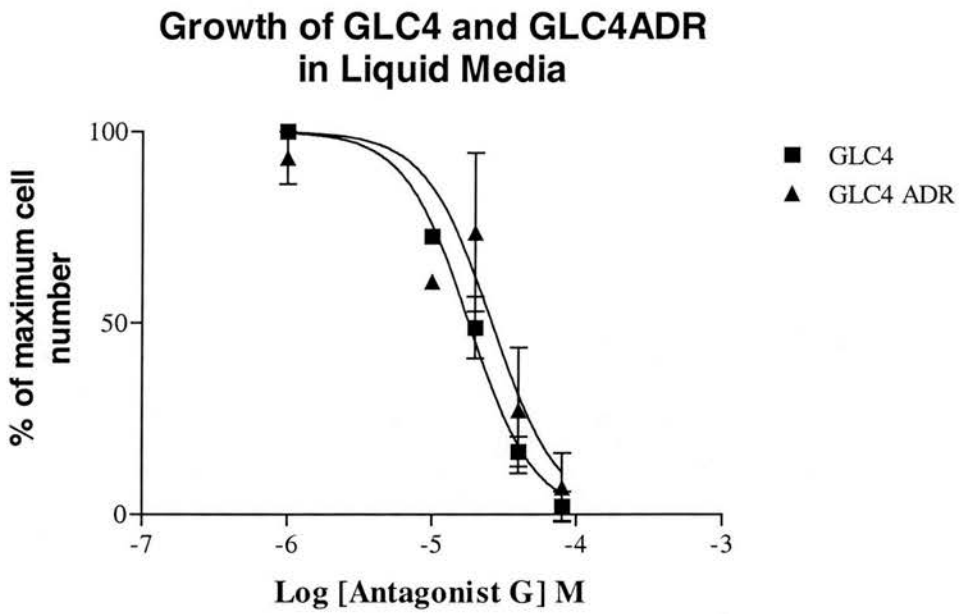


Fig 5.5. Proliferation of the GLC4 and GLC4ADR SCLC cell lines in liquid culture.
Cells were grown in SITA media in the presence or absence of the indicated concentrations of antagonist G. Cell number was determined using a coulter counter following 7 days of growth. Graphs represent the mean of two independent experiments performed in triplicate. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

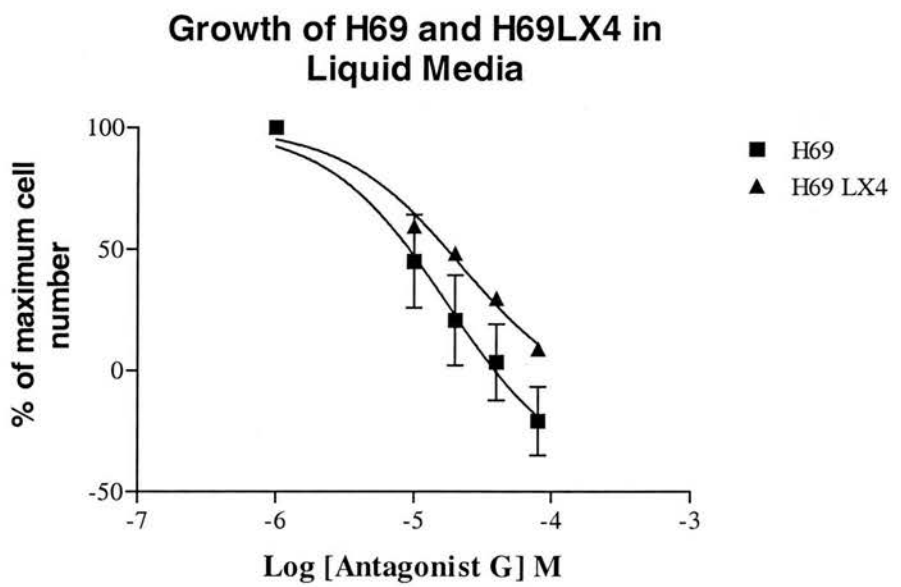


Fig5.6. Proliferation of the H69 and H69LX4 SCLC cell lines in liquid culture.
Cells were grown in SITA media in the presence or absence of the indicated concentrations of antagonist G. Cell number was determined using a coulter counter following 7 days of growth. Graphs represent the mean of two independent experiments performed in triplicate. Curve fitting was carried out using non-linear regression curve fitting in Prism software.

5.5 Chemotherapy Resistant SCLC Cells with Increased Responsiveness to Substance P Analogues, also Show Increased Responsiveness to Bombesin/GRP.

In view of the findings of the previous chapter, which suggested that cell lines that were sensitive to growth inhibition by antagonist G exhibited increased expression of Bombesin/GRP receptors, it was decided to compare the responsiveness of the SCLC cell lines GLC16 and GLC19, to bombesin/GRP.

The mobilisation of intracellular calcium by100nM bombesin was used as an indicator of bombesin responsiveness. The GLC16 cell line showed very little response to stimulation with supra-maximal concentrations of bombesin with a change in intracellular calcium levels of <10nM. FCS was used as a positive control and stimulated the mobilisation of >200nM intracellular calcium, indicating that defects in the mechanisms of calcium mobilisation did not account for this lack of responsiveness to bombesin. The GLC19 cell line, which showed increased sensitivity to growth inhibition by substance P analogues, exhibited a much greater response to stimulation with 100nM bombesin. In this cell line, supra-maximal concentrations of bombesin stimulated the mobilisation of 150nM intracellular calcium. (Fig 5.7). Other members of our group have shown that the GLC19 cell line can be stimulated by bombesin, to form colonies in semi-solid media to a significantly greater degree than the GLC16 cell line (data not shown). The above data is in accordance with the data presented in chapter 4 and supports the hypothesis that cell lines which are sensitive to growth inhibition by substance P analogues show relatively higher expression of GRP/Bombesin receptors.

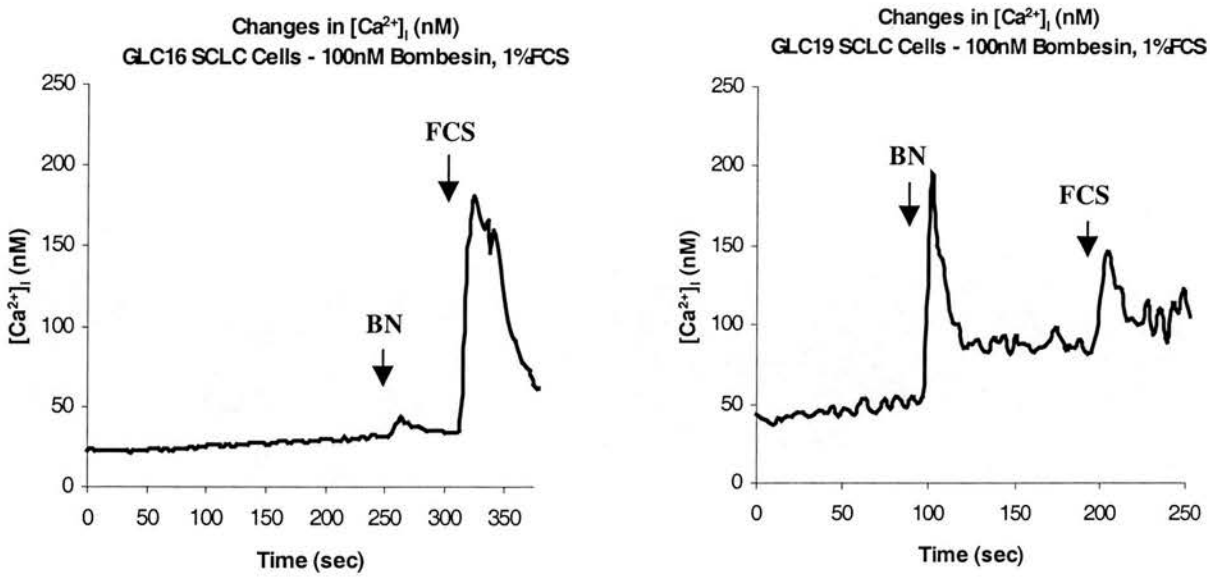


Fig 5.7. Calcium Mobilisation in SCLC GLC16 and GLC19 Cells in Response to Stimulation with Bombesin. Calcium mobilisation was detected as described in materials and methods. SCLC cells, grown in SITA media, were loaded with the calcium indicator FURA2-AM then exposed to 100nM bombesin or 1%FCS as indicated. Traces are representative of three independent experiments.

5.6 Discussion of Results

This work was carried out in order to investigate the effect of pre-exposure to conventional chemotherapy on the sensitivity of SCLC cell lines to growth inhibition by substance P analogues. Four pairs of cell lines were used for this study. The GLC16 cell line was derived from a SCLC recurrence in the lung and its partner, the GLC19 cell line was derived from the same tumour following the onset of chemotherapy resistance. The LS274 and LS310 SCLC cell lines were obtained in similar circumstances with the LS310 cell line being resistant to further chemotherapy treatment. The GLC4ADR and the H69LX4 SCLC cell lines had been exposed to chemotherapeutic compounds *in vitro* in order to create multi-drug resistant cell lines which were compared to their parent cell lines.

The GLC19 cell line showed increased sensitivity to both antagonist G and D when compared to the GLC16 cell line. In accordance with data presented in chapter 4, the

GLC19 cell line showed increased responsiveness to bombesin, as measured by the mobilisation of intracellular calcium.

The second pair of SCLC cell lines were the LS274 and LS310 cells, the LS310 cells being a model of *in vivo* derived chemotherapy resistance. Both of these cell lines displayed similar sensitivities to both antagonist G and D induced growth inhibition. There was also no significant correlation between sensitivity to chemotherapy and sensitivity to substance P analogues in the two pairs of cell lines used as models of *in vitro* derived chemotherapy resistance (GLC4/GLC4ADR and H69/H69LX4). Taken together, these results suggest that cell lines which have been exposed to conventional chemotherapy and have developed resistance to such treatments, are not more resistant to growth inhibition by substance P analogues. This implies that patients bearing tumours with multi-drug resistant phenotypes may be benefited by treatment with substance P analogues, either as an adjunct to conventional treatments or during such times when further chemotherapy will have no therapeutic effect.

SCLC growth is sustained by multiple paracrine and autocrine loops involving a variety of neuropeptide growth factors (Woll and Rozengurt, 1989; Sethi and Rozengurt, 1992). A plausible extension of this hypothesis is that the clinically aggressive, drug resistant, SCLC cells that emerge after chemotherapy might have a more extensive network of neuropeptide regulation and therefore may display increased sensitivity to neuropeptide antagonists. In support of this theory, the GLC19 cell line, which displayed increased sensitivity to substance P analogues, also showed increased responsiveness to bombesin.

Due to the lack of available cell lines derived from longitudinal follow ups from single patients, it is difficult to draw conclusions from these observations and it will be necessary to further investigate this topic in order to clarify the situation.

Chapter 6

An Investigation into the Agonist Activity of Substance P Analogues

The precise mechanism of action of substance P analogues is more complex than was previously appreciated. It has been clearly demonstrated that these compounds can inhibit many neuropeptide stimulated signalling events such as neuropeptide receptor binding (Woll and Rozengurt, 1988; Bepler et al., 1988), calcium mobilisation (Sethi and Rozengurt, 1991; Bunn et al., 1994; Sethi and Rozengurt, 1992), and MAPK activation (Mitchell et al., 1995; Seckl et al., 1996). The inhibition of these neuropeptide stimulated events can be reversed by the addition of high concentrations of neuropeptides which suggests that substance P analogues are competitive antagonists of neuropeptide signalling (Seckl et al., 1996). It has recently been hypothesised that substance P analogues induce apoptosis and growth inhibition via a mechanism which is independent of competitive neuropeptide antagonism. This is demonstrated by the fact that high concentrations of neuropeptides are not capable of reversing the pro-apoptotic and growth inhibitory effects of substance P analogues (MacKinnon et al., 1999). In a study by Bunn et al it was found that substance P analogues were capable of inhibiting SCLC cell growth whereas a range of specific neuropeptide antagonists did not inhibit cell growth (Bunn et al., 1994). This suggests that the mechanism of growth inhibition by substance P analogues involve some activity distinct from, or in addition to, competitive neuropeptide antagonism.

Studies in this lab, and others, have demonstrated that antagonists D and G are capable of activating c-Jun N-terminal Kinase (JNK) in both SCLC cells and in fibroblasts and that this is a neuropeptide receptor mediated event (MacKinnon et al., 1999; Jarpe et al., 1998). Thus, in addition to acting as classical receptor antagonists, antagonists G and D are capable of initiating cellular events which are independent of neuropeptide antagonism. Activation of JNK may play an important role in stimulating apoptosis in SCLC cells (Xia et al., 1995) and in this lab it has previously been shown that antagonist G induced JNK activation is dependant upon the generation of reactive oxygen species and that antagonist G induced apoptosis is inhibited by hypoxic conditions (MacKinnon et al., 1999).

Thus, it is apparent that substance P analogues have agonist activities in addition to classical antagonist activity. In a recent paper by Jarpe et al (Jarpe et al., 1998), both bombesin and antagonist D were shown to activate JNK however, calcium mobilisation was stimulated by bombesin but not antagonist D. This led the authors to speculate that antagonist D was activating the bombesin receptor but it was activating some available downstream pathways and not others. Bombesin behaves as a classical agonist, so the term "biased agonist" was introduced as a way of explaining the behaviour of antagonist D (Fig 6.1). The biased agonist theory suggests that antagonist D favours association with R*2 over R*1. This model can be extended to include as many activation states of the receptor as there are available G protein-coupling states. The data published by Jarpe et al suggests that antagonist D may cause neuropeptide receptors to couple to G12 but not to Gq. This hypothesis is based on the data of other groups who show that JNK activation, via G protein coupled receptors, can be through activation of G12 family of G proteins (Prasad et

al., 1995). Jarpe et al also show that antagonist D induces cytoskeletal reorganisation in Swiss 3T3 cells via a Rho dependant pathway. This pathway has previously been shown to be via the activation of G12(Buhl et al., 1995).

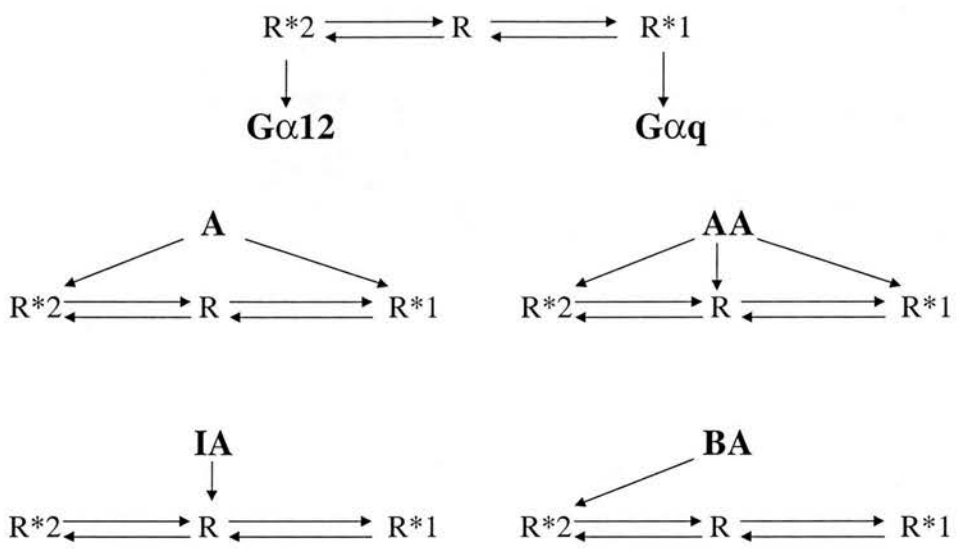


Fig 6.1 Biased agonist hypothesis for the activation of receptors by antagonist D A receptor, R, is capable of spontaneously cycling between two or more active conformational states, here shown as R*1 and R*2, and one energetically favoured inactive state, R.
R*1 represents the conformation of GRPR that activates Gαq and R*2 represents the conformational state of the GRPR that activates Gα12. An agonist (A) binds to and stabilises both active states of the receptor. An antagonist (AA) binds to all three states, competing with the agonist. It may or may not stabilise the active states. If an antagonist stabilises an active state of the receptor it is termed a partial agonist. An inverse agonist (IA) binds to and further stabilises the inactive state of the receptor. A biased agonist (BA) binds preferentially to one of the active states and stabilises it, thus initiating one set of signal transduction events downstream of the receptor, without activating others.
(adapted from (Jarpe et al., 1998))

Binding of a biased agonist to the receptor inhibits the binding of full agonists so when bombesin stimulated Gq events such as calcium mobilisation are measured, it appears that antagonist D is acting as a competitive receptor antagonist. This theory fits previous experimental data by other groups but so far there is no direct evidence of this biased agonist activity.

For this reason it was decided to further investigate the agonist activities of antagonist D in fibroblast models. Firstly, it was necessary to confirm that the

agonist activity of antagonist D was, indeed, via neuropeptide receptors and not via another mechanism. It was intended to compare agonist signalling events, such as the activation of JNK by antagonist D, in the Rat-1 fibroblasts and the BOR15 cell line (Rat-1 fibroblasts stably expressing the bombesin receptor). The agonist effects were also investigated in a panel of Balb 3T3 rat fibroblasts which were stably transfected with either wild type GRPR or with various GRPR mutants. Secondly, the signal transduction pathways involved in the agonist activity of antagonist D were compared to those of the full agonist, bombesin. Lastly, the physiological relevance of the agonist effects of antagonist D were investigated, in terms of the growth inhibitory effects of this class of compounds.

This work was initiated in order to look at JNK activation by antagonist D, however, it soon became clear that antagonist D also had profound stimulatory effects on MAPK signalling. It was decided to focus on the activation of MAPK by antagonist D in an attempt to fit this phenomenon into the biased agonist theory and to determine its physiological relevance.

6.1 Antagonist D, Antagonist G and Bombesin activate MAPK

Interestingly, in addition to the activation of JNK, it became apparent that antagonists G and D were potent activators of MAPK (Fig 6.2). Using SDS-PAGE and western blotting followed by detection of phosphorylated ERK1 and ERK2, it was found that relatively low concentrations (10μM) of antagonists G and D, produced a potent stimulation of MAPK phosphorylation. This phosphorylation was comparable to the degree of phosphorylation found with maximal concentrations (3nM) of bombesin.

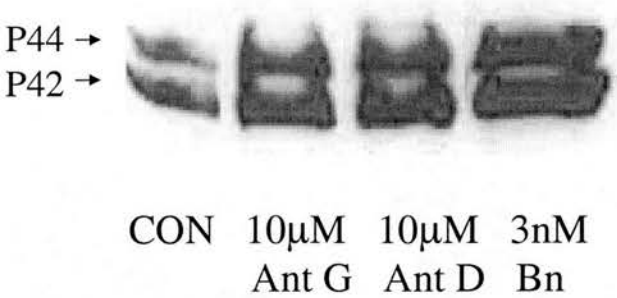


Fig 6.2. Antagonist G, Antagonist D and Bombesin activate MAPK in BOR15 cells. Confluent, quiescent cultures of BOR15 cells were incubated with the indicated treatments for 5 minutes. Samples were prepared as described in materials and methods and proteins were separated using SDS-PAGE. Western blots were probed with anti-phospho ERK1 and 2 (p44,p42) primary antibody and then an anti-mouse-HRP secondary antibody. Proteins were visualised using ECL detection. Blot is representative of three independent experiments.

6.2 Activation of MAPK by antagonist D in BOR15 cells is via the Bombesin/GRP Receptor.

It was unclear whether the observed MAPK activation by substance P analogues occurs via the activation of neuropeptide receptors, or by some other mechanism. To address this question, the extent of MAPK activation by antagonist D was analysed in the bombesin receptor expressing, BOR15 cell line, and compared to MAPK activation in the parent Rat-1 fibroblasts.

Firstly, the expression of bombesin receptors and other neuropeptide receptors were analysed in these two cell lines. By detecting the mobilisation of intracellular calcium in response to bombesin and other neuropeptides, the neuropeptide receptor status of these cell lines could be determined. This served the purpose of confirming the presence of the bombesin receptor in the BOR15 cells and also determining which other neuropeptide receptors may be present on the cell lines (Fig 6.2). Using

high concentrations of a range of neuropeptides (100nM) it was found that the Rat1 cells had no large responses to any neuropeptide tested. There was a small but reproducible response to 100nM bradykinin in these cells. The BOR15 cells responded to stimulation with 100nM bombesin and showed an increase in intracellular calcium concentration of 400nM. As in the BOR15 cells, the Rat-1 cells responded to no other neuropeptide tested, other than with a small response to bradykinin.

This lack of neuropeptide receptors in the Rat-1 cells was confirmed by testing the ability of a range of neuropeptides (100nM) to induce the phosphorylation of ERK1 and ERK2 (Fig 6.4). In accordance with the above data, only bradykinin and FCS stimulated any significant increase in MAPK phosphorylation. These data show that, apart from low levels of bradykinin receptor, the Rat-1 fibroblast cell line does not normally express any of the tested neuropeptide receptors and that transfection of the bombesin receptor into these cells creates a cell line that is able to respond to stimulation with bombesin.

Secondly, the extent of MAPK activation by antagonist D and bombesin was compared between the Rat-1 fibroblasts and BOR15 cells. This work was carried out using a kinase assay which measures the ability of immuno-precipitated p42ERK2, to phosphorylate myelin basic protein in the presence of radio-labelled [^{33}P]ATP. This method of measuring MAPK activation is quantitative and allowed direct comparisons of MAPK activity between the two cell lines. The cells were treated with increasing concentrations of either antagonist D or bombesin and the activation state of ERK2 was determined (Fig 6.5). It was found that bombesin stimulated a dose dependant increase in MAPK activity in the bombesin receptor expressing

BOR15 cells (EC_{50} 0.59 +/- 0.18nM) but not in the parent Rat-1 cell line. Antagonist D stimulated a dose dependant increase in MAPK activity in both cell lines however, this activity was much greater in the bombesin receptor expressing BOR15 cells (EC_{50} 4.19 +/- 0.6μM) than in the Rat-1 cells (EC_{50} 22.5 +/- 2.6μM). This data suggested that the presence of the bombesin receptor potentiates MAPK activation by antagonist D.

To confirm this observation, MAPK activity and phosphorylation were measured, in the presence or absence of the specific bombesin antagonist RC3940-II (Hca^{6},$Leu^{13}\psi[CH_2N]Tac^{14}$-BN(6-11)) (Koppan et al., 1998). It was found that 1μM RC3940-II inhibited MAPK activity and phosphorylation by both antagonist D and bombesin (Fig 6.6). This data confirms that MAPK activation by antagonist D is at least partly via the bombesin receptor in BOR15 cells.

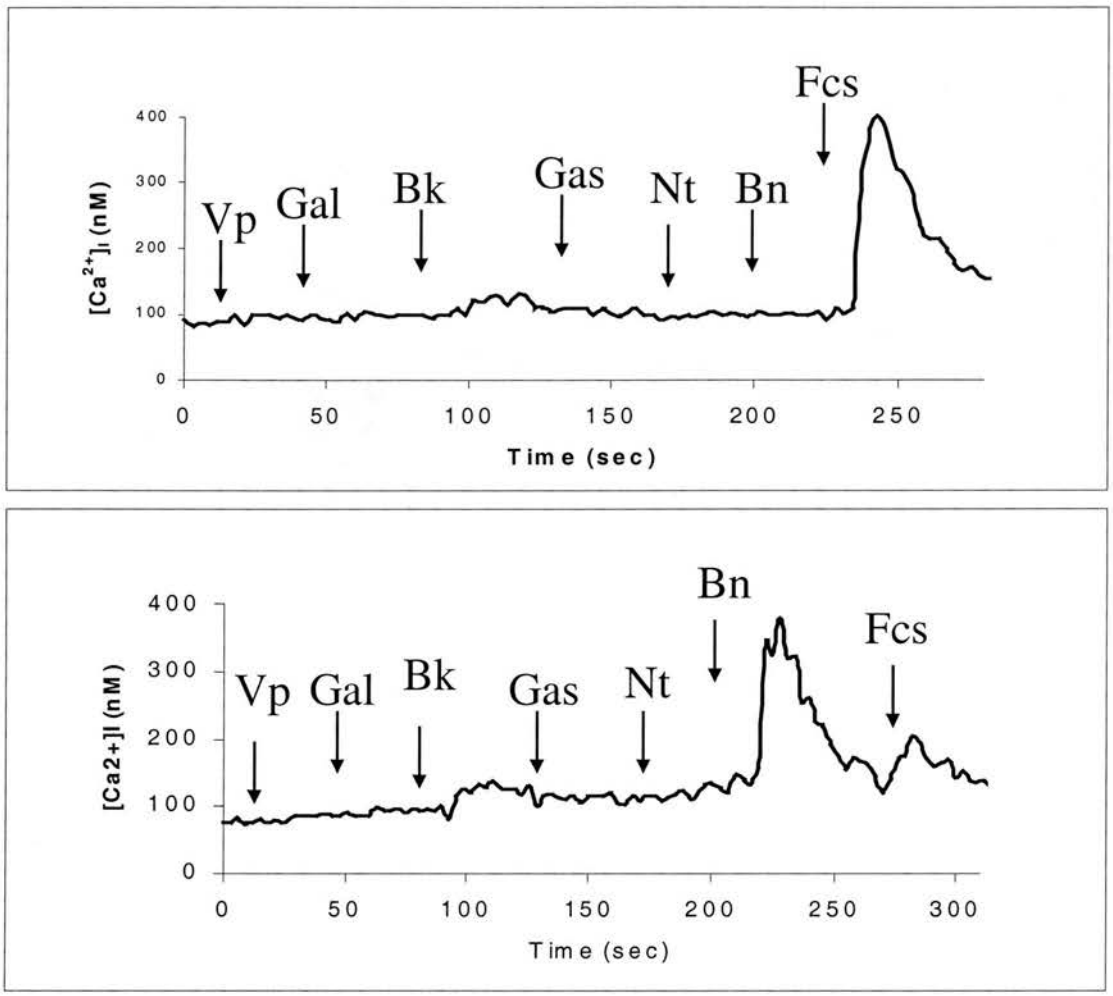


Fig 6.3. Calcium mobilisation in Rat-1 and BOR15 cells in response to stimulation with neuropeptides. Calcium mobilisation was detected as described in materials and methods. Confluent, quiesced cultures of Rat-1 or BOR15 cells were loaded with the calcium indicator FURA2-AM then exposed to the indicated neuropeptides. Neuropeptides were used at 100nM. 1% FCS was used as a positive control. Results are representative of three independent experiments.

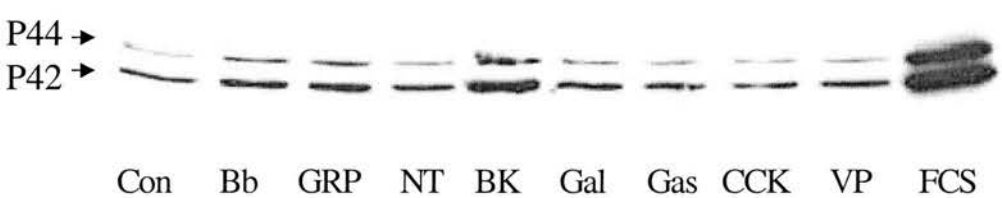


Fig 6.4. Phosphorylation of ERK1 and ERK2 in RAT1 cells in response to stimulation with neuropeptides. Confluent, quiescent cultures of BOR15 cells were incubated with the indicated treatments (100nM neuropeptides or 1%FCS) for 5 minutes. Samples were prepared as described in materials and methods and proteins were separated using SDS-PAGE. Western blots were probed with anti-phospho ERK1 and ERK2 (p44,p42) primary antibody and then an anti-mouse-HRP secondary antibody. Proteins were visualised using ECL detection. Blot is representative of three independent experiments.

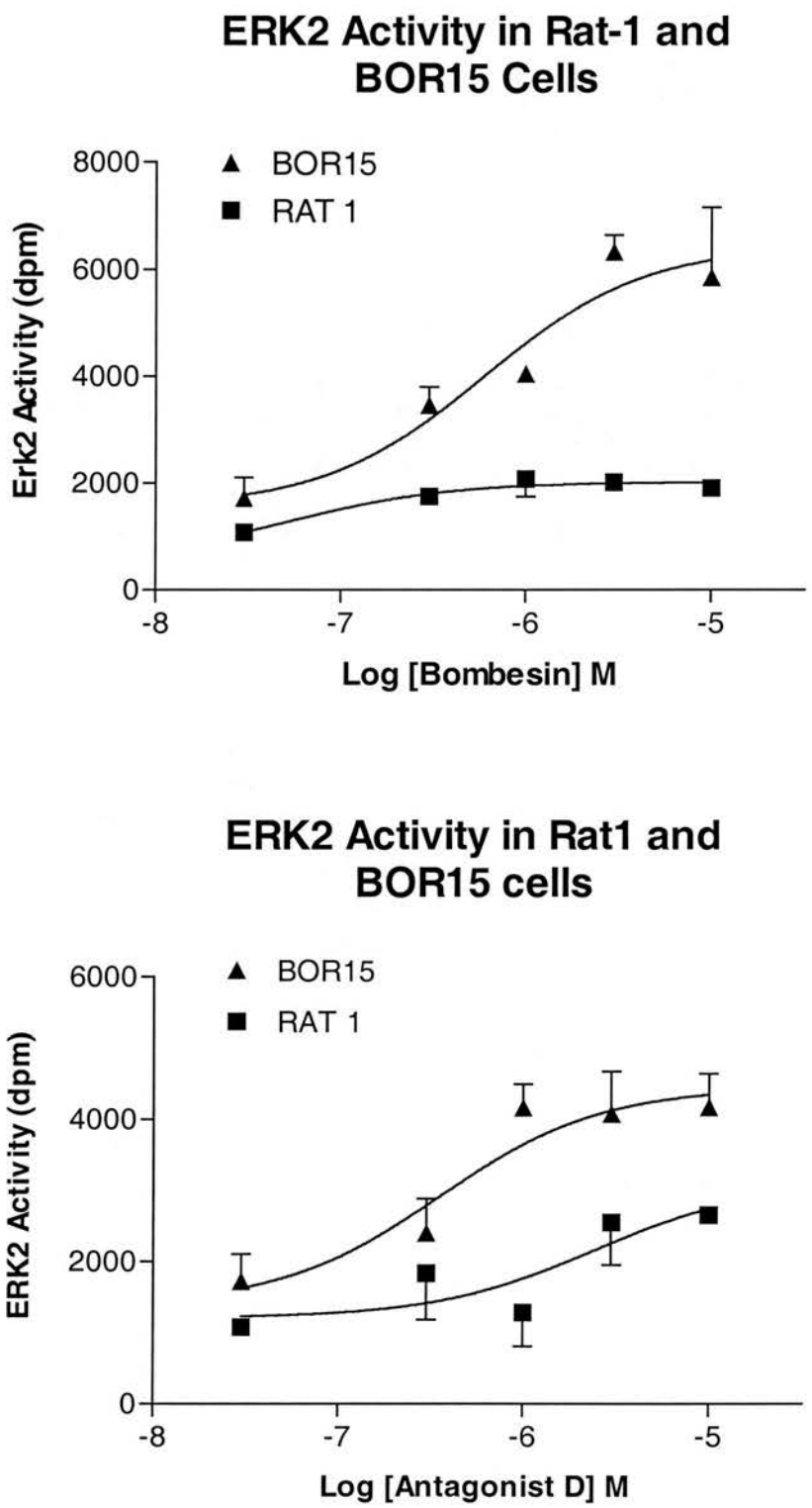


Fig 6.5. ERK2 activity stimulated by bombesin or antagonist D in Rat-1 fibroblasts and BOR15 cells. MAPK activity was assessed as described in materials and methods. Bombesin stimulates a dose dependant increase in MAPK activity in the BOR15 cells but not in Rat-1 cells (top). Antagonist D stimulates a dose dependant increase in MAPK activity in both cell lines but this increase is potentiated in the BOR15 cells (bottom). Data represents the means of 3-4 independent experiments, each performed in duplicate.

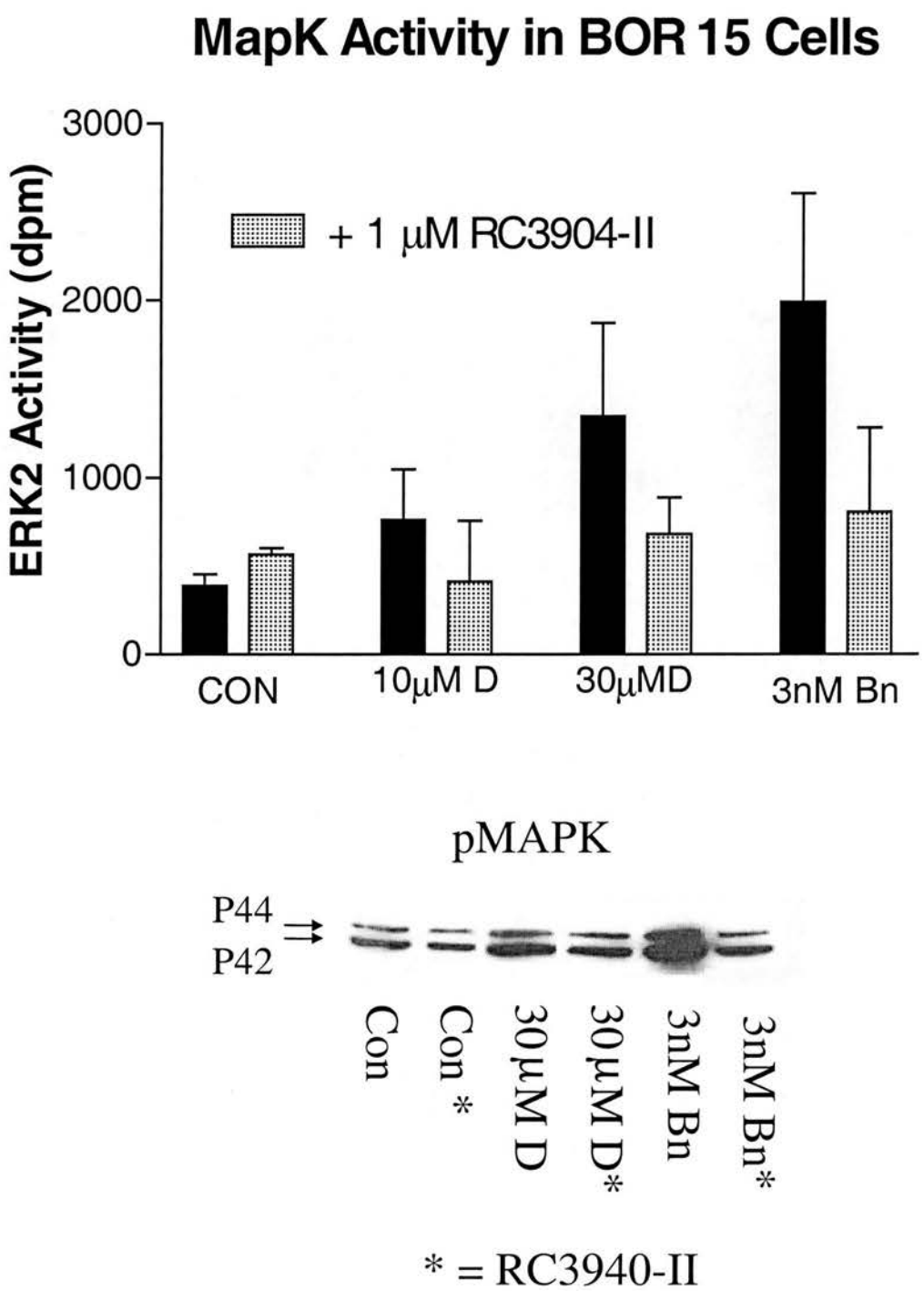


Fig 6.6. ERK2 Activity in BOR15 cell is attenuated by the bombesin receptor antagonist RC3940-II. MAPK activity was determined as described in materials and methods. Confluent, quiescent cultures of BOR15 cells were incubated for 5 minutes with the indicated concentrations of antagonist D or bombesin, with or without 1μM RC3940-II. The increase in ERK2 activity seen with antagonist D and bombesin was markedly attenuated in the presence of RC3904-II. (top). Data represents the means of 3 independent experiments.
Representative western blot probed with anti-phospho ERK1 /ERK2 antibody, showing inhibition of antagonist D and bombesin stimulated MAPK phosphorylation in the presence of RC3940-II.

6.2.1 MAPK activation by antagonist D is inhibited in balb3T3 fibroblasts expressing mutant GRP receptors.

In order to confirm that the MAPK activation by antagonist D is via bombesin / GRP receptors, MAPK activation by antagonist D was measured in two, balb3T3 fibroblast derived, cell lines. The 5ET4 cell line was provided stably transfected with the wild type mouse GRP receptor. The R288H cell line was provided stably transfected with a mutated mouse GRP receptor. The mutations in the cDNA encoding the R288H GRP receptor were created by site-directed mutagenisis. Amino acid residues in the wild type GRP receptor were swapped for corresponding residues from the bombesin receptor subtype-3 (BRS-3) which has low affinity for bombesin. The resulting receptor has a 1000 fold reduction in bombesin affinity and a 100 fold reduction in bombesin stimulated inositol phosphate hydrolysis (Akeson et al., 1997). The neuropeptide receptor status of the parent balb3T3 cell line was determined by measuring the phosphorylation of ERK1 and ERK2 in response to high concentrations of various neuropeptides (100nM). It was found that there was no large responses to any neuropeptide tested but 100nM bombesin, GRP and galanin produced small increases in MAPK phosphorylation (Fig 6.7). It may well be the case that using such high concentrations of agonist produced non-specific effects.

ERK2 activity was compared in the 5ET4 GRPR expressing cell line and the R288H, mutant GRPR expressing cell line. It was found that in the 5ET4 cells, antagonist D produced a dose dependant increase in ERK2 activity whereas in the R288H cells antagonist D was unable to produce a response (Fig 6.8). Antagonist D induced phosphorylation of ERK1 and ERK2 was also measured in these two cell lines (Fig

6.9). Again in the 5ET4 cells, antagonist D produced a dose dependant increase in ERK1 and ERK2 phosphorylation whereas in the R288H cell line there was no increased phosphorylation except with 60μM antagonist D. There is a slight inconsistency between the results of the kinase (ERK2 activation) assay and the phospho ERK1 and ERK2 detection. At 60μM, antagonist D did not cause any ERK2 activation in the R288H cells, as measured by the kinase assay, but did produce an increase in ERK1 and ERK2 phosphorylation as measured by western blotting. In any case, both experiments show that there is no dose dependant increase in MAPK activation by antagonist D in cells expressing mutant GRPR but there is a dose dependant increase in MAPK activation in cells expressing wild type GRPR.

These experiments confirm findings in the previous section and lend support to the hypothesis that antagonist D induces MAPK activation via the gastrin releasing peptide receptor.

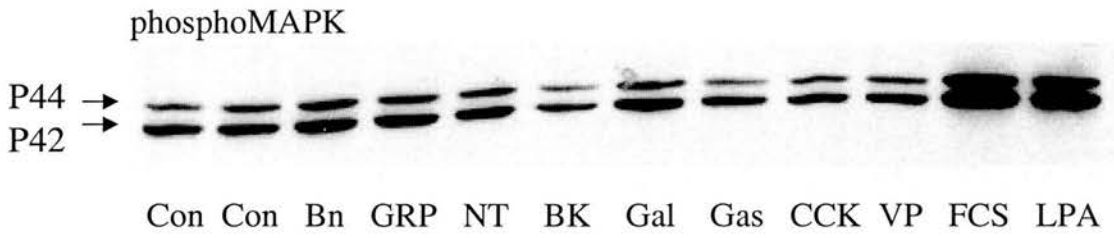


Fig 6.7 MAPK phosphorylation in balb3T3 cells. phosphorylated ERK1 (p44) and ERK2 (p42) were detected as described in materials and methods. Confluent, quiescent cultures of balb3T3 cells were exposed to either 100nM of the indicated neuropeptides, 1%FCS or 10μM LPA for 5 min at 37^{0}C. The balb3T3 cells were slightly responsive to 100nM bombesin, GRP and galanin. FCS and LPA were included as positive controls and both stimulated the phosphorylation of ERK1 and ERK2. Blot is representative of three independent experiments.

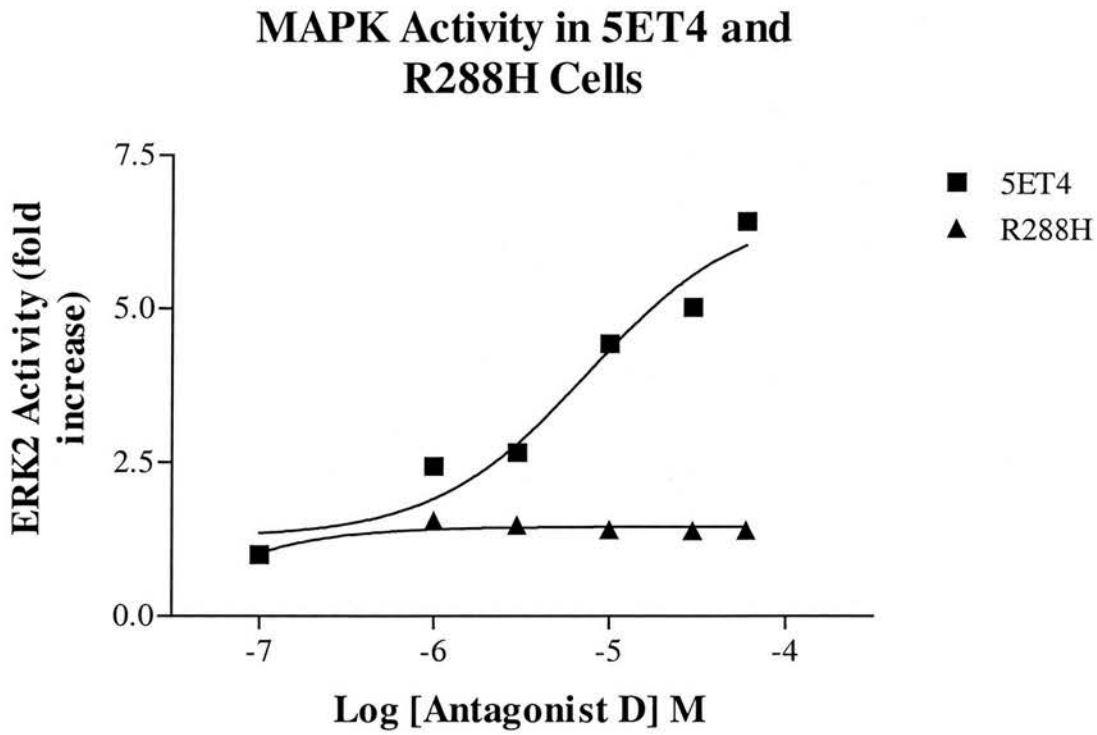


Fig 6.8 ERK2 activity in 5ET4 cells and R288H cells. ERK2 activity was measured as described in materials and methods. Confluent, quiescent cultures of 5ET4 cells (balb3T3 cells expressing wild type GRPR) or R288H cells (balb3T3 cells expressing mutant GRPR) were exposed to increasing concentrations of antagonist D (1-60μM) for 5 min at 37^{0}C. Antagonist D stimulated a dose dependant increase in ERK1 and ERK2 phosphorylation in the 5ET4 cells. The R288H cells were unable to respond to antagonist D. Graph is representative of three independent experiments.

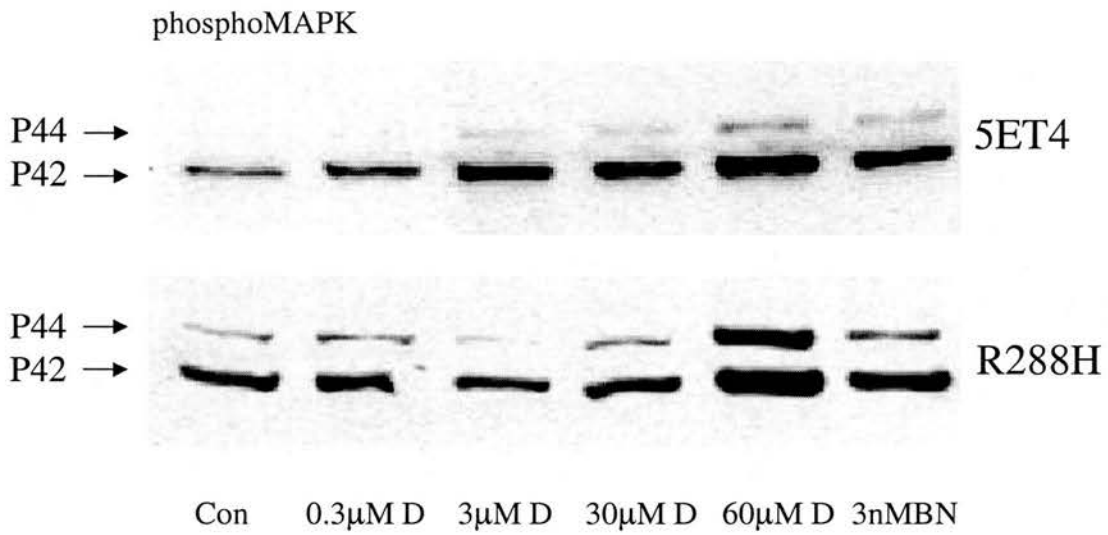


Fig 6.9 MAPK phosphorylation in 5ET4 and R288H cells. Phosphorylated ERK1 (p44) and ERK2 (p42) were detected as described in materials and methods. Confluent, quiescent cultures of 5ET4 cells (balb3T3 cells expressing wild type GRPR) or R288H cells (balb3T3 cells expressing mutant GRPR) were exposed to increasing concentrations of antagonist D (0.3-60μM) or 3nM bombesin for 5 min at 37^0C. The 5ET4 cells were responsive to bombesin and antagonist D stimulated a dose dependant increase in ERK1 and ERK2 phosphorylation. The R288H cells were unable to respond to bombesin and antagonist D did not cause phosphorylation of ERK1 and ERK2 except at 60μM. Blots are representative of three independent experiments.

6.3 Antagonist D activates MAPK in the absence of calcium mobilisation and can inhibit bombesin stimulated calcium mobilisation in BOR15 cells.

It is apparent that both bombesin and antagonist D activate MAPK via the bombesin receptor. It is accepted that bombesin can activate MAPK via the mobilisation of intracellular calcium and the activation of PKC (Zugaza et al., 1997; Herget and Rozengurt, 1994) but it was unclear whether antagonist D utilised the same mechanism. The ability of both bombesin and antagonist D, to stimulate the mobilisation of intracellular calcium, was assessed in the BOR15 cells using the previously described method. Bombesin (10nM) stimulated an increase of intracellular calcium concentration of ~400nM whereas high concentrations (30μM) of antagonist D failed to elicit a response. (Fig 6.10).

The inhibition of neuropeptide stimulated calcium mobilisation by substance P analogues, has been well characterised in both SCLC cells and in swiss3T3 fibroblasts (see introduction for review). In order to confirm that antagonist D was capable of inhibiting calcium mobilisation in BOR15 cells, dose response curves for calcium mobilisation by bombesin in the presence or absence of antagonist D were carried out (Fig 6.11). Bombesin stimulated a dose dependant increase in calcium mobilisation (EC50 1.5nM) which was inhibited by 30μM antagonist D (EC50 10.4nM). These results show that antagonist D activates MAPK via a non-calcium dependant pathway and can inhibit calcium mobilisation stimulated by bombesin.

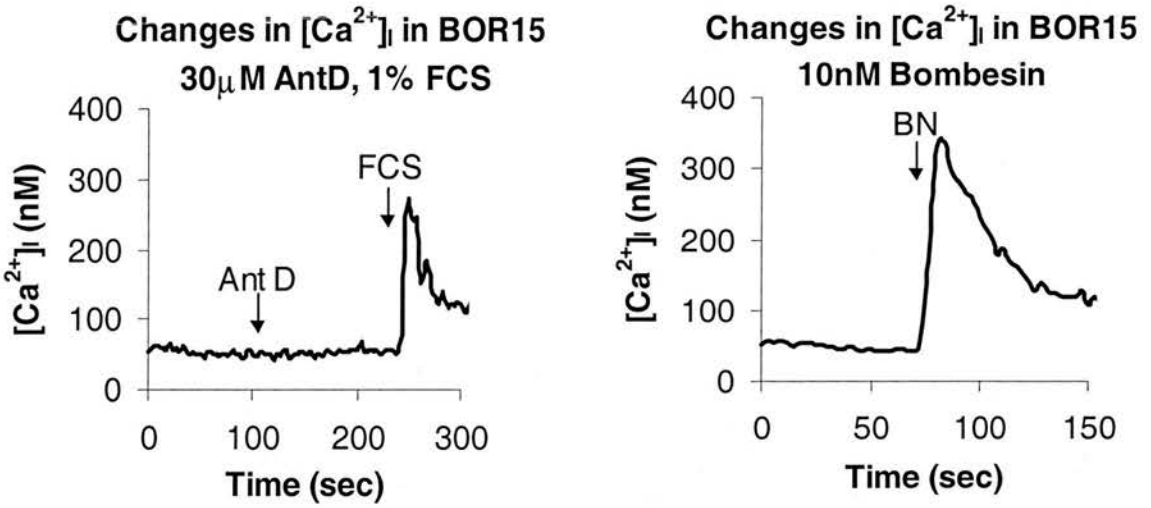


Fig 6.10 Bombesin but not antagonist D stimulates mobilisation of intracellular calcium in BOR15 cells.

Measurement of intracellular calcium was carried out as described in materials and methods. 30μM antagonist D does not stimulate mobilisation of calcium. Bombesin stimulated an increase of ~400nM. Data is representative of three independent experiments.

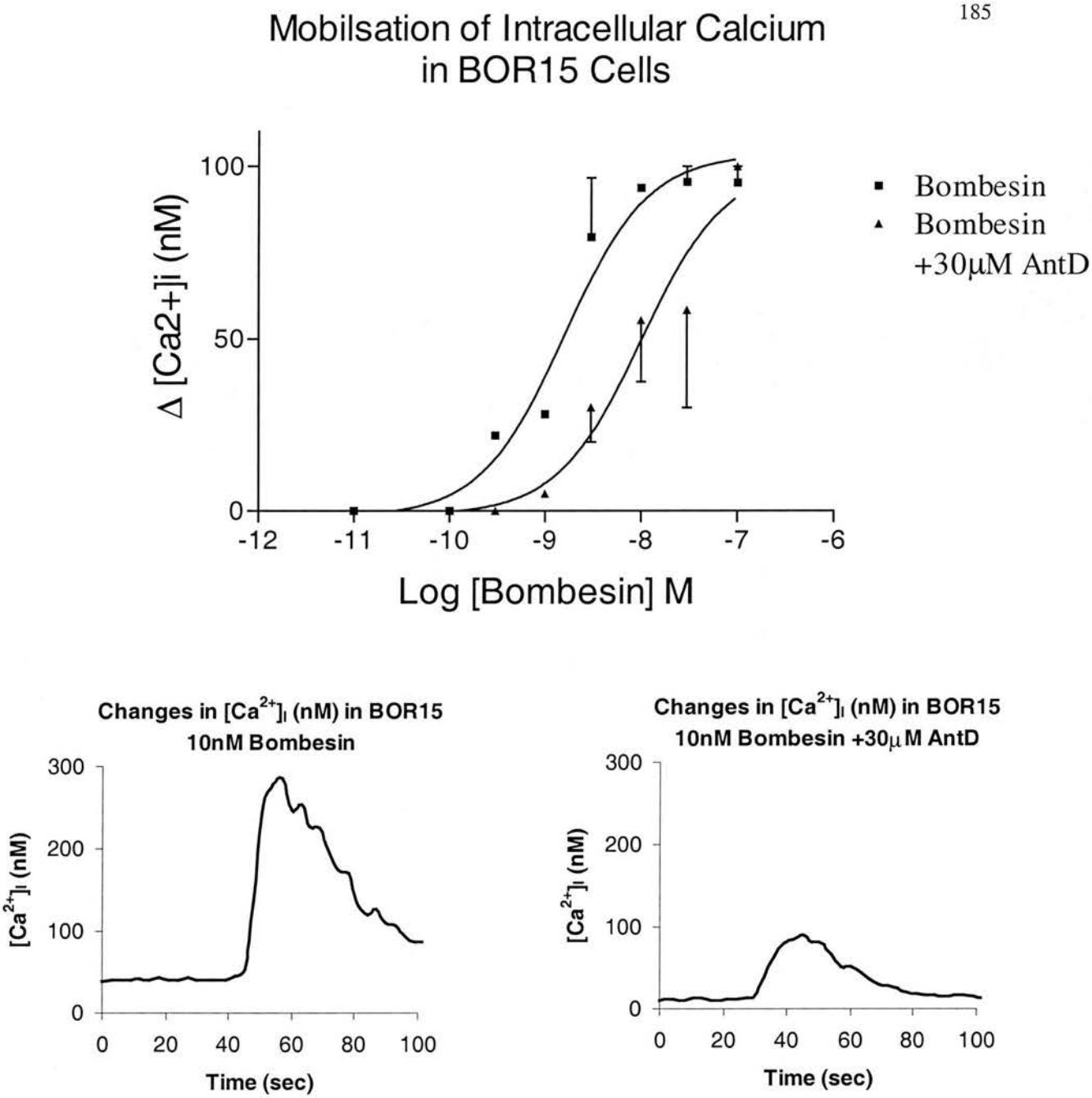


Fig 6.11 Antagonist D inhibits bombesin induced calcium mobilisation in BOR15 cells. Calcium mobilisation was carried out as described in materials and methods. Cell suspensions were treated with increasing concentrations of bombesin in the presence or absence of 30μM antagonist D. Data represents the mean of three independent experiments.
Below are representative calcium traces showing real time calcium mobilisation in response to 10nM bombesin (left) and 10nM bombesin in the presence of 30μM antagonist D (right).

6.4 Antagonist D does not activate MAPK via a PKC dependant pathway.

It has previously been established that, in some cell lines bombesin activates MAPK via a calcium mediated, PKC dependant pathway (Zugaza et al., 1997; Herget and Rozengurt, 1994). The above data show that the activation of MAPK by both bombesin and antagonist D is via signalling from the GRPR but that MAPK activation by antagonist D is independent of intracellular calcium mobilisation. To investigate the involvement of PKC in antagonist D and bombesin induced MAPK activation in BOR15 cells, the PKC antagonist GF109203-X was used. This antagonist is a bisindolyalemide which acts as a competitive inhibitor of ATP binding and is specific against PKC (Toullec et al., 1991). GF109203-X treatment alone, increased basal MAPK phosphorylation. This may have masked some of the potential inhibitory effects against antagonist D or bombesin stimulated MAPK phosphorylation. It appeared that GF109203-X only partially inhibited the phosphorylation of ERK1 and ERK2 by bombesin but had no effect upon the phosphorylation induced by antagonist D (Fig 6.12). These results suggest that while bombesin stimulated MAPK activity may be partially dependent upon PKC activation in BOR15 cells, antagonist D stimulated MAPK activity does not involve PKC.

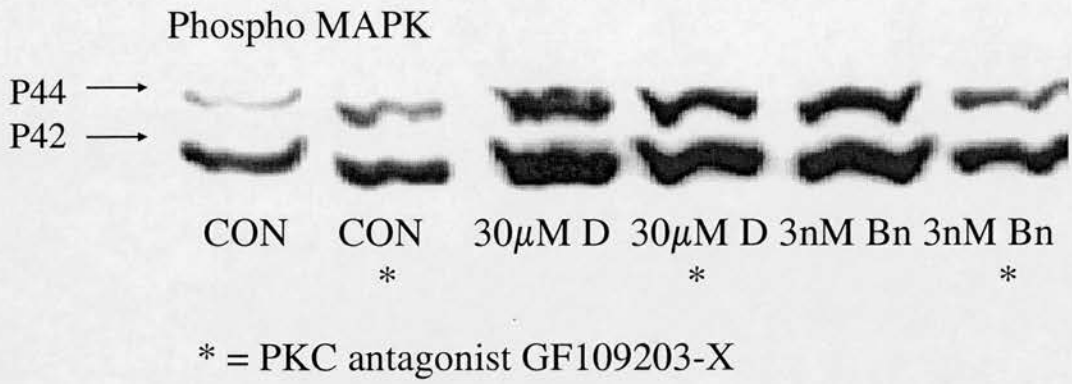


Fig 6.12 Antagonist D activates MAPK via a PKC independent pathway. SDS PAGE and western blotting was carried out as described in materials and methods. Confluent, quiescent cultures of BOR15 cells were incubated for 5 min at 37^{0}C, with either 30μM antagonist D or 3nM bombesin in the presence (*) or absence of 5μM GF109203-X. Blot is probed with anti-phospho ERK1 and ERK2 primary antibody and an anti-mouse HRP secondary antibody. Blot is representative of three independent experiments.

6.5 Bombesin and antagonist D both activate MAPK via the bombesin receptor but via differential activation of G-proteins.

Bombesin stimulation of phospholipase C (PLC) which leads to calcium mobilisation, PKC activation and MAPK activation has been previously shown to be via the Gq family of G-proteins (Offermans et al., 1994; Wang et al., 1996; Piiper et al., 1997). The above data shows that antagonist D, unlike bombesin, activates MAPK via a mechanism that is independent of intracellular calcium mobilisation. Antagonist D induced MAPK activation is also independent of PKC whereas bombesin stimulated MAPK activation may be partially dependent upon PKC activation in BOR15 cells. It was likely therefore, that antagonist D stimulates a pathway downstream of the bombesin receptor that does not involve the Gq family of G-proteins. Several reports have shown that MAPK activation by G-protein coupled

receptors can be via Gi family of G-proteins and this stimulation of MAPK does not involve either calcium flux or PKC activation (Faure et al., 1994; Mochizuki et al., 1999). It has also been shown that the bombesin receptor is capable of coupling to Gi G-proteins, in addition to Gq G-proteins (Profrock et al., 1992; Letterio et al., 1986). Hence, it was decided to determine whether MAPK activation by antagonist D was sensitive to treatment with pertussis toxin, a bacterial toxin which inhibits the activation of Gi/0 G-proteins by ADP ribosylation of the α subunits. Using ERK2 activity and ERK1 and ERK2 phosphorylation, as indicators of GRPR activation by bombesin and antagonist D, it was attempted to block receptor signalling by stimulating BOR15 cells in the presence or absence of 100ng/ml of pertussis toxin (activated by incubation with an equal volume of 100mM DDT at room temperature for 1hr). It was found that antagonist D stimulated MAPK phosphorylation and activity were attenuated in the presence of pertussis toxin whereas the effects of bombesin were not significantly affected (Figs 6.13 and 6.14).

This work shows that antagonist D-induced MAPK activation occurs partially via G-proteins of the Gi/0 subtype.

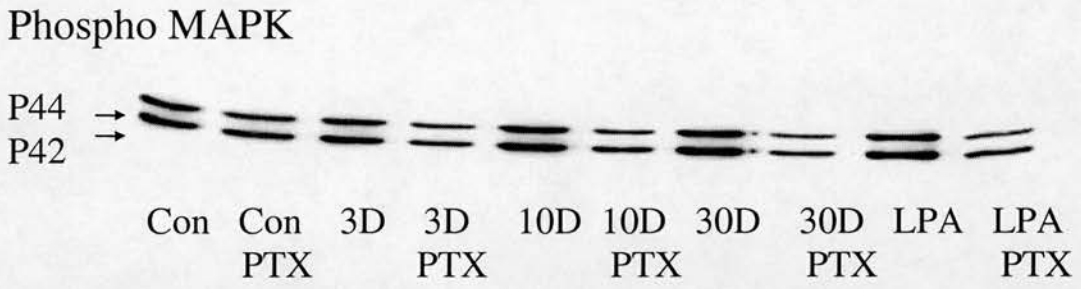


Fig 6.13 ERK1 and ERK2 phosphorylation by antagonist D is inhibited by pertussis toxin. SDS PAGE and western blotting was carried out as described in materials and methods. Confluent, quiescent cultures of BOR15 cells were pre-incubated for 18hrs with 100ng/ml activated pertussis toxin (PTX) then PTX treated or untreated cells were incubated for 5 min at 37^{0}C, with either indicated concentrations of antagonist D or 10μM LPA. Blot is probed with anti-phospho ERK1 and ERK2 primary antibody and an anti-mouse HRP secondary antibody. Blot is representative of three independent experiments.

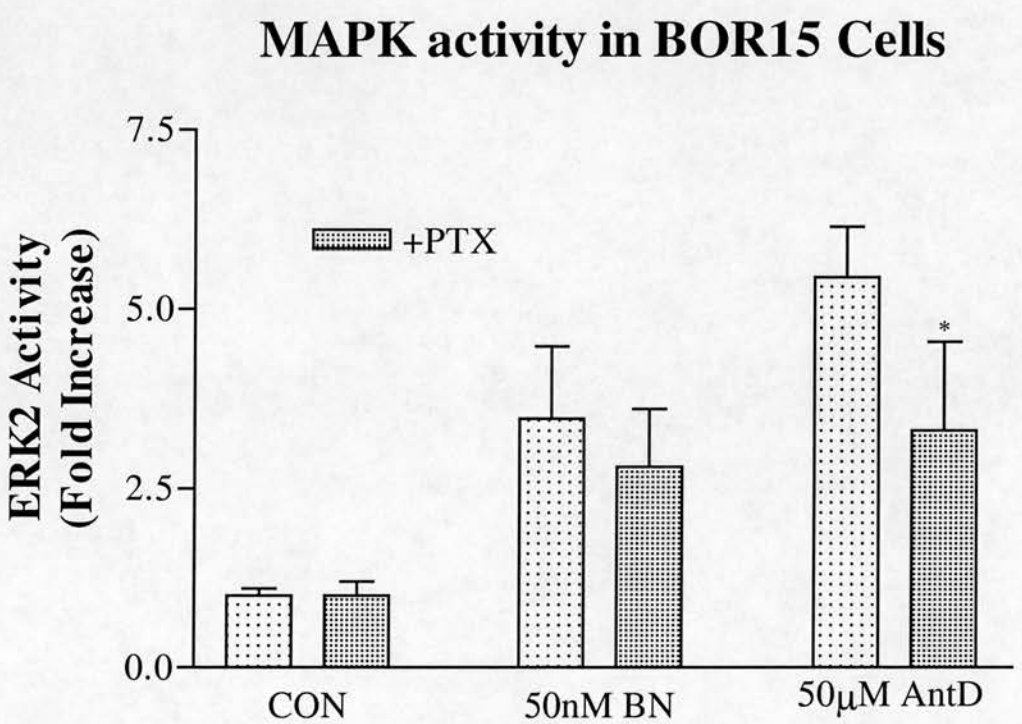


Fig 6.14 ERK2 activation by antagonist D is inhibited by pertussis toxin. ERK2 activation was measured as described in materials and methods. Confluent quiescent cultures of BOR15 cells were pre-treated for 18hrs with 100ng/ml activated PTX. PTX treated and untreated cells were incubated for 5 minutes at 37°C with either 50nM bombesin or 50μM antagonist D. Data is the mean of three independent experiments performed in duplicate. (* AntD + PTX treated group is significantly different from Ant D treated group, $P<0.05$)

6.6 MAPK activation by bombesin and antagonist D involves differential activation of tyrosine kinases.

In addition to PKC/calcium mediated MAPK activation, the MAPK cascade can also be activated via pathways involving various tyrosine kinases. MAPK activation by both pertussis toxin sensitive and pertussis toxin insensitive G proteins has been shown to utilise the tyrosine kinase Src. This Src activation is thought to be mediated by G protein β/γ subunits and does not involve the activation of PKC or the mobilisation of intracellular calcium (Igishi and Gutkind, 1998; Della Rocca et al., 1999b). The activation of Src family tyrosine kinases can be downstream of several pathways but seems to follow the recruitment of Ras-GTP exchange factors to the membrane in most cases. The recruitment of Ras is dependent upon the formation of a Ras activation complex, which consists of tyrosine phosphorylated adapter proteins such as Shc and Gab1 and involves the recruitment of Grb-2 and Sos. It has been proposed that GPCR mediated activation of the MAPK cascade by Ras is catalysed by either transactivation of tyrosine kinase receptors such as the EGF receptor to provide a scaffold for Src interaction with the Ras activation complex, or by the activation of focal adhesion components such as FAK which then mediate Src interaction with the Ras activation complex. The preferred scaffold seems to be determined by cellular context in which the receptor in question is expressed (Della Rocca et al., 1999b).

With this in mind it was decided to investigate the involvement of Src kinases, Ras and the tyrosine kinase EGF receptor in MAPK activation by antagonist D and bombesin in the BOR15 cells. Using the prazolopyramidine Src kinase inhibitor PP2, it was attempted to inhibit the activation of MAPK by both antagonist D and

bombesin. Figure 6.15 shows that bombesin stimulated MAPK phosphorylation is blocked by pre-treatment with 5μM PP2. Antagonist D stimulated MAPK phosphorylation was also inhibited by PP2, indicating that both compounds stimulate MAPK activity using pathways involving Src family tyrosine kinases.

Src activation can be involved in MAPK activation via both receptor tyrosine kinase scaffolds and focal adhesion scaffolds. As both bombesin and antagonist D activation of MAPK appeared to be dependant upon the activation of Src kinases it was of interest to determine whether both compounds used Src kinases in the same signalling context. It has been shown that Bombesin can stimulate the activation of FAK but it has not been demonstrated that MAPK activation stimulated by bombesin is mediated via this focal adhesion event (Seufferlein et al., 1996b). Thus, MAPK activation by bombesin may be regulated by another tyrosine kinase pathway. The role of receptor tyrosine kinases were a likely starting point for investigation. BOR15 cells were treated with the EGF receptor specific tyrosine kinase inhibitor AG1478 prior to incubation with either antagonist D or bombesin. It was found that MAPK activation by bombesin but not antagonist D was inhibited by AG1478 (Fig 6.16). This suggests that the MAPK activation by bombesin involves transactivation of the EGFR whereas MAPK activation by antagonist D employs some other mechanism involving Src kinase activation.

The activation of Ras follows the activation of Src kinase by GPCRs. It has previously been shown that stimulation of BOR15 cells with bombesin results in the activation of Ras and activates MAPK via Ras mediated Raf activation (Charlesworth and Rozengurt, 1997). In order to determine whether antagonist D utilised Ras in order to activate MAPK, ERK2 activity was measured in the presence

or absence of the ras antagonist, S-farnesylthiosiacyclic acid (FTS), which prevents Ras membrane association and the subsequent activation of Raf-1 and MAPK (Gana-Weisz et al., 1997). As expected, it was found that 50μM FTS inhibited ERK2 activation by both bombesin and antagonist D (Fig 6.17) indicating that both activate the MEK/MAPK cascade via Ras in BOR15 cells.

Further studies to confirm these findings were carried out using a GST fusion protein containing the ras binding domain of c-raf in order to pull out activated Ras from cell lysates (see materials and methods). Unfortunately, these experiments were inconclusive due to problems which were encountered with anti-pan-Ras and anti-H-Ras antibodies (data not shown).

These results suggest that although both bombesin and antagonist D stimulate MAPK activation via the GRP/bombesin receptor, both compounds differentially utilise tyrosine kinase scaffolds in order to activate Src and Ras, upstream of MAPK activation. These findings support previous data which show that antagonist D and Bombesin activate MAPK via different G-proteins thus activating different downstream signalling pathways.

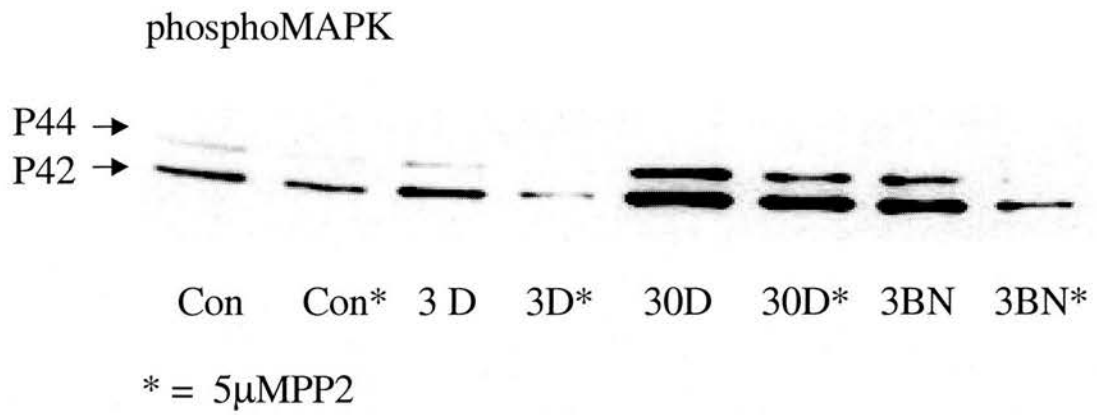


Fig 6.15 ERK1 and ERK2 phosphorylation by antagonist D and bombesin involves Src family kinases. SDS PAGE and western blotting was carried out as described in materials and methods. Confluent, quiescent cultures of BOR15 cells were pre-incubated for 20min with the Src inhibitor PP2. PP2 treated or untreated cells were incubated for 5 min at 37^{0}C, with either 3 or 30μM antagonist D or 3nM bombesin. Blot is probed with anti-phospho ERK1 and ERK2 primary antibody and an anti-mouse HRP secondary antibody. Blot is representative of three independent experiments.

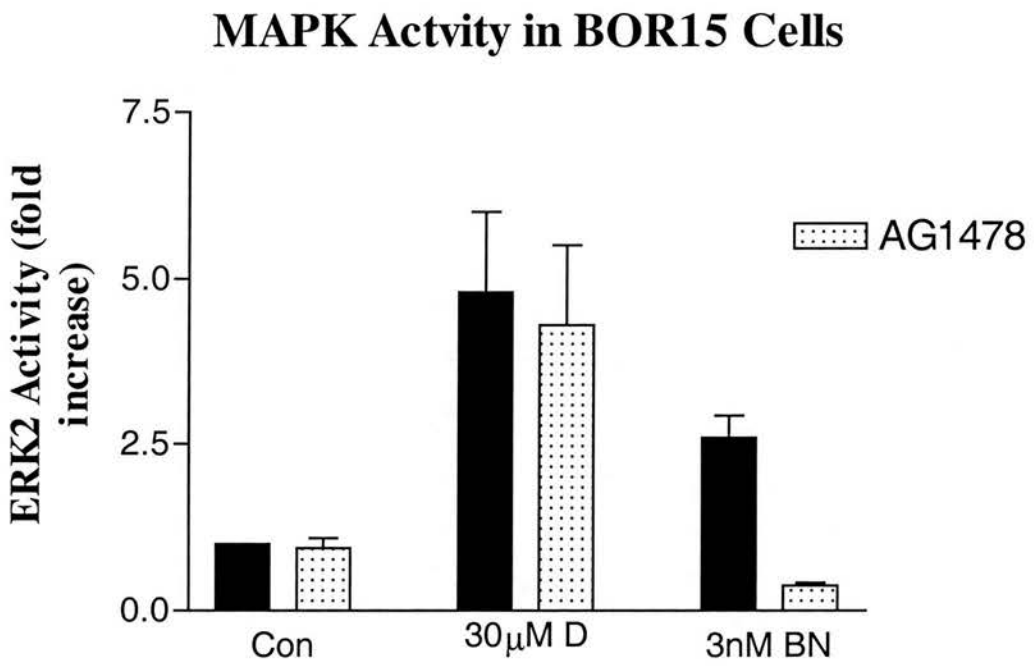


Fig 6.16 ERK2 activity in BOR 15 cells. ERK2 activity was measured as described in materials and methods. Untreated or AG1478 (5μM) treated confluent quiesced cultures of BOR15 cells were treated with 30μM antagonist D or 3nM bombesin. Results are representative of three independent experiments performed in duplicate.

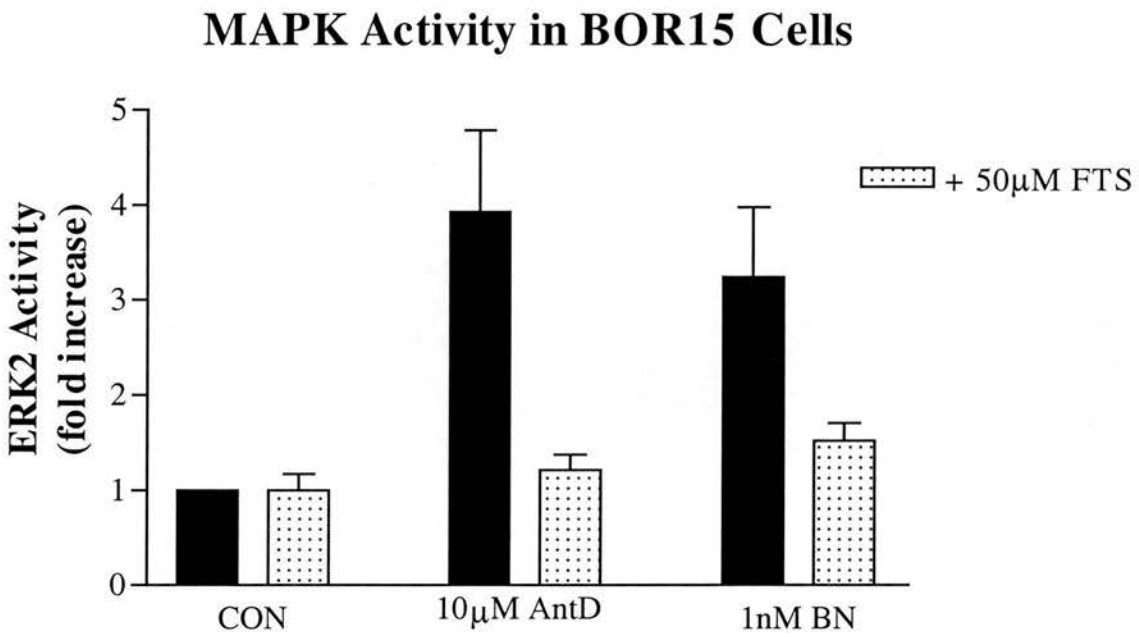


Fig 6.17 ERK2 activity in BOR 15 cells. ERK2 activity was measured as described in materials and methods. Untreated or FTS treated (for 18hrs) confluent quiesced cultures of BOR15 cells were treated with 10μM antagonist D or 1nM bombesin. Results are representative of three independent experiments performed in triplicate.

6.7 Antagonist D does not alter the expression of cell cycle inhibitors $P27^{KIP}$ and $P21^{WAF}$ via sustained activation of MAPK.

So far it has been established that antagonist D is a potent stimulator of MAPK. This MAPK activation is via stimulation of Gi dependant pathways downstream of the bombesin receptor. It was of importance to investigate the relevance of this MAPK activation in terms of the mechanism of growth inhibition by antagonist D. Several publications have highlighted the fact that inappropriate activation of the Ras/MAPK pathway in SCLC cells can result in the upregulation of cell cycle inhibitors (Ravi et al., 1998; Ravi et al., 1999) and that sustained activation of the Ras/MAPK signalling cascade can have adverse effects on cell growth in several other cell types (Pumiglia and Decker, 1997). In light of these findings a time course of MAPK phosphorylation by both antagonist D and bombesin was carried out in order to highlight any differences in MAPK activation kinetics by these two compounds. Ravi et al show that in SCLC cells activation of MAPK by constituitively active Ras protein results in the upregulation of various cell cycle inhibitor protein levels including $P27^{KIP}$ and $P21^{WAF}$. Using BOR15 cells, stimulated with antagonist D over a 4hr time course, the levels of $P27^{KIP}$ and $P21^{WAF}$ were analysed by SDS PAGE and western blotting.

The time course of MAPK phosphorylation by antagonist D and bombesin were very similar over the four hour period (Fig 6.18). In addition, there were no apparent differences in the levels of the $p27^{KIP}$ cell cycle inhibitor (Fig 6.19) or the $p21^{WAF}$ cell cycle inhibitor (data not shown) during this time period. These results indicate that in BOR15 cells antagonist D does not influence cell growth via transcriptional regulation of $P27^{KIP}$ and $P21^{WAF}$ resulting from sustained activation of MAPK.

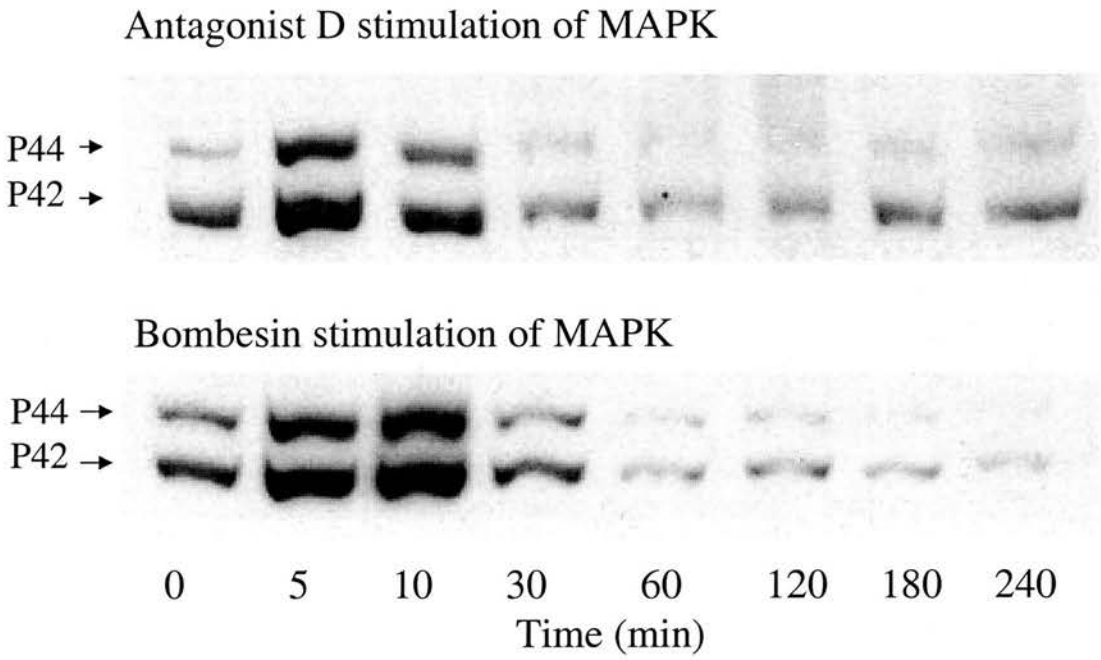


Fig 6.18 Timecourse of MAPK phosphorylation by Antagonist D and Bombesin. Confluent, quiesced cultures of BOR15 cells were exposed to either 30μM antagonist D (top) or 3nM bombesin (bottom). SDS PAGE and western blotting was carried out as described in materials and methods. Blot is probed with anti-phospho ERK1 and ERK2 primary antibody and an anti-mouse HRP secondary antibody. Blot is representative of three independent experiments.

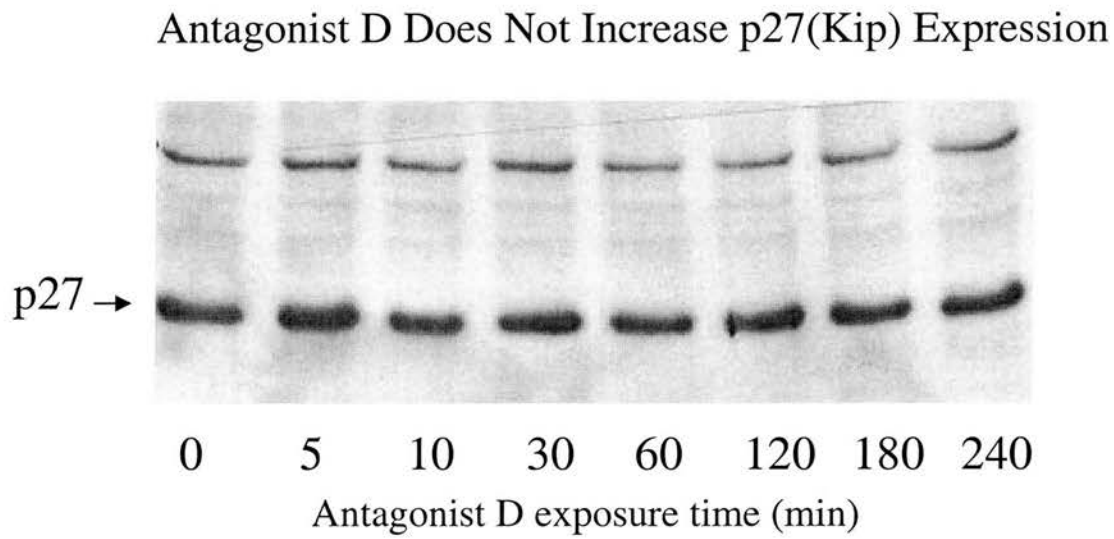


Fig 6.19 Antagonist D does not increase expression of $P27^{KIP}$ in BOR15 cells.
Confluent, quiesced cultures of BOR15 cells were exposed to 30μM antagonist D over a four hour time course. SDS PAGE and western blotting was carried out as described in materials and methods. Blot is probed for $P27^{(KIP)}$. Blot is representative of three independent experiments

6.8 MAPK activation by antagonist D is integral in its pro-apoptotic effects.

It has been shown that antagonist D can stimulate apoptosis in SCLC cells (see chapter 3). To investigate the involvement of MAPK activation by substance P analogues it was decided to look at the effects of blocking MAPK activation on the ability of these compounds to induce apoptosis. Using annexin V binding as an indicator of apoptosis, the pro-apoptotic effects of antagonist D in BOR15 cells was investigated in the presence or absence of the MEK inhibitor PD098059. It was found that antagonist D stimulated apoptosis in BOR15 cells and the apoptosis was partially inhibited by PD098059. This experiment shows that the activation of the MAPK cascade by antagonist D, is somehow related to its ability to induce apoptosis. Our group have also shown that PD098059 inhibits antagonist D and G induced apoptosis in the SCLC cell line, H69 (data not shown). As the time course of MAPK activation by antagonist D and bombesin are similar, it is most probably the cellular context of other signalling events that change the activation of MAPK from a proliferative signal into an inhibitory signal. The antagonist D induced, activation of JNK and the inhibition of calcium flux, are likely candidates that alter the normal cellular context of MAPK activity.

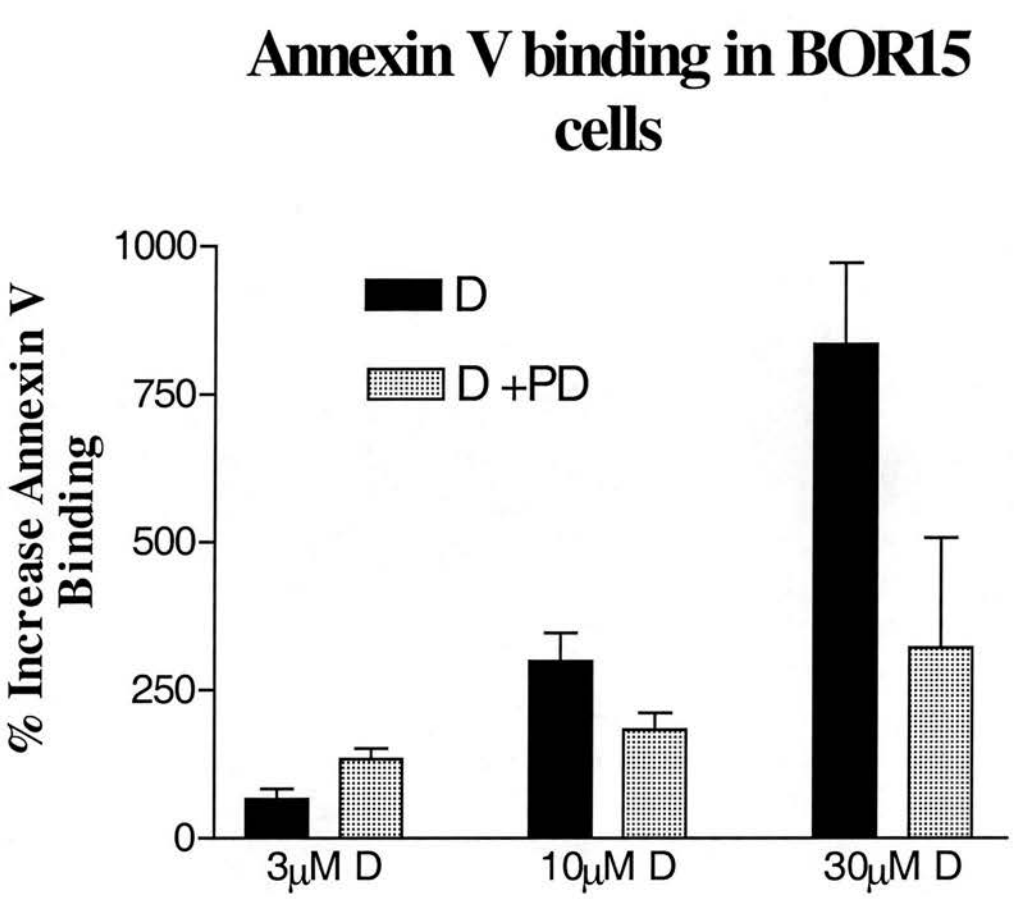


Fig 6.20 MEK inhibitor PD098059 inhibits antagonist D induced apoptosis. Annexin binding was carried out as described in materials and methods. Confluent, quiescent cultures of BOR15 cells were incubated for 30min with 10μM PD098059 then either PD098059 treated or untreated cells were incubated for 24hrs with the indicated concentrations of antagonist D. Data represents the mean of two independent experiments performed in triplicate.

6.9 Discussion of Results.

The data presented in this chapter show that antagonist D and G both activate MAPK in SCLC and Rat-1 fibroblasts. The activation of MAPK in Rat-1 fibroblasts is potentiated by the presence of the bombesin/GRP receptor indicating that antagonist D can activate these receptors. Some antagonist D induced MAPK activation occurred in the parent Rat-1 cell line. This is likely to be due to the broad-spectrum activity of antagonist D upon the bradykinin receptor which is present in this cell line. Rat-1 cells have been shown to express relatively high levels of endothelin receptors (Ambar and Sokolovsky, 1993; Cadwallader et al., 1997). Substance P analogues are able to inhibit binding of endothelin to its receptor and also block calcium mobilisation via endothelin receptors (Fabregat and Rozengurt, 1990) so it is possible that some of the MAPK activation by antagonist D is via activity at these receptors. The MAPK activation via the GRP receptor is sensitive to partial inhibition by the specific bombesin antagonist RC3940-II. Again, the lack of complete inhibition of MAPK activation may be due to some of the total MAPK activity being stimulated via other receptor types or via another mechanism. Further experiments in our group have shown that the MAPK activation by antagonist D is also sensitive to inhibition by another specific bombesin antagonist [$Leu^{13}\psi(CH_2NH)$-Leu^{14}]-Bombesin (data not shown). Conformation of the hypothesis that antagonist D activates MAPK via the GRPR, is provided by the fact that MAPK activation by antagonist D occurs in balb3T3 fibroblasts stably transfected with the human GRP receptor and this activation is markedly attenuated in balb3T3 cells transfected with the human GRP receptor with a mutated ligand

binding site. This cell line bearing the GRP receptor with a mutant ligand binding site is also unable to respond to bombesin.

As discussed previously, bombesin acts upon the bombesin/GRP receptor resulting in MAPK activation via the activation of Gq family of G-proteins, stimulating PLC mediated mobilisation of intracellular calcium and the activation of PKC. This work has shown that antagonist D activates the same receptor as bombesin but initiates a different signalling cascade resulting in MAPK activation, which does not involve the mobilisation of calcium or the activation of PKC. Antagonist D, unlike bombesin, stimulates the activation of the Gi/0 family of G-proteins as determined by the sensitivity of antagonist D induced MAPK activation to pertussis toxin treatment. It is likely that the G-protein involved is Gi and not G0 as the bombesin receptor has been shown to functionally couple to Gi (Profrock et al., 1992; Letterio et al., 1986). It is thought that this Gi stimulated MAPK activation is via Gi β/γ subunits and may involve the activation of p21 Ras (Koch et al., 1994).

In accordance with experimental data in this chapter, the activation of MAPK via Gi has been shown to be independent of PKC activation. Activation of MAPK via Gq has been shown in many cases to be PKC dependent (Hawes et al., 1995). This current work has shown that, in Rat-1 cells, MAPK activation by bombesin is, at most, partially dependent upon PKC as the phosphorylation of ERK1 and ERK2 was only slightly inhibited by the PKC inhibitor GF109203X. It has previously been shown that bombesin stimulation of MAPK in Rat-1 cells, is independent of PKC activation (Charlesworth and Rozengurt, 1997) whereas in swiss3T3 cells bombesin stimulated MAPK activation is PKC dependent (Seufferlein and Rozengurt, 1996a).

The investigation into the involvement of p21 Ras in the MAPK activation by antagonist D and bombesin showed that MAPK activity was inhibited in both cases, by the Ras antagonist FTS. It has previously been demonstrated that bombesin stimulates the GTP-loading of p21 Ras in BOR15 cells (Charlesworth and Rozengurt, 1997), however in swiss3T3 cells bombesin stimulation of MAPK is completely dependent upon PKC and independent of Ras activation (Seufferlein et al., 1996b; Mitchell et al., 1995). These findings stress the importance of cell context when defining the signal transduction pathways which activate MAPK and indicate that the involvement of PKC and Ras in signal transduction pathways is very much determined by cell type and not completely by the nature of the agonist.

The ras dependent activation of MAPK by GPCRs appear to involve a scaffold which provide docking sites whereby ras can interact with other proteins of the ras activation complex, mSOS and Grb2 (Luttrell et al., 1997; Della Rocca et al., 1999a). These sites have been shown to be provided by both integrin-based focal adhesion complexes and receptor tyrosine kinases, again, depending on cell type. It appears that Src family of non-receptor tyrosine kinases present a point of convergence for signals originating from either scaffold. The data presented in this chapter shows that MAPK activation by both bombesin and antagonist D is partially inhibited by the Src kinase inhibitor PP2.

It has been proposed that MAPK activation via Gq coupled receptors occurs by the activation of one or both, tyrosine kinase independent pathways (PKC mediated) and the activation of receptor tyrosine kinases such as the EGF receptor (Della Rocca et al., 1999a). In the present study, MAPK activation by bombesin was inhibited by the EGF receptor specific, tyrosine kinase antagonist, AG1478. Della Roca et al propose

that Gi mediated MAPK activation can be dependant upon the formation of focal adhesions as a scaffold for MAPK activation. It has been show that in several cell types, bombesin can activate FAK (Rodriguez-Fernandez and Rozengurt, 1998; Salazar and Rozengurt, 1999) but that the activation of MAPK by bombesin is not dependant upon this event (Luttrell et al., 1999). It can therefore be suggested that, in BOR15 cells, antagonist D may stimulate Gi mediated MAPK activation via a focal adhesion scaffold whereas bombesin stimulated MAPK activation involves the Gq mediated transactivation of the EGF receptor scaffold and in addition, the activation of a non-receptor tyrosine kinase pathway involving calcium mobilisation and the activation of PKC. Both Gi and Gq mediated tyrosine kinase pathways involve the activation of Src and result in the activation of Ras.

It is therefore apparent that the signal transduction pathways which are stimulated by antagonist D are mostly in accordance with the biased agonist mechanism of action proposed by Jarpe et al. Antagonist D activates Gi mediated signalling pathways in favour of the Gq mediated pathways which are stimulated by bombesin. However, according to the biased agonist theory, the full agonist bombesin should activate all available, receptor-G-protein coupling states. Although bombesin stimulated MAPK activation in BOR15 cells is independent of Gi, there are several publications which suggest that mitogenic signalling and DNA synthesis by bombesin partly involve Gi family of G-proteins. It has been shown that pertussis toxin inhibits bombesin stimulation of DNA synthesis in Swiss 3T3 cells, but does not effect the mobilisation of intracellular calcium or the activation of PLC (Zachary et al., 1987). However there is contradictory evidence which suggests that Gi does not play a major role in transducing mitogenic signals from the bombesin receptor (Taylor et al., 1988). It

has been proposed that a pertussis toxin sensitive G-protein may mediate cross talk between transmembrane signalling pathways in response to bombesin stimulation (Rozengurt et al., 1990). It is apparent that the role of Gi in mitogenic signalling by bombesin deserves more focused attention in future studies.

Similar examples of receptor promiscuity of G-protein coupling leading to activation of multiple second messenger pathways have been described before where both or just one of these can be blocked by an inverse agonist (Perez et al., 1996). However antagonist D is the first compound shown to be capable of simultaneously activating and inactivating different arms of the signal transduction pathways normally activated by an agonist.

It has recently been shown by our group that JNK activation by antagonist D is via the G12 family of G-proteins. JNK activation by antagonist D in COS7 cells is inhibited by the transient transfection of dominant negative G12 (unpublished results from our laboratory). Thus the mechanistic model for antagonist D can be extended to include the activation of both Gi and G12 and the inhibition of Gq receptor coupling.

The physiological relevance of the signalling events triggered by antagonist D was investigated. In a recent paper it was shown that the stimulation of the endothelin receptor with endothelin-1 in Rat-1 cells results in the activation of both MAPK and JNK. It was demonstrated that the inhibition of either PKC activation or intracellular calcium mobilisation results in potentiated and sustained activation of JNK by endothelin (Cadwallader et al., 1997). These findings may be applicable to the mechanism of JNK activation by antagonist D. Stimulation of the bombesin receptor with bombesin results in the mobilisation of calcium, the activation of PKC and the

activation of JNK. Antagonist D stimulation of the bombesin receptor results in the inhibition of calcium mobilisation and PKC and the sustained activation of JNK. Thus, by inhibiting the mobilisation of intracellular calcium, antagonist D may cause discordant activation of other pathways downstream of the receptor.

Several studies have shown that in SCLC cells and some fibroblasts, activation of elements of the MAPK signalling pathway can produce a growth inhibitory effect (Ravi et al., 1998; Ravi et al., 1999; Serrano et al., 1997). SCLC cells very rarely have oncogenic Ras mutations and the authors show that in SCLC cells, constitutive or sustained activation of proliferation signals involving Ras, activates dormant tumour suppressor pathways involving upregulation of cell cycle inhibitor proteins including $P16^{INK,}$ $P27^{KIP}$and $P21^{WAF}$. With these findings in mind, a time course of MAPK activation by antagonist D and bombesin was carried out to determine whether antagonist D induced MAPK activation was sustained in comparison to bombesin induced MAPK activation. It was found that both time courses were similar which indicated that sustained MAPK activation was not responsible for the growth inhibitory effects of antagonist D. Antagonist D induced upregulation of $P27^{KIP}$and $P21^{WAF}$ was also investigated over the same time period. No changes in protein levels were detected following antagonist D treatment. It would be of interest to repeat these experiments in SCLC cells as it is possible that the activation of Ras signalling pathways in SCLC cells will have different consequences than those observed in Rat-1 fibroblasts.

Both the activation of MAPK and JNK are likely to be important in the growth inhibitory effects of substance P analogues. There has been considerable research implicating JNK in the onset of apoptosis in a variety of cell types including SCLC

(Butterfield et al., 1997), see (Chen and Tan, 2000; Leppa and Bohmann, 1999) for review. Our group have shown that the JNK activation by antagonist D is sustained in comparison with the JNK activation by bombesin (Mackinnon et al., 2000) and this may be pivotal in the pro-apoptotic effects of this class of compound. In the current study, it has been shown that the activation of MAPK is also partially required for the pro-apoptotic effects of antagonist D. The dose dependant increase in antagonist D induced apoptosis, in BOR15 cells, is partially inhibited by the MEK inhibitor PD098059. Our group has also shown this to be the case in the SCLC cell line H69 (data not shown).

In summary, it is likely that the combined effects of sustained JNK activation and the discordant activation of MAPK, alongside the inhibition of neuropeptide stimulated calcium mobilisation, produce a growth inhibitory / pro-apoptotic effect (Fig 6.21). It can be hypothesised that cells presenting a neuroendocrine phenotype are sensitive to the biased agonist activation of signalling pathways by antagonist D and are thus sensitive to growth inhibition by such compounds.

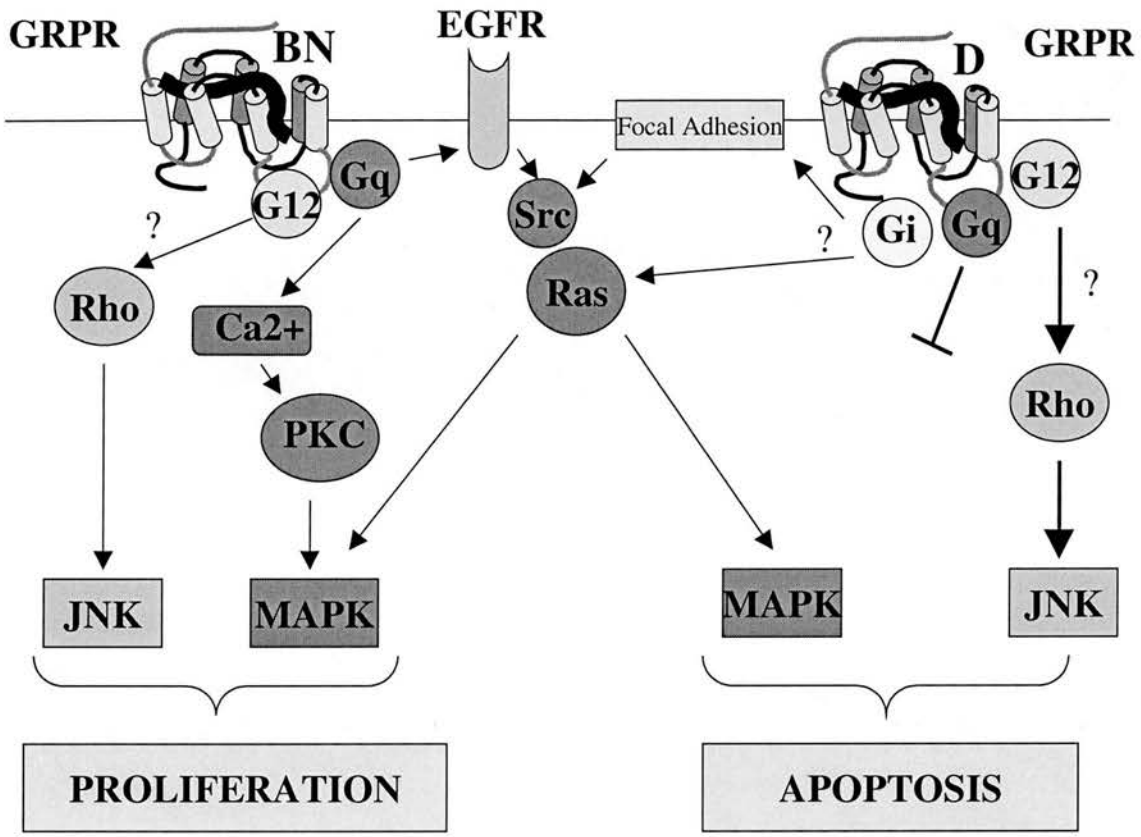


Fig6.21 Proposed model for the modulation of signal transduction pathways by antagonist D in comparison to bombesin. Both antagonist D and bombesin activate the GRP/bombesin receptor. Bombesin stimulates the activation of Gq mediated signal transduction pathways whereas antagonist D inhibits the activation of Gq mediated pathways. These bombesin stimulated pathways result in the mobilisation of intracellular calcium and the activation of PKC and in addition, bombesin stimulates the trans-activation of the EGFR, the activation of Src and Ras, all of which are upstream of the activation of MAPK. Antagonist D also activates MAPK kinase but rather than via Gq mediated signals, it stimulates the activation of Gi. Upstream of MAPK activation by antagonist D is the activation of Src and Ras. This is via a tyrosine kinase scaffold other than the EGFR and may involve focal adhesions or another receptor tyrosine kinase. Both antagonist D and bombesin stimulate the activation of JNK, most likely via the activation of G12 G proteins and possibly involving the small GTP binding Rho (further experiments in progress in our lab). However, the JNK activation by antagonist D is of greater magnitude and duration than the activation by bombesin.
Taken together it is likely that the differential activation of G proteins coupled to the GRPR, results in discordant activation of MAPK and JNK and the inhibition of calcium mobilisation. This shift from the normal signalling of the full agonist bombesin results in growth inhibition or apoptosis.

Chapter 7

General Discussion and Suggestions for Future Work

This project was designed to address several issues that can be loosely divided into two categories – pre-clinical studies and mechanistic studies. The pre-clinical studies investigated the range of tumour types which are sensitive to growth inhibition by substance P analogues, looked for the expression of neuropeptide receptors which may confer sensitivity and addressed the question of whether resistance to conventional chemotherapy alters the effectiveness of treatment with substance P analogues. The mechanistic studies focussed on the nature of the interaction of substance P analogues with cells and explored the effects that these compounds have on signal transduction pathways. These studies also addressed the physiological relevance of the observed cellular effects, in terms of growth inhibition and apoptosis.

A panel of tumour cell lines of various origins was used to investigate the range of sensitivity to antagonist G. It was found that in addition to SCLC cell lines, a non-SCLC cell line, two colo-rectal cancer cell lines and an ovarian cell line were sensitive to growth inhibition by pharmacologically achievable concentrations of antagonist G (<40μM). Several cell lines in the cell panel (a colo-rectal cancer cell line, a pancreatic cancer cell line and a SCLC cell line) showed marked resistance to growth inhibition by antagonist G which indicated that this compound acts via some specific mechanism rather than non-specifically disrupting cell growth. RT-PCR studies on the tumour cell panel, and experiments using GRPR/bombesin receptor bearing fibroblast models, showed that high expression of the gastrin releasing

peptide receptor confers increased sensitivity to antagonist G. Other tumour types such as breast cancer (Halmos et al., 1995) and prostate cancer (Bologna et al., 1989; Aprikian et al., 1996) have also been shown to express GRP receptors. It would be of interest to test the sensitivity of cell lines from other GRP receptor expressing tumours to fully explore the range of cancers which may be treatable with substance P analogues. The presence of the GRP receptor may be a reflection of the general neuroendocrine phenotype of the cancer cells or it may be the case that the interaction of substance P analogues with the GRPR initiates a growth inhibitory signal which does not occur upon interaction with other neuropeptide receptors.

These findings have important implications for the clinical use of substance P analogues. Antagonist G is currently entering a phase two clinical trial where its effectiveness will be tested in SCLC patients, but ultimately, compounds of this type may also be suitable for the treatment of other tumour types. Detecting expression of the GRP receptor from biopsy samples may be a useful indicator to identify patients for whom such treatment may be effective, therefore, it is important to further clarify this situation. It would be of interest to re-attempt the transfection of Rat-1 fibroblasts with other neuropeptide receptors such as the vasopressin receptor and look for effects on sensitivity to substance P analogues. In addition to using a Rat-1 fibroblast model, these findings should be confirmed using GRP receptor non-expressing neuroendocrine cancer cell lines as recipients for the transfection of GPR receptors. Also, anti-sense molecules could be designed to knock out the GRP receptor on a cell line which naturally expresses high levels of GRP-R in an attempt to create a more resistant cell line. In addition to this work, it would be useful to confirm the correlation between sensitivity to antagonist G and GRP receptor

expression in murine xenograft models. Several of the cell lines used in this study are already available in our lab as xenografts so this investigation would be feasible. The final question which was investigated as part of the pre-clinical aspect of this project, was whether or not cell lines which had been exposed to conventional chemotherapy and subsequently developed resistance to these treatments, displayed any differences in sensitivity to substance P analogues. This work was initiated in order to help define the clinical setting of these compounds. Should they be used in addition to standard chemotherapy or would they be more effective when used after tumours have relapsed and cells are resistant to currently available treatments? A panel containing pairs of SCLC cell lines derived before and after the onset of chemotherapy resistance were tested for sensitivity to antagonist G. A major drawback in this work was the unavailability of paired cell lines which had been created before and after exposure to chemotherapy *in vivo*. As a result two of the four pairs of cell lines used, were models of *in vitro* derived resistance. It is not known whether the mechanisms of drug resistance derived *in vitro,* accurately represent chemotherapy resistance as it occurs in an *in vivo* tumour environment. The results of this work did not confirm the hypothesis which suggested that tumours which are resistant to chemotherapy display increased neuropeptide responsiveness and increased sensitivity to substance P analogues, however, this theory did hold true in one of the two *in vivo* derived resistance models. None of the chemotherapy resistant cell lines tested showed increased resistance to antagonist G or D. This finding is encouraging as many chemotherapy resistant cell lines show cross resistance to multiple treatments. It is therefore likely that tumours which show resistance to conventional therapies may be treatable with substance P analogues.

Access to more cell lines from longitudinal patient follow ups would allow further work to be carried out on this matter. It would also be of interest to create in vivo derived chemotherapy resistant cell lines in murine xenograft models. These cell lines would be more representative of the situation found in cancer patients. Finally, it is not known whether prolonged treatment with substance P analogues will result in the development of resistance to these compounds. It would be of interest to try and derive resistance to substance P analogues, and attempt to identify any resistance mechanisms involved. Techniques which analyse differences in RNA species between two cell lines (differential display) could be employed to detect changes between the parent cell line and the resistance cell line. It is unknown whether substance P analogues are substrates for the p180 membrane pump which is responsible for much of the multi-drug resistant (MDR) phenotypes which are common in SCLC tumours. Further work in this area would be useful.

The second part of this project involved studies in order to increase the understanding of the mechanism of action of substance P analogues. Several lines of evidence had shown that substance P analogues may not simply act as a competitive neuropeptide antagonists and that in addition to blocking neuropeptide signalling, antagonists D and G had agonist effects which resulted in the activation of c-Jun-N-terminal Kinase (JNK) (MacKinnon et al., 1999). The pre-clinical studies had highlighted the GRP/bombesin receptor as candidate factor in the mechanism of growth inhibition and for this reason it was decided to investigate the nature of the interaction of antagonist D with GRP/bombesin receptor expressing fibroblast models. Studies were initiated to investigate the agonist effects of substance P analogues. It was discovered that both peptides were potent activators of MAPK and

it was decided to focus on this novel observation. It was unclear whether the activation of MAPK was receptor mediated or via some other mechanism and the signal transduction pathways involved were unknown. It was found that the activation of MAPK by antagonist D was indeed, via the GRP/bombesin receptor as shown by increased MAPK activity in the bombesin receptor expressing BOR15 cells compared to the parent cell line, the inhibition of this MAPK activity by specific bombesin antagonists and the inability of antagonist D to activate MAPK in fibroblasts expressing GRP receptors with mutated ligand binding sites.

Investigations into the signal transduction pathways employed by both bombesin and antagonist D showed that bombesin stimulated the mobilisation of intracellular calcium whereas antagonist D did not. In fact antagonist D inhibited calcium mobilisation by bombesin. Bombesin activation of MAPK was shown to be via a pathway which involves the transactivation of the EGF receptor and the activation of Ras and Src. Subsequent studies showed that the activation of MAPK by antagonist D, also involves the activation of Ras and Src but not the transactivation of the EGF receptor. Further experiments to determine how antagonist D stimulates MAPK should be carried out. The use of agents such as cytochalasin D, which prevent activation of tyrosine kinases via focal adhesions by disrupting the actin cytoskeleton, may be useful in determining whether or not MAPK activation occurs via focal adhesion associated proteins. Also immuno-precipitation of tyrosine phosphorylated proteins and subsequent probing for transactivated receptor tyrosine kinases may be necessary to find out if antagonist D induced MAPK activation involves a receptor tyrosine kinase scaffold other than the EGF receptor.

MAPK activation by antagonist D was shown to be via a pertussis toxin sensitive G protein, most likely of the Gi class. This contrasted with the natural receptor agonist, bombesin, which activates MAPK via the Gq family of G proteins. It was apparent, therefore, that antagonist D and bombesin both activate different signal transduction pathways from the same receptor by the differential activation of G-proteins. Our group has also shown that antagonist D and G activate JNK via the G12 family of G proteins and that this JNK activation is of greater magnitude and duration that JNK activation by bombesin. It is likely that this discordant activation of MAPK and JNK and the inhibition of Gq mediated signals, such as the mobilisation of calcium, is responsible for the growth inhibitory effects of substance P analogues. Further work to characterise the differential signalling by neuropeptides and substance P analogues is now necessary, to determine which of several aspects are integral in the growth inhibitory / pro-apoptotic effects of this family of peptides. In this study it is shown that inhibition of antagonist D induced MAPK activation results in a reduction in the ability of antagonist D to induce apoptosis. It is of great interest to determine the exact mechanism by which this activation of MAPK creates an inhibitory signal. Is it dependent upon the concurrent activation of JNK and/or the inhibition of intracellular calcium? Experiments using dominant negative JNK expression to block JNK activation or constituitively active Gq to prevent the inhibition of calcium mobilisation, could be carried out to further investigate this issue.

It is not clear as to whether these receptor effects are unique to the GRP receptor (which would explain the correlation between sensitivity to antagonist G and GRP receptor expression) or whether substance P analogues have the same "biased agonist" activity at other neuropeptide receptors. There is evidence to suggest that

the signal transduction pathways downstream of the GRP/bombesin receptor may be slightly different than those stimulated by other neuropeptide growth factor receptors. In order for SCLC cells to proliferate in response to neuropeptide growth factors they require stimulation with an additional growth factor such as insulin. Bombesin/GRP differs in this respect in that it is the only neuropeptide that can act as a sole mitogen for SCLC cells. It may be useful to further explore these ideas if it is shown that the mechanism of growth inhibition by substance P analogues is solely dependent upon their biased agonist effects on the GRP/bombesin receptor. Interestingly, it has been shown that antagonist D has highest affinity for the GRP/bombesin receptor whereas antagonist G has higher affinity for the vasopressin receptor and in terms of growth inhibition and pro-apoptotic effects, antagonist D is more potent than antagonist G. It would be interesting to compare the growth inhibitory potency of the complete range of substance P analogues with their affinity for the GRP/bombesin receptor. It is of vital importance to this line of drug development to answer these questions, as it may be the case that the broad spectrum activity of substance P analogues is not as important as their relative affinity to the GRP/bombesin receptor. This will undoubtedly influence future drug design.

The current study has highlighted the fact that the mechanism of action of substance P analogues is more complex than was previously appreciated. The novel mechanism of "biased agonism" resulting in differential activation of signal transduction pathways, is worthy of further characterisation and it must be determined whether this aspect of receptor pharmacology can be exploited by other types of compound.

The potential clinical setting for substance P analogues needs to be defined. The current range of substance P analogues may not be an immediate clinical success due to their relatively low potency (μM range) and high cost of production, however the ongoing drug development programme to search for more potent analogues may find more hopeful candidate compounds. Further research to pinpoint the exact mechanism of action by which substance P analogues inhibit the growth of cancer cells will allow drug structure / function relationships to be investigated, perhaps culminating in the design of effective non-peptide drugs which would be of much greater clinical impact.

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List of Publications

MacKinnon,A.C.; Armstrong,R.A.; Waters,C.M.; Cummings,J.; Smyth,J.F.; Haslett,C.; Sethi,T. (1999)
[Arg6,D-Trp7,9,NmePhe8]-substance P (6-11) activates JNK and induces apoptosis in small cell lung cancer cells via an oxidant-dependent mechanism. Brit. Jnl. Cancer 80(7), 1026-1034.

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Bombesin and substance P analogues differentially regulate G-protein coupling to the GRP receptor: Direct evidence for biased agonism. (Accepted to J Biol Chem).

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Gastrin releasing peptide receptor expression confers sensitivity to SP-G induced growth inhibition in tumour cells. (Ready for submission).