A STUDY OF EGF-MEDIATED EARLY AND LATE SIGNALING EVENTS IN RELATION TO EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE ACTIVITY IN THE HUMAN BREAST CANCER CELL LINE, MDA 468

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A STUDY OF EGF-MEDIATED EARLY AND LATE SIGNALING EVENTS IN RELATION TO EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE ACTIVITY IN THE HUMAN BREAST CANCER CELL LINE, MDA 468

by

Soma Mandal, M. Sc. (Physiology and Biochemistry)

A thesis submitted to the School of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Division of Basic Medical Sciences
Faculty of Medicine
Memorial University of Newfoundland

May 2001

St. John’s Newfoundland Canada
“Even he with the worst of karma (past action) who ceaselessly meditates on Me quickly loses the effects of his past bad actions. Becoming a high-souled being, he soon attains perennial peace; know this for certain: the devotee who puts his trust in Me never perishes!”

Bhagavad Gita

Dedicated to my Almighty beloved whose constant inner guidance and blessings vanished all obstacles along the journey and led me to the understanding of true love
ABSTRACT

The human breast cancer cell line, MDA 468 overexpresses the epidermal growth factor receptor, the EGFR to 1-2 x 10^6 receptors per cell. Receptor phosphorylation influences the EGF-mediated signaling events initiated at the cell surface. In this study we used a protein tyrosine kinase inhibitor (PTK) to study the effects of inhibition of EGFR kinase activity on EGF-dependent effects on phosphatidylinositol (PI) turnover, cell viability, and cell proliferation.

In the initial phase of the study, we tested some of the frequently used PTK inhibitors for their ability to inhibit the EGF-stimulated EGFR autophosphorylation. MDA 468 cells exhibited a differential sensitivity to inhibition of receptor autophosphorylation with these PTK inhibitors. Of the inhibitors tested, only the dihydroxybenzene moiety containing inhibitor lavendustin A (LA) effectively inhibited (~ 95% at 1 μM) the EGF-stimulated EGFR autophosphorylation in intact cells and in crude membrane preparations in a time and dose dependent manner. Exposure of cells to 1 μM LA beyond 10 h caused morphological changes such as nuclear condensation and membrane blebbing. Morphological changes and occurrence of a subdiploid peak at higher concentrations are consistent with the fact that LA produced cell death by apoptosis.

Earlier experiments from this laboratory had shown that EGF stimulates an increase in the total PI turnover and that a major portion of the metabolites was being converted to a component whose elution time did not correspond to the that of the elution times of non-cyclic inositol phosphates. This is an outcome of EGF-stimulated EGFR autophosphorylation. This metabolite was acid-labile and comprised over 50% of the PI turnover components in control untreated cells. Treatment of cells with EGF increased the levels of this component, while 1 μM LA treatment decreased it. Using the technique of electrospray ionization tandem mass spectrometry, we identified this metabolite to be myo-inositol 1,2-(cyclic) monophosphate (ciP) through the specific fragmentation pattern as compared to the standard. Change in the total PI turnover by stimulation (with EGF) or inhibition (with LA) of EGFR phosphorylation could be entirely accounted for by alterations in the percentage of ciP generated by these cells. This type of PI turnover profile is atypical and unlike that generated by phospholipase C-γ. ciP is the major constituent of EGF-stimulated PI turnover and it is the metabolite which is most closely linked to changes in phosphorylation of EGFR in MDA 468 cells.

MDA 468 cells are growth inhibited by 10^6 M EGF between 48-72 h of exposure. Previous work has shown that this growth inhibition is due to a pronounced G_1 growth arrest. Using a PTK inhibitor we hoped to see how changes in this relationship would affect the proliferative response in MDA 468 cells. At 0.2 μM, LA reduced cell proliferation (as estimated by cell number) and DNA synthesis ([^3]H-thymidine uptake). Growth inhibition was accompanied by perturbations in the cell
cycle. LA, EGF, and EGF+LA produced a pronounced G1 arrest. LA and EGF+LA-induced arrest was reversed 24 h after their removal, but EGF-treated cells showed a more persistent arrest. MDA 468 cells possess the p53\textsuperscript{273.\text{Hs}} mutant, which positively enhances cell proliferation, but it transforms into an alternate "pseudo-wild type" conformation during EGF-mediated G\textsubscript{1} arrest as demonstrated by previous work in this laboratory. In fact, p53 in cells treated with EGF, LA, or both, showed an altered subcellular localization at 24 h. The p53 in the nucleus lost reactivity to PAb 240 (it reacts with mutant p53), but reacted positively to PAb 1620 (it reacts with wild-type p53). This altered conformational shift of p53\textsuperscript{273.\text{Hs}} from mutant to wild-type was also accompanied by a strong nuclear localization of p21\textsuperscript{WAF1/CIP1} and an absence of nuclear localization of cyclin dependent kinase 2 (cdk2). Thus we hypothesize a role of p53\textsuperscript{273.\text{Hs}} dependent participation of p21\textsuperscript{WAF1/CIP1} and cdk2 in G\textsubscript{1} arrest in response to EGF, LA alone or both in combination. The augmented growth inhibitory response in presence of both EGF and LA is intriguing and is probably indicative of a segregated relationship between receptor phosphorylation and proliferative response.

In this study we provide evidence of EGF-dependent changes associated with phosphorylation in the levels of an unusual PI metabolite, whose levels cause changes in total PI turnover. Apparently, this receptor phosphorylation-mediated changes in clP during short-term exposure do not correlate with changes in proliferative response. Time and concentration dependent inhibition of receptor kinase activity by LA is reflected in later times by growth inhibition. Furthermore, the data presented suggests a function of p53\textsuperscript{273.\text{Hs}} in growth arrest.
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LIST OF ABBREVIATIONS

BSA—bovine serum albumin
CAD MS/MS—collision activated dissociation tandem mass spectrometry
ciP—cyclic inositol 1:2 monophosphate
-ciP_2—cyclic-inositol 1:2, 4,5-trisphosphate
cdc2/cdk1—cyclin dependent kinase 1
cdk2—cyclin dependent kinase 2
DMEM—Dulbecco’s minimum essential medium
DMSO—dimethyl sulfoxide
ECL—enhanced chemiluminescence
EGF—epidermal growth factor
EGFR—epidermal growth factor receptor
EM—electron microscope/microscopy
ER—endoplasmic reticulum
ESI MS/MS—electrospray ionization mass spectrometry
FACS—fluorescence activated cell sorter
FBS—fetal bovine serum
FITC—fluorescein isothiocyanate
G_0/G_1—gap 0 and 1 phase
HBEGF—heparin-binding epidermal growth factor
HPLC—high pressure liquid chromatography
HGF—hepatocyte growth factor
HRP—horse radish peroxidase
IP—inositol monophosphate
IP_2—inositol bisphosphate
IP_3—inositol trisphosphate
IP_4—inositol tetrakisphosphate
LA—lavendustin A
LB—lavendustin B
L15—Leibovitz medium
M—mitotic phase
MAb—monoclonal antibody
MPF—maturation promoting factor
MS—mass spectrometry
MTT—3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
PBS—phosphate buffered saline
PCD—programmed cell death
PCR—polymerase chain reaction
PL3′K—phosphatidylinositol 3′-kinase
PI-phosphatidylinositol
PIP-phosphatidylinositol 1-phosphate
PIP$_2$-phosphatidylinositol 4,5-bisphosphate
PLC-phospholipase C
PKC-protein kinase C
PMSF-phenylmethylsulphonylfluoride
PTK-protein tyrosine kinase
rpm-revolutions per minute
RTK-receptor tyrosine kinase
S phase-synthetic or the DNA synthesis phase
SDS-sodium dodecyl sulphate
SH2 and SH3-Src homology (domains) 2 and 3
TBS-T-Tris base, sodium chloride and Tween$^{20}$
CHAPTER I

INTRODUCTION
Signal transduction is key to life as we know it. Unicellular organisms had mechanisms by which one cell could influence the behavior of another. With the evolution of multicellular organisms, elaborate inter and intracellular signaling mechanisms enable cells to communicate to coordinate their mutual behavior for the benefit of the whole organism. Cellular signal transduction processes are coordinated by growth factors, differentiation factors, and hormones which are the primary components of a signaling system in the multicellular organisms that also largely coordinate their growth (Ullrich and Schlessinger, 1990). One of the many important phenomenon in signal transduction is tyrosine phosphorylation.

Phosphorylation is considered to be a major currency in signal transduction pathways (Hunter, 1995). Protein tyrosine kinases (PTKs) are associated with receptor and non-receptor proteins which are activated by tyrosine phosphorylation. Tyrosine phosphorylation is central to many cellular processes, including regulation of intracellular calcium concentration \([\text{Ca}^{2+}]\) (Marhaba et al., 1996), synapse formation (Catarsi et al., 1995), induction of ornithine decarboxylase activity in parotid glands (Kinoshita et al., 1996), acrosome reaction in sperm head, mammalian fertilization (Meizel and Turner, 1996), and in many developmental processes such as establishment of ventral cell fates and production of cuticle in *Drosophila* (Perrimon and Perkins, 1997).

Activation of receptor tyrosine kinase (RTK) signaling pathways is one of the common routes via which extracellular signals regulate gene expression. The effects of
many growth factors are mediated by high-affinity RTKs. RTKs catalyze the transfer of the γ-phosphate of ATP to specific tyrosine residues in certain protein substrates (Burke, 1992). In mammalian systems, the binding of ligand to the extracellular domain activates the tyrosine kinase in the cytoplasmic domain ultimately leading to the downstream activation of a number of common signaling proteins, including phospholipase C-γ (PLC-γ), phosphatidylinositol-3 kinase (PI3'K), GTPase-activating protein (GAP) etc. (Fantl et al., 1993). RTKs regulate processes such as cell growth, differentiation, migration, viability, and homeostasis (Perrimon and Perkins, 1997). Early events of growth factor receptor interaction following receptor phosphorylation include a transient increase in free calcium, the temporary expression of several genes like the nuclear-located proto-oncogene products c-myc and c-fos and a stimulation of monovalent ion-transport across the plasma membrane (Church et al., 1989). Several distinct subfamilies of RTKs, though diverse, share common features (Fantl et al., 1993). Site-directed mutagenesis within the insulin receptor or the epidermal growth factor receptor which eliminate their PTK activities nullify their biological activity. Thus specific tyrosine residues responsible for the tyrosine kinase activity are essential for the signaling action (Gazit et al., 1989).

In the present study we have examined the influence of EGFR phosphorylation on epidermal growth factor mediated signaling in MDA 468, a human breast cancer cell line.
1.1 MECHANISM OF GROWTH FACTOR RECEPTOR ACTION

Growth factor receptors possess an extensively glycosylated, protease-resistant extracellular ligand-binding domain, a single hydrophobic transmembrane region and a cytoplasmic domain which contains the tyrosine kinase catalytic domain. Thus, the RTKs possess a topology by which the ligand binding domain and the protein kinase activity are separated by the plasma membrane. Therefore, receptor activation due to extracellular ligand binding must be translated across the membrane barrier for the activation of the intracellular domain functions.

Ligand-induced activation of the receptor kinase domain is mediated by receptor oligomerization. Receptor oligomerization is universal among growth factor receptors in living cells, isolated membranes, and purified receptors. Oligomerization may be induced by monomeric ligands like EGF or bivalent ligands like PDGF and CSF-1. Monomeric ligands induce conformational changes and bivalent ligands mediate dimerization of neighbouring receptors. Oligomerized growth factor receptors have elevated protein tyrosine kinase activity and enhanced ligand binding affinity (reviewed in Ullrich and Schlessinger, 1990).

Binding of growth factors to receptors triggers the formation of complexes between receptors and cellular components; these complexes are responsible for the generation of second messengers. Tyrosine phosphorylation in the receptor forms links to the other intracellular reactions. Growth factors such as EGF, PDGF and insulin
enhance the phosphorylation of their own receptors in their tyrosine residues. By this phosphorylation, certain cellular serine/threonine kinases (such as PKC, Raf-1, MAP kinase, PI3'K) interact with the receptors in a ligand-dependent fashion through their SH (src homology) domains. The SH regions preferentially bind to the tyrosine phosphorylated sites (Koch et al., 1991; Pawson, 1995). A mitogenic signal transduction from the cell membranes to the nucleus involves the participation of adaptor proteins SHC and Grb2, which mediate the activation of the Ras/mitogen-activated protein (MAP) kinase pathway (Daub et al., 1996).

1.2 EPIDERMAL GROWTH FACTOR (EGF) AND THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

EGF is a 53 amino acid polypeptide. It is a powerful mitogen which acts in a variety of cells both in culture and in vivo (Cohen et al., 1980; Carpenter and Cohen, 1990). EGF stimulates the proliferation of fibroblasts and most types of epithelial cells (Wu et al., 1995). EGF exerts its tissue specific actions through its membrane-bound receptor, EGFR. EGF also regulates functions such as inhibition of gastric acid secretion and causes premature eye opening in newborn mice when injected daily (Gregory, 1975). EGF, along with HGF (hepatocyte growth factor), act as powerful mitogens in liver regeneration in vivo (Stolz and Michalopoulos, 1994).

EGFR is a 170 kD transmembrane phosphoglycoprotein (Xu et al., 1984;
Maiorano et al., 1998) consisting of 1186 amino acid residues and an N-linked oligosaccharide (40 kD) (Carpenter and Cohen, 1990). It can regulate the cell cycle in a large number of target cells including stratified squamous epithelia, salivary and mammary gland epithelial cells, glial cells, and endothelial cells (Maiorano et al., 1998).

EGFR has an NH$_2$-terminal extracellular domain (621 residues) (Weber et al., 1984) separated from a COOH-terminal intracellular domain (542 residues) by 26 preponderantly hydrophobic amino acids which form a membrane-spanning region. The cytoplasmic side of the transmembrane domain consists of 9-15 extremely basic amino acids; this feature is common to many membrane-spanning segments. The extracellular region has 51 cysteine residues which are clustered in two regions, each of about 170 amino acids. These regions might form a two-folded structure with an EGF-binding cleft; this so-called "duplicated" structure could be involved in ligand-mediated receptor oligomerization (Hunter, 1984). EGFR exits in two active states on the cell surface as demonstrated by the Scatchard analysis of EGF binding. These are the high-affinity and low-affinity states of the receptor (with respect to their EGF binding affinity) respectively.

Although the nature of such difference in the existence of the receptor populations is not clear, it has been suggested that the dimeric form (the active form) of the receptor represents the high-affinity state (Sorokin et al., 1994).

EGFR dimerization is thought to be a critical early event in the response to stimulation with EGF. It has been shown that coexpression of wild-type EGFR with a
mutated or a truncated receptor lacking most of the cytoplasmic domain results in the suppression of signaling by the normal receptor (Honnegar et al., 1990; Kashles et al., 1991). The importance of EGFR dimerization was elegantly demonstrated by Sorokin et al. (1994). They generated a mutated form of the EGFR by inserting a cysteine residue in the extracellular domains of two receptors via disulfide bond formation. The mutation resulted in the ligand-induced appearance of covalently linked EGFR dimers and, in parallel, increased the number of high-affinity receptors present on the surface of cells expressing the mutated EGFR. The dimeric form of the EGFR is essential for the activation of the kinase moiety of the receptor and dimer formation is associated with the appearance of high-affinity binding EGFRs on the cell surface (Sorokin et al., 1994). Thus the EGFR dimer represents the active form of the receptor.

When the EGFR interacts with ligands such as EGF and transforming growth factor-α (TGF-α) (Modjtahedi et al., 1993), phosphorylation of tyrosine residues 992, 1068, 1148, 1173 and 1183 respectively results. Binding sites for adenosine triphosphate (ATP) and phosphate acceptor substrate proteins are located within the kinase core domain. The ATP-binding domain is highly conserved and contains the essential lysine residues (Chen et al., 1987); if these lysine residues are replaced by neutral amino acids, then the ligand-stimulated protein kinase activity of EGFR is abolished (Carpenter and Cohen, 1990; Pandiella et al., 1989). Interestingly, the Drosophila homolog of the EGFR contains both the EGF binding and the tyrosine
kinase domains; this indicates that both these activities have been present in the same molecule for over 800 million years (Livneh et al., 1985). The erbB oncogene protein of the avian erythroblastosis virus lacks the extracellular ligand-binding domain (Xu et al., 1984). EGFR can act as an oncoprotein not only when it lacks the restraining structural features but also when there is an excessive expression (amplification) of structurally normal receptors (Filmus et al., 1985).

The EGFR belongs to the ErbB type I family of RTKs. The other members of this family include ErbB2, ErbB3 and ErbB4. These receptors play an important role in regulating cell growth and differentiation. Several growth factors including EGF, TGF-α, amphiregulin, HBEGF (heparin binding EGF), epieregulin, and betacellulin bind and activate the EGFR (Modjtahedi et al., 1996). Since the coexpression of EGF related peptides and their own receptors frequently occur in human carcinomas, it has been suggested that autocrine activation of the receptor might play a role in cancer cell growth (Jannot et al., 1996). EGF and TGF-α are able to directly activate EGFR; interaction of EGFR with signaling proteins such as transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and protein kinase C (PKC) influence stimulation (PDGF, PKC) or inhibition of cell replication (Maiorano et al., 1998).

1.2.1 EGFR and tyrosine phosphorylation

Signaling from the activated EGFR, leading either to cell proliferation or
transformation, requires the enzymatic activity residing in the receptor itself; the
activated receptor triggers further signaling events. Briefly, after EGF binds to its
receptor, the activated receptor dimerizes and autophosphorylates its tyrosine
residues. Phosphorylated tyrosine residues on the receptor are recognized by signaling
proteins with SH2 domains. These are the Src homology regions 2 and 3 which are
highly conserved non-catalytic domains (they were first found in the Src protein) in
certain endogenous substrates. These regions form tight associations with specific
peptide sequences of phosphorylated tyrosine residues and appear to direct protein-
protein interactions, such as binding of substrate to the PTK. Mutations in the SH2
regions have been shown to lead to the activation of the transforming potential (Burke,
1992). SH2 domains recognize phosphorylated tyrosine and enable proteins containing
them [such as PLC-γ, Ras GTPase (guanosine triphosphatase)-activating protein,
PI3'K, and Src-like tyrosine kinases that have been transiently phosphorylated on
tyrosine residues]) to bind to the activated receptor as well as to other intracellular
signaling proteins (Koch et al., 1991). EGFR tyrosine kinase associates with PLC-γ
through the high-affinity interaction between tyrosine 992 in the EGFR and the SH2
domain of PLC-γ (Koch et al., 1991; Rotin et al., 1992). EGFR and other growth factor
receptor kinases also bind to protein phosphatases with SH2 domains (Sun and Tonks,
1994) thus causing rapid dephosphorylation of receptor kinases; in EGFR, tyrosine
residue 1173 was mapped to be the major binding site for SHP-1, through a SH2
domain in this protein tyrosine phosphatase (Keihack et al., 1998).

Research has shown that the action of oncogenic proteins and other molecules which constitute the "signal transduction machinery of the cell" can contribute to excessive proliferation of cells. The concept is based on the fact that interfering with signal transduction interferes with proliferation. It is therefore widely believed that specific inhibitors of signal transduction might constitute the next generation of anticancer chemotherapy regimes (Levitzki and Gazit, 1995; Corbly et al., 1996). Since the mitogenic action of EGF is mediated through the ligand-induced autophosphorylation of the EGFR (Wakeling et al., 1996), overexpression of EGFR in a variety of human tumors makes these receptors promising targets for directed tumour therapy (Schmidt and Wels, 1996).

1.3 INHIBITION OF EGFR PROTEIN TYROSINE KINASE

Overexpression of the EGF receptor is a hallmark in several solid tumors. Thus its inhibition may be an important strategy for selectively targeting therapeutic agents. EGFR and its ligands such as EGF and TGF-α have been associated with poor prognosis and metastatic spreading. Pioneering studies have defined two important facts, namely, that the receptor for EGF is a transmembrane tyrosine kinase (Carpenter and Cohen, 1990) and that ligands such as TGF-α could activate the receptor in an autocrine fashion (Taketani, and Oka, 1983). Thus the EGFR is an attractive target for
the delivery of therapeutic agents. Use of anti-EGFR monoclonal antibodies (MAb) or EGFR-specific tyrosine kinase inhibitors are some of the extensively studied therapeutic strategies, with the aim of achieving inhibition of EGFR activity and subsequent regression of several types of tumors with EGFR overexpression. It has been shown that PTK inhibitors or monoclonal antibodies produce the same biological changes, namely inhibition of EGF-stimulated EGFR phosphorylation and inhibition of growth. Their sites of action are different. The PTK inhibitor inhibits the cytoplasmic tyrosine kinase domain, whereas the antibody blocks EGF binding in the extracellular domain (Modjtahedi et al., 1998). Such therapeutic tools have been tested in vitro in cell lines of tumors overexpressing EGFR, in animal studies and even in clinical trials alone or in conjunction with other modes of treatment.

1.3.1 PTK Inhibitors and EGFR inhibition

The EGFR protein tyrosine kinase was one of the first tyrosine kinases chosen as a target for the development of a new class of inhibitors, the protein tyrosine kinase (PTK) inhibitors (Traxler, 1997). Since then many potent PTK inhibitors for both receptor and non-receptor tyrosine kinases have been developed. Many currently available anticancer drugs including taxol, platins, and adriamycin are cytotoxic possessing highly toxic side effects. On the other hand, hormonal agents such as antiestrogens and antiandrogens are generally well tolerated and effective against hormone-dependent
tumors, but are ineffective often when such tumors become hormone-independent. Hence the medical need for new well-tolerated treatments for common solid tumors is extremely urgent. Although growth factor receptor mediated signal transduction via inhibition of protein tyrosine kinase is not a novel idea, its inhibition as a clinical approach to the regulation of cell proliferation is not yet proven. It can be speculated that PTK inhibitors might be able to inhibit or reverse the growth of tumor cells and slow down disease progression because in the absence of positive growth signals, some of the cancers may differentiate or die via various mechanisms of cell death such as apoptosis or necrosis (Traxler, 1997). Several active compounds such as erbstatin, herbimycin A and staurosporine, flavonoids and isoflavonoids have been isolated from biosources. Some of the synthetic compounds that have been synthesized are derivatives of natural PTK inhibitors. One such compound is methyl 2,5-dihydroxycinnamate, a well-known PTK inhibitor, which is an erbstatin derivative (Umezawa et al., 1990). Many synthetic compounds have been synthesized: these include dianilinophthalimides (Trinks et al., 1994), anilinoquinazolines (Wakeling et al., 1996), hydroxycinnamides, thiazolidinediones, phenylaminopyrimidines (DeWitte et al., 1993) and tyrphostins (Posner et al., 1993). All these classes of compounds have been reported to inhibit the growth of cells in culture and also in vivo by interference with PTK activity (DeWitte et al., 1993).

Lavendustin A (LA) is one of the most potent natural products to possess a PTK
inhibitory activity with a reported IC₅₀ value of 12.0 nM in vitro (Onoda et al., 1989). It is a microbial metabolite which was isolated from Streptomyces griseolavendus and is selective for the inhibition of tyrosine kinases relative to serine/threonine kinases. It is an exceptionally potent inhibitor of ATP binding to the EGFR kinase (Onoda et al., 1989). LA contains multiple aromatic rings and also has the 2,5-dihydroxyphenyl group of erbstatin (Burke, 1992) and has been shown to be a "mixed-type" inhibitor (Hsu et al., 1991). Some other natural PTK inhibitors include quercitin (flavone) and genistein (isoflavone) which have both been used extensively for the purpose of studying EGFR tyrosine kinase inhibition.

1.3.2 EGFR inhibition—other strategies

Besides the ongoing search for various EGFR protein tyrosine kinase inhibitors, both natural and synthetic (as is discussed in the previous section), various other strategies have been used to inhibit the EGF receptor tyrosine kinase activity. Strategies of EGFR inhibition are based on two functional aspects of the receptor, binding of EGF to the receptor, and secondly, the protein tyrosine kinase activity of the receptor (Gill et al., 1984).

Extensive studies with monoclonal antibodies (MAb) to the EGF receptor have been done as a means for inhibiting the EGFR. During the past 20 years or so, a number of MAbs have been synthesized and tested against human cell lines
overexpressing EGFR.

Mouse MAb 425 and 528 blocked ligand interaction with EGFR, reduced anchorage-independent growth of NS2T2A1 (a cell line derived from normal human breast epithelial cell culture transformed by SV40-T antigen, and possessing increasing levels of EGFR, TGF-α, amphiregulin), and reduced the mean tumor mass after NS2T2A1 cells were grafted in nude mice (Ma et al., 1998). MAb C225 in combination with an 18-mer antisense oligonucleotide HYB 190 (targets expression of the RIA regulatory subunit of c-AMP dependent protein kinase I) has been found to inhibit the growth of human renal cancer cell lines, 769-P, ACHN, A498 and SW839 in soft agar (Ciardiello et al., 1998). MAb 225 caused a G1 arrest and programmed cell death (apoptosis) in DiFi colorectal adenocarcinoma cells by inhibiting receptor tyrosine kinase (Wu et al., 1995). Mouse MAb 225, 528 and 579, prepared from EGF receptor protein from the A431 carcinoma cell line, have been shown to be competitive against EGF binding and they antagonize EGF-stimulated protein tyrosine kinase activity as assayed in intact A431 cells or by using an exogenous peptide substrate in solubilized membranes. Many such competitive antagonist MAbs can be used to control the concentration of receptors activated by EGF (Gill et al., 1984; Mendelsohn, 1997).

A most promising aspect of MAbs has been their use in phase I or phase II clinical trials in patients with head, neck, lung or brain cancers (Modjtahedi et al., 1996). The purpose of such studies was to determine whether the treatment produced life-
threatening toxicities by their binding to EGFR expressed by normal tissues. Two mouse MAbs, 225 and 528, which have been extensively characterized for their biological and preclinical properties, represent the first series of antiEGFR blocking reagents that have entered clinical evaluation in cancer patients (Ciardiello et al., 1998). Rat MAb ICR62 had been safely administered in patients with unresectable squamous cell carcinoma in phase I clinical trials. This antibody was found to be safe and also localized efficiently in the metastatic sites. In this study the safety of administration (and not its therapeutic potency) was assessed (Modjtahedi et al., 1996).

Other strategies that have been used to inhibit the EGFR activity include use of chimeric molecules comprising of a natural ligand (such as the chimeric molecule consisting of heparin and EGF, a ligand that will bind to the EGF receptor) with high affinity to the EGFR attached to a cytotoxic protein (ribosome-inactivating protein saporin, a plant mitotoxin) to deliver specific toxins to the cells (Chandler et al., 1998). Another approach is tumor gene therapy. Human breast cancer MDA-MB-231 cells transfected with EGFR in the antisense orientation, showed decreased EGFR RNA expression which resulted in the suppression of the transformed phenotype, which, in turn correlated with the decrease in the expression of EGFR (Hong et al., 1998).

1.4 APOPTOSIS

Apoptosis or “programmed cell death” (PCD) is an active process of cell
destruction in the normal development of an organism (Alison and Sarraf, 1992). This name was derived from a Greek word meaning a flower losing its petals or a tree dropping its leaves (Wyllie, 1993). Apoptosis occurs in developing embryos where genetically controlled cell death occurs at precise developmental stages in the process of organogenesis. In normal healthy adult tissues cell death (or loss) must keep pace with cell production in the great renewal systems (bone marrow, gastrointestinal tract, skin) in order to maintain tissue homeostasis between cell proliferation and loss. Certain cellular environments such as serum-starvation or growth factor deprivation, (Moyer et al., 1997) also trigger apoptosis. Apoptosis is regarded as "physiological" cell death since it is a genetically determined cellular program (Raff, 1992). In apoptosis, cells shrink, their chromatin is pushed to the periphery of the nucleus, and the cells lose their plasma membrane integrity. The remnants of apoptotic cells are eliminated through phagocytosis by the neighboring cells and macrophages (Alison and Sarraf, 1992).

Apoptosis is a phenomenon which has been shown to be of immense importance in determining the efficacy of various anticancer agents because many kill tumor cells by apoptosis. Also much attention has been focussed on understanding factors which cause cells to acquire or lose their sensitivity to chemotherapeutic agents (Lowe et al., 1993). Many cellular targets including p53 have been identified which influence the sensitivity of cells to apoptosis. Evidence suggests that expression of
oncogenes such as adenovirus early region 1A (E1A) and c-myc can also increase the cellular susceptibility to PCD (Lowe et al., 1993). On the other hand, oncogenes like bcl-2 have been found to prevent apoptosis (Fairbarin et al., 1993) by promoting survival of cells in the non-cycling state (Schmandt and Mills, 1993). Apoptosis thus involves interplay between repressor and inducer genes (Buja et al., 1993).

1.5 PHOSPHOINOSITIDE BREAKDOWN-A SECOND MESSENGER SYSTEM IN CELLULAR SIGNALING

Coordination of cell growth and division is mediated by specific growth factor receptors and intracellular messengers. This is true when growth is considered under physiological (fetal growth, wound healing and tissue regeneration) and pathological (cancer) conditions. Receptors for Ca$^{2+}$-mobilizing hormones, neurotransmitters and growth factors utilize a well-studied second-messenger system involving the hydrolysis of phosphatidylinositol (PI) (Rhee, 1991) to elicit at least part of their biological responses (Hurley, 1995). The very first version of the phosphoinositide system was elucidated by Hokin and Hokin (1964). They proposed that phosphoinositide (inositol-containing phospholipids) was hydrolyzed in response to cell stimulation with an increase in phosphatidic acid, whose phosphate moiety was not directly derived from phosphatidylinositol, but from diacylglycerol (DAG), formed as a result of phosphoinositide breakdown (phosphorylation of DAG produces the phosphate moiety
in phosphatidic acid; this reaction is catalyzed by the enzyme diacylglycerol kinase). Through subsequent accumulation of research data a well-studied sequence of events have been shown to occur as a result of phosphoinositide breakdown.

Phosphoinositides in cell membranes varies from 2-12% in different tissues, the proportion of PI being highest among them. Most PI is located in the inner membranes, whereas phosphoinositides which contain the most phosphate groups [including phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP₂)] are preferentially located in the plasma membrane (Michell, 1975). PI-specific phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositols, namely phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂), produces inositol mono, di, and trisphosphates (IP, IP₂, IP₃) respectively with diacylglycerol (DAG) as the common product (Rhee et al., 1989). Figure 1.1A shows the interconversion of the phosphoinositides by the stepwise phosphorylation of PI by the respective kinases. Phosphoinositides which are substrates for PLC usually contain stearic (at the first position of glycerol) and arachidonic (at the second position of glycerol) acids (Tkachuk, 1998). IP₃ and DAG are the primary intracellular second messengers produced as a result of the PLC-mediated PI (PIP₂) hydrolysis (Kim et al., 2000) (Figure 1.1B). DAG plays an integral role in the activation of protein kinase C (PKC), and IP₃ diffuses from the plasma membrane through the cytoplasm to intracellular calcium stores to release
Figure 1.1 Phosphoinositides and the generation of second messengers (reproduced from Alberts et al., 1994).

Figure 1.1A describes the formation of polyphosphoinositides (PIP and PIP$_2$) by the stepwise phosphorylation of phosphatidylinositol (PI).

Figure 1.1B describes the generation of the second messengers IP$_3$ and DAG. All three classes of PLC ($\beta$, $\gamma$, and $\delta$) hydrolyse PIs. PLC-$\gamma$ is activated by receptor tyrosine kinases, including EGF receptor tyrosine kinase.
A
fatty acid chains of outer
lipid monolayer of plasma membrane

B
fatty acid chains of outer
lipid monolayer of plasma membrane
intracellular Ca\textsuperscript{2+} (Phillippe, 1994). Ca\textsuperscript{2+} combines with a calcium-binding protein calmodulin; the calcium-calmodulin complex is responsible for activating Ca\textsuperscript{2+}-dependent protein kinases (Bourne and DeFranco, 1989).

PLC plays an essential role in the PI signaling pathway utilized by many receptors. Mechanisms of PLC regulation that have been clearly identified include those that are G-protein mediated and those linked through a tyrosine kinase. The presence of multiple isoforms of PLC conforms to the notion of differential regulation of these enzymes (Cockcroft and Thomas, 1992). Three families of PLC have been identified. These are the \( \beta \), \( \gamma \), and \( \delta \) families respectively. The classification of the families of PLC has been based on the deduced amino acid sequences. The \( \beta \) family has three subtypes, namely, \( \beta_1 \), \( \beta_2 \), and \( \beta_3 \) respectively. The \( \gamma \) family has two subtypes, \( \gamma_1 \) and \( \gamma_2 \), while the \( \delta \) family has three subtypes, \( \delta_1 \), \( \delta_2 \), and \( \delta_3 \) respectively. In addition, the existence of at least two more families, namely, PLC-\( \alpha \) and PLC-\( \epsilon \) has been predicted. PLC-\( \beta \) is regulated by G proteins whilst PLC-\( \gamma \) is under the control of tyrosine kinases (Cockcroft and Thomas, 1992).

Hydrolysis of phosphoinositide results in the generation of non-cyclic and cyclic inositol phosphates. When PLC cleaves PI, it leads to the production of cyclic inositol 1:2 monophosphate (cIP) (Kim \textit{et al.}, 1989). Current evidence suggests that the percentage of cyclic inositol phosphates to non-cyclic phosphates generated decrease in the order of PLC-\( \alpha > \) PLC-\( \beta > \) PLC-\( \delta > \) PLC-\( \gamma \) (Kim \textit{et al.}, 1989; Rhee \textit{et al.}, 1989). As
such, DAG, IP, IP$_2$, and IP$_3$ constitute 90-95% of all the PLC-γ1-mediated PI products (Berridge and Irvine, 1989; Majerus et al., 1990; Rhee and Choi, 1992), with cyclic phosphates of inositol considered to comprise only the remaining 5-10% (Berridge and Irvine, 1989; Kim et al., 1989; Majerus et al., 1990; Putney and Bird, 1993). It is unclear as to what factor determines the ratio of cyclic compounds to their non-cyclic counterparts (Majerus, 1992). In some species, such as the intracellular bacterial pathogen _L. monocytogenes_, for instance, a specific PLC produces only cIP and DAG from PI breakdown (Camilli et al., 1991). Increased production of cyclic inositol phosphates have also been noted in various tumor cell lines.

All cyclic inositol phosphates are metabolized through cyclic 1:2-inositol phosphate hydrolase (cIPH). Therefore, it is likely that this enzyme plays a key role in the regulation of cellular concentrations of cIP (Ross et al., 1990). The enzymatic breakdown of cIP was first demonstrated by Dawson et al. (1971). It was later identified that the enzyme responsible for this reaction is cIPH. Annexin III, a calcium-dependent, phospholipid-binding protein was identified as having an enzymatic property consistent with cIPH (Ross et al., 1990; Tait et al., 1993). This identity was widely accepted until recently, when a dissociation of cIPH activity from that of annexin III was demonstrated (Sekar et al., 1996; Perron et al., 1997).
1.5.1 The inositol phosphates

The production of the two second messengers, IP₃ and DAG, are the key steps in the signaling pathways initiated by the breakdown of PIP₂ by PLC-γ. The deactivation of this pathway is necessary to terminate the increase in free [Ca²⁺], as well as the recycling of inositol for the restoration of precursor inositol stores (Putney and Bird, 1993). Various PI turnover metabolites, from inositol monophosphate to hexaphosphate (IP, IP₂, IP₃, IP₄, IP₅, IP₆), are produced by the rapid phosphorylation and dephosphorylation cycles by the actions of various inositol phosphate kinases and phosphatases.

Myoinositol is one of the nine stereoisomeric hexahydroxycyclohexanes, collectively termed inositols. This molecule is an essential component of membrane lipids down the evolutionary scale from eubacteria to eukaryotes. Inositol is essential; S. cerevisiae mutants unable to synthesize inositol show a defect in the acid-hydrolase secreting system. Even though most mammalian tissues other than brain and testis, make small amounts of inositol, the ubiquitous presence of inositol in the diet makes it quite difficult to render mammals inositol deficient (Michell, 1997).

Inositol phosphates also have important functions. IP₃ (inositol 1,4,5 trisphosphate) has an identified role as a potent second messenger, which leads to the mobilization of [Ca²⁺] stores (Furuichi and Mikoshiba, 1995). Inhibition of IP₃-induced Ca²⁺ release by PIP₂-specific antibodies has been shown to greatly lengthen the
duration of the cell cycle in microinjected two-cell stage *Xenopus* embryos. Synaptotagmin II, an integral membrane protein of synaptic vesicles binds to IP₄ (inositol-1,3,4,5 P₄) suggesting it has an important role in the regulation of neurotransmitter release (Niinobe *et al.*, 1994). Calciotropic compounds like IP₃ and IP₄ are also natural chelators of Ca²⁺ and may thus be partly responsible themselves, for providing a basis for the diverse biological actions of the metal ion. The coupling of chelation with enzyme or receptor interactions offers a general mechanism for the divalent cation control of diverse biological processes. Inositol monophosphate 1-phosphatase and inositol polyphosphate 1-phosphatase are the two enzymes which may conform to this mechanism (Luttrell, 1994). IP₅ and IP₆ have been suggested to have roles as antioxidants (Graf *et al.*, 1987). IP₆ has also been suggested as an intracellular iron transporter which binds iron during its transport through cytosol or cellular organelles (Hawkins *et al.*, 1993). IP₆ has been shown to promote Ca²⁺ uptake and modulate neurotransmitter release in cultured cerebellar granule cells (Nicoletti *et al.*, 1989). Recently IP₆ has been shown to have an antineoplastic effect. It decreases cell proliferation by causing G₁ arrest of human colon carcinoma cells by upregulating the tumor suppressor genes p53 and p21⁵⁵⁶⁴⁶⁵ (Saied and Shamsuddin, 1998).

Cyclic inositol 1:2, 4, 5-trisphosphate (cIP₃) has been shown to have an intracellular Ca²⁺-mobilizing role, although this must be reconciled with its reported relatively long half-life of ≈10 min (Putney and Bird, 1993). In *Limulus* photoreceptor
cells, cIP_3 was five times more potent than IP_3 in increasing Ca^{2+} conductance (Wilson et al., 1985). Cyclic inositol phosphates accumulate in stimulated cells for longer periods than their non-cyclic counterparts. This is suggestive of their importance in evoking sustained or delayed responses, including stimulation of cell growth (Ross et al., 1990).

1.6 TYROSINE PHOSPHORYLATION AND PI TURNOVER

The link between tyrosine phosphorylation and PI turnover is through PLC-γ (Haugh et al., 1999). EGF stimulation causes autophosphorylation of the EGFR in cells expressing the receptor; the activated receptor tyrosine phosphorylates its substrate, PLC-γ, which, in turn, is responsible for enhanced PIP_2 breakdown. Thus, the EGF-mediated receptor activation is linked to PI turnover via PLC-γ in EGF-stimulated cells.

PLC-γ is a substrate of the PDGF and EGF receptor kinases. It has been suggested that this phosphorylation is responsible for the increase in PLC-γ activity in the cell types where these growth factors stimulate PI turnover (Meisenhelder et al., 1989). In a high percentage of mammary carcinomas, increased phosphotyrosine content of PLC-γ1 has been observed resulting in amplification of signaling steps in receptor tyrosine kinase stimulated signal transduction pathways (Arteaga et al., 1991). Phosphorylation of PLC-γ on tyrosine residues has been shown unequivocally in cells treated with growth factors EGF and PDGF. Both these growth factors induce PI
turnover in 3T3, A431 (Meisenhelder et al., 1989), MDA 468 cells and R1hER cells (Church et al., 1992). In MDA 468 cells autophosphorylation of the EGFR, its association with and activation of PLC-γ (by tyrosine phosphorylation) leads to an increase in the total PI turnover (Church et al., 1992).

1.7 THE CELL CYCLE

"All things began in order, so shall they end, and so shall they begin again; according to the ordainer of order and mystical mathematics of the city of heaven."

Sir Thomas Browne, 1658

This statement is truly applicable for the cell cycle. Cells never re-duplicate their chromosomes before sister chromatids have been segregated at the previous mitosis, never start mitosis before DNA duplication is completed, nor attempt to segregate sister chromatids until all pairs have been aligned on the mitotic spindle at metaphase (Nasmyth, 1996). Thus cells have mechanisms by which the events related to cell division occur in the right sequence. The cell cycle is a mechanistic explanation for the proliferative nature in cells (López-Sáez et al., 1998). Therefore the balance among the cell populations in an organism is controlled by regulating the rates of proliferation, differentiation, and cell death (Collins and Rivas, 1993).

Looking back at the history of the elucidation of cell cycle events, it was recognized from early microscopic studies that cell division was preceded by mitosis
(M phase), during which cells condensed their chromosomes, aligned them on a microtubular spindle and segregated sister chromatids to opposite poles of the cell. Not much knowledge could be gained by simple observation, of the interval between succeeding mitoses (known as interphase) except that there was an increase in cell volume. Chromosome duplication was detected and shown to occur during a narrow window during interphase. Interphase was thus split into three intervals: G₁, the gap between mitosis and the onset of DNA replication; S phase (synthetic phase), the period of DNA synthesis; G₂ the gap between S and M phases. G₁, S, G₂, and M gradually were thought of as major cell cycle states (Nasmyth, 1996). The gap phases, G₁, and G₂, represent the pre- and post-replication periods respectively between the replication and segregation of DNA. Through the cell cycle there is growth of cellular mass, as well as the replication and segregation of DNA in S and M phases respectively.

In general, in response to an effective stimulus, the cell starts proliferating by initiating a cycle. This is the G₀ to G₁ transition. In the presence of signals to proliferate, cells first leave G₀, a state of quiescence, and commit themselves to start the cycle in G₁. The proliferative potential of a tissue is often expressed by its G₁ to G₀ ratio (López-Sáez et al., 1998). In G₁ cells contain a 2N or diploid DNA content between division and S phase, and G₂ is when the cells have a tetraploid DNA content, separating the S phase from mitosis. Many events which are essential for the progression of cells through
the cell cycle occur during these two gap phases. In normal cycling cells \( G_1 \) is the longest phase, resulting in the preponderance of 2N cells seen in the typical DNA histogram produced by flow cytometry of asynchronous, cycling cells. During \( G_1 \), the cell size, protein content, and RNA content increase markedly because cells must ultimately divide again maintaining a characteristic size. If protein synthesis is prevented, cells do not reach \( S \) phase from \( G_1 \). In addition, histone H1 is progressively phosphorylated together with a variety of other proteins, such as the retinoblastoma protein (Rb). During \( G_2 \), the tetraploid cells have to prepare for mitosis, and again, protein and RNA synthesis is required for progression. In \( G_2 \) the cell gets the time to check for any damage that might have occurred due to irradiation, thus allowing repair of any damaged DNA.

\( S \) phase is important not only for DNA replication, but also because it is responsible for the maintenance of the genomic integrity during cellular proliferation for the continued viability of the organism. When the cells bypass normal restrictions to entering \( S \) phase, often imposed by conditions which cause DNA damage, the continued replication of such cells can contribute to cancer formation (Hartwell and Castan, 1994). Howard and Pelc (1951) showed that \(^{32}\text{P}\) orthophosphate was incorporated into the roots of \textit{Vicia} seedlings during a particular stage of interphase, which is now known to be in the \( S \) phase or the DNA synthesis phase. Synthesis of more specific DNA label, \(^{3}\text{H}\)-thymidine (Taylor \textit{et al.}, 1957), was then used to study
DNA synthesis. Only cells in the process of synthesizing DNA take up and retain the \[^{3}\text{H}]-\text{thymidine}. A number of specific enzymes increase markedly, including thymidine kinase, ribonucleotide reductase, and DNA polymerase. These enzymes are associated into a large multienzyme complex called repliase, which may provide a high concentration of precursors for DNA synthesis at the replication site. Then the entire DNA content of the human chromosome is replicated. In addition to DNA synthesis, structural elements of the chromosomes, such as histones are also duplicated. S phase, like mitosis, is not a natural point of replication, because cells do not pause at this point unless inhibited by agents such as hydroxyurea or iron chelators, both of which interfere with ribonucleotide reductase, a rate-limiting enzyme for DNA synthesis (Bybee and Thomas, 1991).

### 1.7.1 Regulation of the cell cycle

Progression through several major checkpoints in the cell cycle is controlled by multiple protein kinases, generally a regulatory cyclin and a catalytic unit, making up a cyclin dependent kinase (Chen and Hitomi, 1999). The first molecular models of cell cycle regulation focused on how a single enzymatic oscillator comprised of a regulatory cyclin (cyclin B) and a catalytic cyclin-dependent kinase (cdk) controlled mitotic entry and exit. The cdks provide the enzymatic activity of this complex. An important element of this cyclin-regulated cell cycle is the rapid induction of expression of the cyclins and
their facile destruction as the cell progresses into the next phase of the cell cycle (Rodriguez-Puebla et al., 1999). It was recognized later that cdks have roles in multiple cell cycle events, including DNA replication, mitosis, centrosome/spindle pole body duplication, and cell morphogenetic changes (Roberts, 1999). The activity of these kinases is regulated by the expression level of each component, its phosphorylation status, and the presence of specific inhibitory proteins (Hall and Peters, 1996). The cdks are constitutively expressed throughout the cell cycle. They are the promoters of the progression through the cell cycle, and they are sequentially activated and deactivated.

Several mammalian cyclins have been identified, and their functions at specific points of the cell cycle have been established. The three D-type cyclins (D1, D2, and D3) function during mid-to-late-G, phase. They are regarded as growth factor sensors, because various combinations of them, depending on the cell lineage, are induced in response to various mitogenic stimuli during G, phase. This induction is concomitant with an increase in the catalytic activity of their specific cdk partners, cdk4 and cdk6 respectively. Expression of cyclin E peaks sharply during the G, phase transition (its main partner is cdk2) and is followed by the expression of cyclin A, which maintains cdk2 activity of their specific cdk partners (Rodriguez-Puebla et al., 1999). In mammalian cells, the D cyclins and their catalytic partners cdk4 and cdk6 function as the cells leave G, and progress through G, while cyclin E/cdk2 is activated from G, into
S phase. Cyclin A/cdk2 operates in the S and G₂ phases, whereas cyclin B/cdk1 (or the cell division cyclin in yeast, the cdc2) regulates the G₂M transition (Hall and Peters, 1996). The phases of the cell cycle and the periodicity of expression of the cyclins in the various phases of the cell cycle are shown in Figure 1.2A and Figure 1.2B respectively.

The control of cellular proliferation relies on both accelerating and braking mechanisms, and hence the checkpoint and tumor suppressor pathways are of great importance. Proteins encoded by checkpoint genes are crucial components of these pathways that evaluate the final balance between the mitogenic and non-mitogenic pathways (López-Sáez et al., 1998).

Positive control is brought about by proteins encoded by the cell division cycle (cdc) genes. The cyclin dependent kinases (cdks) are the key regulatory enzymes of the cell cycle. The precise activation and inactivation of CDKs at specific points in the cell cycle are required for cell division to proceed in an orderly fashion.

Cyclin dependent kinase inhibitors such as p21^{WAF1/CIP1} and p27^{KIP1} are actively involved in the negative regulation of cdk activities, playing a role in controlling cell cycle progression and thus acting as tumor suppressors (Hirama et al., 1995; Cheng et al., 1999). Among the genes that are well-known regulators of the cell cycle, the tumor suppressor gene p53 is well recognized as a checkpoint gene which not only regulates the G₁ checkpoint (Kagawa et al., 1997), but also the G₂ phase as well (Schwartz et al.,
Figure 1.2 The cell cycle and expression of various cyclins during the phases of the cell cycle (reproduced from Santa Cruz Biotechnology Inc., catalogue, 1999).

Figure 1.2A describes the various phases of the cell cycle and the involvement of the various cdk-cyclin complexes during the cell cycle.

Figure 1.2B describes the periodicity of expression of the various cyclins during the various phases of the cell cycle.
**Cell Cycle**

- Cyclin A and B + Cdk2
- Cyclin A + Cdk2
- Cyclin D, E, and G + Cdk4, 6, 7, and 8
- G2
- S
- G1
- M

**Cyclin Expression: Periodicity**

- Cyclin A
- Cyclin D
- Cyclin B
- Cyclin E
1997). p21WAF1/CIP1 is induced by p53 (López-Sáez et al., 1998). p21WAF1/CIP1 acts as a downstream effector of p53 in the inhibition of mitotic progression. p21WAF1/CIP1 can also be induced independent of p53 through various regulators, including growth factors PDGF, fibroblast growth factor (FGF), and EGF (Michieli et al., 1994). Like p21WAF1/CIP1, p27KIP1 is another cyclin dependent kinase inhibitor which binds to a number of cdk complexes (Hirama et al., 1995). This protein is abundant in quiescent cells and growth factor stimulation causes its levels to fall (Cheng et al., 1999) and cell cycle to progress.

1.8 GROWTH ARREST

In a properly functioning cell, deprivation of essential growth factors in pathway(s) dependent on the factors, acting as primary mediators of intercellular communication (Schmandt and Mills, 1993), can lead to growth inhibition, leading to growth arrest at the G1 checkpoint, which is known as the restriction point. Cells which are damaged by chemotherapy often arrest at another checkpoint, the G2M phase, in order to enable them to repair DNA. In malignant cells, chemotherapeutic damage signals the cells to pause for repair. In such a case, the requirement for growth factors to allow for the traversal of the cell cycle is converted to the requirement of a growth factor for cell survival. In such a situation, when receptor kinase activity is inhibited (such as the EGFR), cells can no longer survive and undergo apoptosis (Mendelsohn, 1997).
1.9 MDA 468 CELLS

The MDA 468 (MDA-MB-468) cell line was established from pleural perfusion of a breast cancer patient (Pathak et al., 1979). MDA 468 cells express large numbers of EGFRs, \( \sim 1-2 \times 10^6 \) /cell, as a result of an EGFR gene amplification (Filmus et al., 1985; Filmus et al., 1987). This is in comparison to \( \sim 10^5 \) or less receptors/cell in other cells. Although there is a gene amplification, the EGFR in MDA 468 shows characteristics of wild-type receptors; the overexpressed receptor protein appears normal with respect to its binding affinity, synthesis, turnover and autophosphorylation (Kudlow, et al., 1986; Filmus et al., 1987).

1.9.1 EGFR overexpression and growth inhibition in MDA 468 cells

Elevated levels of EGFRs are commonly associated with breast cancers (Gusterson, 1992), which suggests a critical role for EGF in transformation. It has been suggested that increased EGFR levels may confer a growth advantage in situations of limiting EGF concentrations (Gill et al., 1985). Further in vitro studies have demonstrated that EGF inhibits cellular proliferation at concentrations above 1 nM (Filmus et al., 1985). A431 is a human epidermoid carcinoma cell line which also exhibits EGFR amplification (Gill and Lazar, 1981) and overexpress EGFRs upto 1-2 \( \times 10^6 \) /cell (Merlino et al., 1984). Similarly, studies with A431 cells indicated that EGF induced growth inhibition could be observed at nanomolar concentrations, while it
stimulated cell proliferation at picomolar concentrations (Kawamoto et al., 1983).

Both MDA 468 and A431 cells overexpress the EGF receptor and are growth inhibited by nanomolar concentrations of EGF. Taking advantage of this fact studies have been performed with clonal variants of both cell lines to study the relationship between EGF receptor number and growth. Filmus et al. (1987) have isolated six clonal variants of MDA 468 cells. The six had EGFR levels similar to normal human fibroblasts, had lost EGFR gene amplification and were resistant to EGF-induced growth inhibition observed in the parental cell line. Karyotype analysis showed that MDA 468 cells had an abnormally banded region (ABR) in chromosome 7p, which was not present in the variants. Five of the six variants were able to generate tumors in nude mice with a lower growth rate from that of the parental cell line. Only one variant S4 was unable to generate tumors in nude mice and was unable to grow independently in soft agar in the absence of EGF (Filmus et al., 1987). The parental cell line was able to generate significantly larger tumors than the nonamplified variants. The basis for this size difference at 9 weeks may have been due to a growth rate advantage, since the parental cells had a shorter doubling time. Other variants most likely did not have a growth advantage as much as the parental cells did, because of their lack of EGFR gene amplification. Therefore, amplification of the EGFR gene provides a progression-related growth advantage (Filmus et al., 1987).
1.10 p53, A TUMOR SUPPRESSOR

p53 is a tumor suppressor gene, whose loss or inactivation is a very common lesion in human neoplasia (Bennett, 1999). Lack of p53 expression, although frequently a late event in tumorigenesis, is seen as a major step towards the development of the neoplastic phenotype. In certain cases, some breast cancer tissues inactivate p53 function without mutation to render the functional wild-type p53 to be non-functional. Examination of breast cancer specimens from 27 breast cancer tissues showed how important wild-type p53 is as a tumor suppressor. 30% (8 cases) had very high levels of p53 in the nucleus, 33% (9 cases) had a complete lack of detectable staining, 37% (10 cases) had a pattern of cytoplasmic staining with sparse nuclear staining. Nucleotide sequence analysis of p53 cDNAs derived from the samples with cytoplasmic staining revealed wild-type p53 alleles only in 6 out of 7 cases. In contrast, the samples containing nuclear p53 contained a variety of missense mutations and nonsense mutations. A normal lactating breast tissue also showed intense cytoplasmic staining for nuclear p53 with sparing nuclear staining. Thus, these data suggest that p53 protein may inactivate its tumor-suppressor activity by sequestering the wild-type p53 protein in the cytoplasm, away from its site of action in the cell nucleus (Moll et al., 1992).

p53 induces both growth arrest and apoptosis, and therefore it was suggested that the same pathways might mediate both responses. While this may be partially true,
it is also true that p53 might mediate apoptosis by pathways distinct from that of growth arrest. The separation of p53-dependent pathways for growth arrest and apoptosis may be based on selective induction of gene products specific to each pathway. For example, p53 has been shown to up-regulate pro-apoptotic genes such as Bax and to suppress anti-apoptotic genes such as bcl-2, by transcriptional activation or repression, thus altering the relative quantities of these genes and shifting the balance towards apoptosis. Also, promoter selectivity of p53 mutants can determine whether p53 induces growth arrest or apoptosis. For example, p53 also directly induces transcription of IGF-bp3, a protein that can inhibit both the mitogenic and anti-apoptotic activity of IGF-1, via a p53 consensus motif in the promoter region (Bennett, 1999).

p53 is a nuclear phosphoprotein, and its nuclear localization is essential for its growth-suppressing activity in late G1 (Shaulsky et al., 1990). The subcellular localization of p53 has been shown to vary throughout the cell cycle in Balb/c 3T3 cells. The newly synthesized protein accumulates in the cytoplasm during the G1 phase. Around the beginning of the S phase, p53 accumulates in the cell nucleus, where it stays for about 3 h. Following this step of DNA synthesis, p53 is no longer found in the nuclear compartment, but accumulates in the cytoplasm. This modulation in the subcellular localization of p53 suggests that the protein is spatially regulated during the cell cycle (Shaulsky et al., 1990).

Mutated forms of p53 have been implicated in the etiology of breast cancer
because mutant p53 is continuously required to maintain the transformed phenotype (Avila et al., 1994). The p53 mutation converting codon 273 arginine to histidine is frequently present in human tumors (Hollstein et al., 1991). This mutant, p53<sup>273.His</sup> has a wild-type epitope map, which is PAb 240 (this antibody recognises mutant p53) negative and PAb 1620 (this antibody recognizes wild-type p53) positive (Slingerland et al., 1993). MDA 468 cells possess a single allele for p53 with a point mutation at codon 273 (p53<sup>273.His</sup>) resulting in the substitution of arginine by histidine (Nigro et al., 1989; Bartek et al., 1990). Evidence suggests that subcellular localization of p53<sup>273.His</sup> has relevance to the growth suppressor function of p53, a function of wild-type p53 (Prasad and Church, 1997).

The p53<sup>273.His</sup> mutant in cooperation with H-ras showed a weak transforming potential in rat embryo fibroblasts even when it was highly expressed from the strong human β-actin promoter (Slingerland et al., 1993). It was concluded that this mutant allele lacked the ability to interfere with wild-type p53 protein in a dominant negative manner. When single copies of wild-type human p53 and p53<sup>273.His</sup> alleles were coexpressed in SAOS-2, an osteosarcoma cell line lacking endogenous p53 protein expression, the wild-type p53 phenotype function slowed growth, decreased saturation density, and reduced tumorigenicity (Chen et al., 1990). However, in the absence of wild-type p53, the mutant promoted cell growth to a higher saturation density than that of parental cells (Chen et al., 1990). In rat embryo fibroblast transformation assays, the
weakly transforming p53\textsuperscript{273His} became strongly transforming when the intact nuclear localization signal site was replaced by the mutated nuclear localization signal site (Slingerland et al., 1993).

This behavior of p53 has been explained in different ways. In the murine system, point mutations exert a common effect on the structure and function of p53. They result in the synthesis of p53 molecules with a different epitope structure. In cells expressing murine p53, a variable fraction of the p53 molecules are in the wild-type state. Bartek et al. (1990) called the p53 molecules with mutations in the primary sequence as "pseudo-wild type." On the other hand they have, on careful examination of cell lines with mutant p53, found a second population of molecules which they call "overtly mutant." These molecules are also present in human cell lines because both PAb 240 positive (overtly mutant) and PAb 1620 positive (pseudo-wild type) forms are derived from the same mRNA (Bartek et al., 1990). Halazonetis et al. (1993) have shown that wild-type p53 can adopt a "mutant" conformation and that conformational changes of wild-type p53 is associated with sequence-specific DNA binding. In fact under certain conditions wild-type p53 can transiently adopt the "mutant" conformation such as during cell division (Halazonetis et al., 1993). Thus p53 (mutant or wild-type) is associated with changes in its conformation under certain conditions and also in a majority of tumors which is manifest in its altered conformation.
1.11 AIM OF THE PRESENT WORK

MDA 468 cells overexpress the EGFR. Binding of EGF (or other natural ligands) causes autophosphorylation of the EGFR, which in turn triggers the binding of several signaling proteins such as PLC-γ, PI3'K, GAP etc. via their SH2 domains. Binding of ligands to the receptor triggers early and delayed biochemical events such as receptor dimerization, phosphorylation of the receptor, PI turnover, and other intracellular protein substrates, ultimately leading to mitogenesis (Modjtahedi et al., 1998). Several lines of evidence suggest that amplification, rearrangements, or abnormal expression of the EGFR gene are associated with neoplasia. Various strategies (discussed in sections 1.3, 1.3.1, and 1.3.2) have been developed to inhibit the activity of the EGF receptor tyrosine kinase.

Blocking the EGFR kinase activity can have growth inhibitory effects in cells leading to growth arrest, or the kinase activity inhibited cells can no longer survive and they ultimately die. Growth arrested cells can still survive as quiescent cells in G₀ when the EGFR pathway is blocked (Moyer et al., 1997). Studies with various monoclonal antibodies against the EGFR have shown that activation of the receptor is required for both progression through the cell cycle and for the prevention of apoptosis (Wu et al., 1995).

Receptor autophosphorylation is one means by which PTK function can be modulated. Taking advantage of this fact we aimed to study the EGF-mediated
signaling events associated with receptor tyrosine kinase activity. Initial experiments showed that EGF stimulated an increase in PI turnover (Church et al., 1992). On careful examination of the EGF-generated PI turnover profile, it became clear that a considerable portion of the metabolites were being converted to a metabolite of unknown identity, which eluted in the early part of the separation profile (Church et al. unpublished observations). Therefore, our initial aim was to identify this metabolite and quantitate it. This metabolite was found to constitute over 50% of the total inositol phosphates. EGF causes a stimulation of EGFR autophosphorylation and an increase in PI turnover; these are the immediate effects of EGF stimulation. It is assumed that activation of PLC-γ by the dimerized EGFR initiates PI turnover. Since EGF caused an increase in the generation of this metabolite, we hypothesized that the mitogenic response of these cells as a long term effect might, in some way, be related to the generation of this metabolite in the shorter term. In order to gain insight into this relationship between PI turnover and proliferative responses, we planned to use a PTK inhibitor to block the receptor autophosphorylation. By using this strategy we hoped to block the signaling pathway initiated by EGFR phosphorylation. Our aim was to examine the effect of receptor phosphorylation by inhibiting this process such that we could examine a response such as PI turnover, which is a direct effect of receptor autophosphorylation. We also wanted to investigate if modulation of receptor autophosphorylation affects the cell's proliferative responses. Receptor phosphorylation
and growth have a positive correlation at picomolar EGF concentration. This correlation however, does not hold good at nanomolar concentrations. In fact, at nanomolar concentrations, the relationship becomes inverse in cell lines overexpressing the EGFR, such as MDA 468 (Filmus et al., 1985), A431 (Gulli et al., 1996), and colorectal DFi cells (Gross et al., 1991). In this way we hoped to study the interdependence between EGF-mediated growth inhibition and EGFR kinase activity in MDA 468 cells and correlate EGF receptor tyrosine kinase activity to growth and progression of cells through the cell cycle.

Proliferation is reflected in the cell’s traversal through the cell cycle; our aim was to further study the role of the cell cycle checkpoint regulators by reflecting on any changes that might be occurring as a result of perturbations in the cell cycle. Previous work by Prasad and Church (1997) has demonstrated a role for p53\(^{273.\text{His}}\) in EGF-mediated growth inhibition. They proposed that EGF was clearly tilting the balance of p53 from mutant-like to more of a wild-type like conformation. They also suggested a conformational flexibility for this p53 mutant, where a dynamic equilibrium between two conformations in the nucleus would determine the G\(_1\)-S progression. In view of these findings, and evidence from the literature, which demonstrates an inhibition of receptor tyrosine kinase activity is responsible for cell cycle arrest, we wanted to further study a link between EGF-mediated signalling, proliferation and the possible role of p53 and other cell cycle proteins influencing the proliferation in MDA 468 cells. Keeping these
objectives in mind, we initially intended to use some of the widely used PTK inhibitors which would effectively block the EGFR kinase activity. We would subsequently choose the appropriate one for further investigation.
CHAPTER II

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Chemicals

Epidermal growth factor (EGF) was obtained from Collaborative Biomedical Products, Bedford, MA. A/G Plus Agarose was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Dimethyl sulfoxide (DMSO), protein A-sepharose beads, and propidium iodide were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. RNase was obtained from Boehringer Mannheim Canada (Laval, Québec).

2.1.2 Tissue Culture Reagents

Tissue culture reagents were all obtained from GIBCO BRL (Burlington, ON). These reagents included Leibovitz-15 (L-15) media, modified with L-glutamine, fetal bovine serum (FBS), methionine-free L-15, inositol-free Dulbecco’s Modified Essential Medium (DMEM), trypsin/EDTA, penicillin (10,000 IU/ml, and streptomycin 10,000 µg/ml.

Tissue culture ware included 75 cm² flasks from Falcon (distributed by Becton Dickinson, Mississauga, ON), 6-well plates, 60 mm, 10 cm and 100 cm dishes from Nalge Nunc International (Naperville, IL), and 8-well chambered glass slides from Nalge Nunc International.
2.1.3 DRUGS

1) Lavendustin A (LA) and lavendustin B (LB) were both obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Drugs were dissolved in DMSO in a concentration of 10 mg/ml and the stock solutions were stored in -20°C. Precautions were taken in handling the drugs because they are light-sensitive. Further dilutions were made in L-15 media (supplemented with or without FBS for tissue culture assays) or in sterile water.

2) Genistein and methyl 2,5-dihydroxycinnamate were purchased from GIBCO BRL. Baicalein was a kind gift from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). All three drugs were dissolved in DMSO and stored at -20°C; during the time of experiment appropriate dilutions were made in L-15 media (supplemented with or without FBS for tissue culture assays) or in sterile water. Genistein, and its analogue baicalein, were sonicated to dissolution in aqueous tissue culture media.

3) Cisplatin (1 mg/ml) was obtained from Faulding (Canada) Inc. (Vaudreuil, Québec).

2.1.4 ANTIBODIES

2.1.4.1 Primary antibodies

1) AntiEGFR, a mouse monoclonal antibody was obtained from Amersham Life
Science, Buckinghamshire, UK. This antibody was used for immunoprecipitation of EGFR from cells.

2) Antiphosphotyrosine (PY20), a mouse monoclonal (IgG2b\textsubscript{a}) antibody was obtained from Upstate Biotechnology, Lake Placid, NY.

4) Antip53 antibodies were obtained from Oncogene Science, Manhasset, NY (distributed by CedarLane, Mississauga, Ontario). These were as follows:
   a) PAb 1801 (Ab 2), a human-specific antibody (IgG\textsubscript{1}), reactive with both wild-type and mutant p53 was obtained from CedarLane (Oncogene Science, Manhasset, NY),
   b) PAb 240 (Ab 3), a mutant-specific and conformation-dependent antibody (IgG\textsubscript{1}) was obtained from CedarLane (Oncogene Science, Manhasset, NY),
   c) PAb 1620 (Ab 5), a human wild-type specific antibody (IgG\textsubscript{2b}K), was from Cedar Lane (Oncogene Science, Manhasset, NY).

5) Antip21\textsuperscript{WAF1/CIP1}, a mouse monoclonal antibody (IgG\textsubscript{2b}) was from Pierce and Biolynx (distributor).

6) Anticdk2, a rabbit polyclonal antibody (IgG), was obtained from Upstate Biotechnology, Lake Placid, NY.
2.1.4.2 Secondary antibodies

1) Sheep anti-mouse IgG horse radish peroxidase (HRP)-linked whole antibody was obtained from Amersham Life Science.

2) Antirabbit IgG-HRP conjugated secondary antibody was obtained from Santa Cruz Biotechnology.

3) Antimouse/antirabbit IgG-FITC (fluorescein isothiocyanate) conjugated secondary antibodies were obtained from Santa Cruz Biotechnology.

2.1.5 RADIOISOTOPES

\(^{3}\text{H}\)-myo-inositol (specific activity 20 Ci/mmol) was obtained from Mandel Scientific, Guelph, ON. \(^{3}\text{H}\)-myo-inositol phosphate mix consisting of inositol1-phosphate, inositol1,4-bisphosphate, and inositol1,4,5-trisphosphate (each 4.5 Ci/mmol) was obtained from DuPont NEN, Boston, MA. \(^{3}\text{H}\)-thymidine (specific activity 85.4 mCi/mmol) was obtained from Mandel Scientific Company.

2.1.6 HPLC MATERIALS

The Partisil SAX 10 column (25 X 0.46 cm) was a kind gift from Phenomenex (CA, USA); this column was fitted with a Partisil SAX 10 (Mandel Scientific Company,
Guelph, ON) direct contact guard cartridge. Standard DL-\textit{myo}-inositol 1,2-cyclic inositol monophosphate and ammonium phosphate (dibasic) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). Readyflow III scintillation cocktail was obtained from Beckman Instruments (Mississauga, ON).

**2.2 METHODS**

**2.2.1 TISSUE CULTURE**

MDA 468 cells were maintained in L-15 modified media supplemented with 10% FBS, 50 IU/ml penicillin, and 50 \(\mu\)g/ml streptomycin at 37°C in a \(\text{CO}_2\)-free incubator. In the case of serum-starvation, cells were transferred to serum-free L-15 media supplemented with antibiotics only.

**2.2.1.1 Routine tissue culture**

MDA 468 cells were routinely maintained in L-15 with penicillin/streptomycin in 75 cm\(^2\) flasks seeded at a cell density of \(1.0 \times 10^6\) cells. Cells were harvested by trypsinization at a 70% confluence weekly, and counted by hemocytometer.

MDA 468 cell stocks were maintained by freezing and then thawing as required. The cells were harvested by trypsinization, counted and a 1 ml (1-2 \(\times\) 10\(^6\) cells/ml) suspension was made. This suspension was transferred to a sterile cryotube and FBS (10% \(v/v\)) and DMSO (10% \(v/v\)) were added to the cryotube. The tube was closed tightly
and transferred to a Nalgene™ Cryo 1°C freezing container (with isopropanol) to achieve a -1°C/min rate of cooling and kept at -70°C overnight. The isopropanol was changed after every five uses and the container was kept at room temperature when not in use.

For thawing cells, cryotubes were taken from liquid nitrogen and transferred to a 37°C water bath for a few min. Then the contents of the tube were transferred to a sterile centrifuge tube and centrifuged. The pellet was washed with 1 x PBS to remove residual DMSO. Cells were then routinely cultured in 75 cm² flask for use in experiments.

2.2.2 IMMUNOPRECIPITATION and WESTERN BLOTTING

2.2.2.1 Drug Treatment

For phosphorylation experiments, 5 x 10⁵ cells were plated in six-well plates. The next day, cells were transferred to serum-free L15 and serum-starved for 48 h, at the end of which cells were treated with the drugs under investigation. After drug treatment, cells were stimulated with 10⁻⁸ M EGF for 10 min at 37°C and harvested for protein extraction. In separate experiments, 5 x 10⁵ cells were seeded in 60 mm dishes, and the next day cells were exposed to LA for a period of 6 h and harvested for protein extraction.
2.2.2.2 Protein extraction

After the various drug treatments, cell monolayers were washed twice with ice-cold 1 x PBS in the presence or absence of phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM pyrophosphate and 100 mM sodium fluoride) and then treated with ice-cold Pawson’s lysis buffer [50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF), 200 μM sodium orthovanadate, 10 mM pyrophosphate, and 100 mM sodium fluoride] (Church et al., 1992). Orthovanadate is a known inhibitor of tyrosine phosphatases (Daniel et al., 1996). Cells were harvested by scraping in lysis buffer using Costar sterile disposable cell scrapers. Cell lysates were collected in 1.5 ml microfuge tubes and kept in ice for 20 min with occasional mixing. Cell lysates were cleared by centrifugation at 13,000 x g for 15 min at 4°C. Supernatants were collected in separate microfuge tubes and protein analysis was done using the BioRad protein detection dye reagent concentrate (Bradford, 1976) in the microassay range (≤25 μg/ml) with bovine serum albumin (BSA) as the standard.

2.2.2.3 Immunoprecipitation

In the case of immunoprecipitation studies, the supernatants were mixed with the appropriate antibody (in a concentration as specified by the manufacturer) and incubated overnight with continual rotation at 4°C. The next day, 40 μl of 50% protein
A-Sepharose was added to the samples with antibodies and incubation was continued for another hour with constant rotation at 4°C. Immune complexes were washed 3 x with cold wash buffer (20 mM Hepes pH 7.5, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, and 1 mM sodium orthovanadate). Sepharose beads were collected each time (after washing) by a microcentrifuge pulse (~20 secs) and the supernatants discarded. The samples were boiled with 40 μl of 10% SDS sample buffer and separated by polyacrylamide gel electrophoresis (PAGE), followed by Western blotting and detection by Amersham's enhanced chemiluminescence (ECL) detection kit.

2.2.2.4 Western blotting

Protein extracts were subjected to PAGE for separation of proteins. Volumes of supernatants (of cell extracts) were adjusted to 20-25 μg per sample which was then loaded onto a gel; this volume was mixed with 2 x 10% SDS sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, β-mercaptoethanol, 0.05% bromophenol blue) heated for a few min at 100°C and loaded on to a 7.5% polyacrylamide gel and electrophoresed in a Mini-PROTEAN® II Dual Slab Cell (BioRad) minigel apparatus. The gel was run at 20 mA (milliamps) for 30 min followed by 30 mA constant current for 2 h to get sufficient protein separation. The stock (5X) electrode buffer used comprised of Tris (25 mM), glycine (250 mM), SDS (1%). This was diluted to 1 x and used as running buffer. At completion, the gel was washed in cold transfer buffer a few times to
remove the SDS in the running buffer. The gel was then ready for transfer.

For transfer, all components of the Western sandwich assembly were collected together. Fibre pads, filter paper, membrane were all soaked in cold transfer buffer (192 mM glycine, 25 mM Tris and 20% v/v methanol) for 15 min. The sandwich was assembled as per the manufacturer’s directions and transfer was done at 60 V (constant voltage) for 1½ h in a BioRad miniblot transfer apparatus at 4°C. The separated proteins were transferred to Hybond™-ECL Nitrocellulose-hybridization transfer membranes (Amersham). After transfer, the sandwich was disassembled and the membrane was blocked for non-specific binding sites by incubating in 5% non-fat milk in TBS-T (Tris-buffered saline and Tween-20) for 1 h or 1% milk/1% BSA (in TBS-T) in some cases as per manufacturer’s directions. The membrane was incubated overnight with the primary antibody (antiphosphotyrosine/antiEGFR) diluted in 5% non-fat milk (in TBS-T) according to the manufacturer’s directions with constant rocking at 4°C. The blot was washed with TBS-T for 1 h at room temperature with at least four changes. The membrane was incubated at room temperature for 1 h with constant rocking with the secondary antibody (sheep anti-mouse whole IgG HRP-linked antibody) diluted in TBS-T. Following this the membrane was washed extensively with TBS-T. After washing, the peroxidase activity was detected with Amersham’s ECL kit (reagent 1 and reagent 2 were mixed in equal volumes and spread over the whole surface area of the blot for 1 min) using Hyperfilm™-ECL high performance luminescence detection
films from Amersham. Exposed films of individual blots were scanned in a Chemiimager™ 4000 model of Low Light Imaging system (DigitallImaging and Analysis Systems, Alpha Innotech Corporation) attached to a Multiimage™ light cabinet for the determination of band intensities.

2.2.3 MEMBRANE EXTRACTION FROM MDA 468 CELLS

MDA 468 cells were grown in 100 cm dishes at 4x10^6 density. Confluent monolayers were then washed twice with ice-cold 1 x PBS after which membrane extraction buffer was added to the dishes. Cells were then scraped with a disposable cell scraper and collected in a homogenization tube. Cells were then homogenized in a variable speed laboratory rotor, TRI-R STIR-R (Model #S63C, Tri-R Instruments, Inc.) for 10-15 strokes. The suspension was then kept in ice for 1 min. This process was repeated five times to ensure complete disruption of cells. The homogenized cell suspension was transferred to 30 ml Corex glass tubes and were then centrifuged at 1,400 x g for 10 min. The supernatant was transferred to 70 Ti rotor ultracentrifuge tubes; the tubes were balanced and the samples were ultracentrifuged at 50,000 x g for 55 min in a Beckman Model L8-70M Ultracentrifuge. The supernatants were discarded and the pellets were resuspended in ice-cold membrane extraction buffer (without PMSF) and the protein content determined. The membrane preparations were aliqoted in cryovials and stored in liquid nitrogen for future use.
2.2.4 ELECTRON MICROSCOPY

2 x 10^5 cells were grown in 60 mm dishes and cells were treated with appropriate drugs in order to examine morphological changes of cell death. Cells were washed with 1 x PBS and harvested by trypsinization. The total population of cells were collected. The attached cell monolayer was washed and trypsinized and fixed.

The EM specimens were processed by a modification of the method of Hyam (1981). Any residual amounts of media were washed off and after one additional wash the cells were fixed by resuspending the cell pellet in 4% paraformaldehyde, 5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. The steps that followed sequentially are as follows:

1) brief wash 2x (5 min each) with 0.1M sodium cacodylate buffer (pH 7.4),
2) 1 wash (10 min) with 1% osmium tetroxide (in 0.1M sodium cacodylate) to fix any lipids in the specimen,
3) 1 wash in 0.1M sodium cacodylate buffer (5 min each),
4) 2 washes in 70% ethanol (5 min each),
5) 2 washes in 95% ethanol (5 min each),
6) 2 washes in absolute ethanol (10 min each),
7) 2 washes in absolute acetone (10 min each),
8) infiltration with 50:50 mixture of Epon (resin):acetone (10 min each),
9) infiltration with 100% Epoxy (Epon) resin-2 x (10 min each),
polymerization in 100% epoxy resin at 70°C overnight.

After polymerization, ultrathin sections (900Å) of the specimens were cut using a Reichert OMU 2 Model ultramicrotome, collected on 300 mesh copper grids and doubly stained in 2.5% uranyl acetate in ethanol and 0.3% aqueous lead acetate. The sections of doubly stained specimens were observed using a JOEL 1200 EX electron microscope and photographed using Kodak Electron Image Film #4489. Prints were made using Kodak Ektomatic black and white paper.

2.2.5 DNA FRAGMENTATION

3.0 x 10⁶ cells were seeded in 100 mm dishes. The next day, cells were treated with LA for 14 h. Adherent cells were washed with 1 x PBS and harvested by trypsinization. Floating cells from the media were pelleted and mixed with trypsinized cells and then the whole population of cells centrifuged. The method used was a modification of Oridate et al. (1996). The pellet was washed a few times with 1 x PBS, resuspended in 0.85% NaCl solution and centrifuged for 5 min. The pellet was then resuspended in nuclear lysis buffer (10% SDS, 20 mg/ml proteinase K and 50 mM Tris/EDTA, pH 8.0) overnight at 37°C. The next day 150 mM NaCl was added and centrifuged, and DNA was precipitated by mixing ethanol (2 volumes) with the supernatant at -20°C. The pellet was washed twice with 70% ethanol, air dried and resuspended with 10 mM Tris/EDTA (8.0) with RNAse (20 µg/ml). Samples were
incubated at 37°C for 2 h for RNA digestion (with RNAse). DNA concentration was determined at 260 nm and 10 μg of DNA was loaded in each well and electrophoresed in a 1.5% agarose gel against 1 kB standard (Gibco BRL). The gel was stained with ethidium bromide, visualized by UV fluorescence, and photographed.

2.2.6 FLOW CYTOMETRIC ANALYSIS (FACS)

To analyze the DNA content of cells and to investigate the percentages of cells in the various phases of the cell cycle, cells were subjected to flow cytometric analysis. 2 x 10^5 cells were seeded in 60 mm dishes. The next day, cells were treated with 0.2 μM LA, 10^-8 M EGF, and 0.2 μM LA+10^-8 M EGF respectively. Cells were treated with the drugs for 72 h; in the case of growth reversibility experiments, the cells were treated for 72 h and at the end of the 72 h period, the drug-containing media was removed and replaced with fresh media for a further 24 h period. In other experiments, cells were treated with 0.125-1 μM LA and incubated for 14 h after which cells were harvested. At the end of the incubation periods, the cells were harvested by trypsinization and the cell pellets were washed 2 x with 1 x PBS and fixed in 50% ethanol (in PBS) for 1 h at -20°C. The cells were centrifuged and the pellet was washed 3 x with 1 x PBS to remove residual ethanol. The cells were then treated with Vindelov's propidium iodide stain (0.01 M Tris pH 8.0, 10 mM NaCl, 700 U RNAse, 0.05 mg/ml propidium iodide, Nonidet P-40) (Krishnan, 1975), which was filtered through 0.45 μM membrane filter and stored
at 4°C. As prepared, the stain is stable for a month after preparation. Cells were treated with propidium iodide for 2h at 4°C. Prior to analysis, the propidium iodide treated cell suspension was passed through 50 micron Filcon syringe type filters (Dako) to remove clumps and the clump-free suspension was transferred to Falcon 6 ml sterile tubes (Becton Dickinson) prior to analysis. Analysis was done in a FACStarplus (Becton Dickinson) fluorescence-activated cell sorter (FACS) fitted with a 50 Watt argon laser source, set at an excitation wavelength of 488 nm. Approximately 10,000 cells were analyzed per sample. The fraction of cells in different phases of the cell cycle, namely G0/G1, S and G2M, was determined using CELL Quest Software (Version 3.1) in a Power Mackintosh 7300/200 computer.

2.2.7 MTT ASSAY

The cytotoxic effect of LA in MDA 468 cells was determined by the 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by a modification of the method of Ford et al. (1989). 1 x 10^4 cells were seeded in each well in a 96-well plate. The next day cells were treated with drugs in doubling dilutions, five wells for each concentration as well as controls (which included untreated control, 0.5% DMSO and a media blank). The cells were exposed to the drug for 24 h. The next day, the drug-containing media was removed and the cells were washed 3x with 1 x PBS and replaced by fresh media for another 24 h. The following day, 0.5 mg/ml MTT dissolved
in L15 media was added to each well and a 4 h incubation period followed. At the end of 4 h, L15 containing MTT was removed and replaced by DMSO and the resulting absorbance was read in a BIORAD Model 3550 Microplate Reader.

2.2.8 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

2.2.8.1 Extraction of inositol phosphates from MDA 468 cells

Inositol phosphates were determined by labelling the cells with \([^{3}H]\)-myo-inositol followed by the separation of the metabolites by high pressure liquid chromatography (HPLC). All samples were extracted under neutral conditions. The method of extraction was as described by Wong et al. (1988). Briefly, 1 x 10^6 cells were seeded in 6-well plates. The next day cells were washed twice with 1 x PBS and switched to inositol-free DMEM under serum-free conditions and 2 \( \mu \)Ci \([^{3}H]\)-myo-inositol was added for 48 h (5% \( \text{CO}_2 \) at 37°C). After 48 h 10 mM LiCl was added and incubated for 30 min (5% \( \text{CO}_2 \), 37°C) followed by the addition of 10^8 M EGF for yet another 20 min under the same conditions.

After the incubation period, the cells were washed 2 x with ice-cold 1 x PBS (1 ml each) and the washes were discarded into radioactive waste. Then 1 ml of quenching buffer (phenol/chloroform/EDTA) was added and left on ice for 20 min. Cells were scraped with Costar sterile disposable cell scrapers and the contents transferred to autoclaved Corex glass tubes. Two more 0.5 ml washes were carried out and
combined in the same Corex tubes. In a second Corex tube, 2 ml mixture of phenol/chloroform/butanol (38:24:1 v/v) was made and the cell washes combined with it. The mixture was vigorously vortexed for 30 secs for proper mixing and then centrifuged at 1370 x g for 10 min. The upper phase was withdrawn for analysis by HPLC.

2.2.8.2 Separation of inositol phosphates by HPLC

Inositol and its charged metabolites were separated by HPLC by the modification of the method of Dean and Moyer (1987). Separation was achieved through a Partisil SAX 10 column (25 x 0.46 cm) with a Partisil SAX 10 C direct contact guard cartridge. All standards and samples were filtered through Millipore syringe type 45 μM filters (Nihon Millipore, Kogyo, Japan) before application to the column. Water (A) and ammonium phosphate (B, pH 3.8) were used to separate the inositol phosphates. A flow rate of 1 ml/min was maintained throughout the gradient. 1 ml fractions were collected in tubes in a Pharmacia FRAC-100 fraction collector. Each fraction was mixed with 8 ml of Readyflow III scintillation cocktail and analyzed for radioactivity by scintillation counting. The resulting data were analyzed by calculating the area under the curves for the individual inositol phosphate peaks using a computer based "INPLOT" software package (Graphpad, San Diego, CA), which uses a cubic spline curve fitting programme. All graphs were done in GraphPad Prism™ (Version
2.2.9 ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY (ESI MS/MS)

HPLC fractions were collected and freeze-dried in a LABCONCO Freeze Dry-5 freeze drying machine. Standard solutions for ESI MS/MS and CAD MS/MS were prepared in a mixture of HPLC solvent grade methanol-water (CH₃OH-H₂O; 60-40%) and analyzed on a Micromass VG Quattro quadrupole-hexapole-quadrupole mass spectrometer equipped with a megaflow electrospray ionization source capable of analyzing ions up to 4000 m/z (mass over charge ratio) operating on a negative-ion mode. A 20 µl aliquot of each sample was introduced into the electrospray ion source with a continuous flow of CH₃OH-H₂O, (60-40%) with a flow rate of 10 µl/min using a Shimadzu LC-10AD pump connected to the Rheodyne injector with a 20 µl loop. The temperature of the ES ionization source was maintained at 75°C. The operating voltage of the ES capillary was 3.00 kV and the high voltage lens was set at 0.40 kV throughout the whole operation. ES mass spectra were recorded with a cone voltage of 25 V. Mass spectra were obtained by scanning in the multi-channel analysis (MCA) mode with a scan time of 1 sec per 250 a.m.u. MicroMass MASSLYNX 3.2.0 Mass Spectrometry Data System software running on a Compaq Desk Pro (Pentium processor 266 MHz) with Windows NT 4 was used for data acquisition and analysis. CAD MS/MS analysis
experiments were conducted using the same instrument. Fragment ion spectra of mass-selected compounds were induced by collision with argon in the RF-only hexapole. The resulting fragments were analyzed by the second quadrupole. A cone voltage varying from $3.5 \times 10^4$ to $6.5 \times 10^4$ mbar (1 bar = $10^5$ Pa) were used in all MS/MS experiments.

2.2.10 GROWTH ASSAYS

Cells were seeded at a density of $2 \times 10^5$ in 60 mm dishes. The next day drug ± EGF were added to the media for the appropriate time (24 or 72 h). In all cases there was a no-treatment control, an EGF-treated control and a 0.5% DMSO control (the concentration of DMSO did not exceed 0.5%).

2.2.10.1 Cell count and [3H]-thymidine assays

At specified time intervals, cells were harvested by trypsinization and counted by hemocytometer. Each treatment group was done in duplicate in four separate experiments.

For DNA synthesis assays, cells were labelled with 3.0 μCi per ml [3H]-thymidine for 24 h. At the end of the incubation period, cells were washed with cold 1x PBS, harvested by trypsinization and precipitated by ice-cold 10% trichloroacetic acid (TCA). The TCA-precipitate was filtered through filters (glass microfibre filters GF/A 21 mm Whatman); the filters were washed twice with cold 10% TCA and counted in 5 ml of
biodegradable counting scintillant (BCS scintillation fluid, Amersham) and in a Beckman counter (Model LS 3801).

2.2.11 IMMUNOFLUORESCENCE EXPERIMENTS

Cells were grown in glass-chambered slides and subjected to appropriate treatments (with $10^{-6}$ M EGF, 0.2 μM LA and EGF+LA) at 37°C. The method used was a modification of the method of Banasiak and Haddad (1998). Following incubation, cells were washed with 1 x PBS in Couplin jars and fixed in methanol (5 min) and acetone (15 min) at -20°C and then air-dried. The cells were washed 2 x with PBS and permeabilized with 0.2% Triton X-100/PBS for 5 min at room temperature in a humidified chamber. The cells were then washed with PBS. The cells were blocked with 3% bovine serum albumin/PBS (BSA/PBS) for 1 h at room temperature in a humidified chamber. The cells were then incubated with primary antibody diluted in 1.5% BSA/PBS overnight in a humidified chamber at 4°C. The next day cells were washed with 1 x PBS (3x) and incubated with 1:50 dilution of fluorescein-conjugated antimouse/antirabbit (as appropriate) secondary antibody for 30 min in the dark in a humidified chamber and washed with PBS (3x) and mounted with 10% glycerol/PBS and viewed under an Olympus BHT System fluorescence microscope fitted to a CoolSNAP digital camera system controlled by the CoolSNAP software (Version 1.1) with a Seanix Computer with Windows 98. All pictures were taken at the original magnification of 200X
(eyepiece-10X X objective lens-20X). Portions of the microscope images were selected in Adobe Photoshop, (Version 5.4), and page-fitted in a page using Corel Draw (Version 7.0). The computer generated images as displayed herein were enlarged by a factor of 3; this produced a final magnification of 600X.
CHAPTER III

INHIBITION OF EGF RECEPTOR KINASE ACTIVITY

BY LAVENDUSTIN A: EFFECTS ON EGF

RECEPTOR PHOSPHORYLATION AND VIABILITY

IN MDA 468 CELLS
3.1 INTRODUCTION

Growth factors produce cellular signals that are critically important for influencing diverse cellular responses including proliferation, differentiation and cell survival. Activation of growth factor receptors at the plasma membrane is the initial step in signal transmission to the cell interior (Resh, 1993). The effects of many growth factors are known to be mediated by receptor tyrosine kinases.

Binding of ligand to the extracellular domain activates the tyrosine kinase in the cytoplasmic domain, thus triggering a sequence of events between receptors and cellular components, which, in turn, are responsible for the generation of second messengers (Fantl et al., 1993). Growth factors such as the epidermal growth factor (EGF), platelet derived growth factor (PDGF), and insulin enhance the phosphorylation of specific cell surface receptors leading to the downstream activation of common signaling molecules. Frequently activated proteins include phospholipase C-γ (PLC-γ), phosphatidylinositol 3'-kinase (PI3'K), GTPase-activating protein (GAP), and MAP kinases (Fantl et al., 1993). The ultimate result of activation of these molecules leads to changes in gene expression and phenotypic state of the cell.

The 53 amino acid polypeptide growth factor, EGF causes the proliferation of a variety of cells (Carpenter, 1984). This proliferative response of EGF is mediated by the 170 kD EGF receptor (EGFR). Increased expression and activity of the EGF receptor has been reported in several types of tumors including squamous cell
carcinomas of the lung, skin, oral cavity, and esophagus, brain tumors (glioblastomas and meningiomas), breast carcinomas (Gross et al., 1991), colon adenocarcinomas (Normanno et al., 1998) and pancreatic adenocarcinomas (Friess et al., 1996). EGFR overproduction has been associated with amplification of the EGF receptor gene in tumors of the head and neck (Grandis et al., 1998). In squamous cell carcinomas of the head or neck, disease-free survival and cause-specific survival was reduced in patients with higher levels of EGFR and TGF-α (Grandis et al., 1998). In the DiFi cell line derived from a hereditary colorectal carcinoma there is overexpression of the EGF-receptor-specific mRNA and overproduction of its membrane-associated EGFR (Gross et al., 1991). Increased level of EGFR expression in tumors, its clear involvement in the malignant process and its accessibility on tumor cell surfaces (Modjtahedi et al., 1998) makes interception of the overexpressed EGFR activity an attractive target for the delivery of therapeutic agents aimed at restricting tumor progression and metastases.

Study of the receptor protein, relationship to growth control and carcinogenesis have often relied on human tumor cell lines overexpressing the receptor. MDA 468 is an estrogen receptor negative human breast cancer cell line which overexpresses the EGFR to approximately $1.5 \times 10^6$ EGFRs/cell due to a 20-fold gene amplification located to an abnormal banding region on one copy of chromosome 7 (Filmus et al., 1985). The properties of the overexpressed receptor appear normal with respect to the binding affinity, synthesis, turnover, and autophosphorylation (Filmus et al., 1987;
Kudlow et al., 1986).

3.1.1 Importance of EGFR in clinical studies

Human breast cancer cell lines, malignant mammary epithelial cells and their surrounding stromal cells are responsible for the synthesis of a large number of growth factors (Ma et al., 1998). The EGF ligand family includes EGF, transforming growth factor alpha (TGF-α), amphiregulin, heparin binding EGF (HBEGF) (Chandler et al., 1998), hepatocyte growth factor (HGF) (Ma and Niederkorn, 1998), and related polypeptides that stimulate the EGFR (Chandler et al., 1998). It has been postulated that the requirement of serum in cell culture is primarily due to the need for specific polypeptide growth factors and growth promoters. Inhibition of cell growth has been suggested to result from the blocking of EGF receptor activation by growth factor present either in the culture medium or produced by the cells in an autocrine fashion (Wu et al., 1995). EGFR overexpression is often accompanied by the production of one or more of its ligands by the same tumors suggestive of an autocrine loop which may be responsible for proliferation (Modjtahedi et al., 1998). It has been seen that in about 20-60% of EGFR overexpressing tumors in vivo, TGF-α is also overexpressed suggesting the presence of an autocrine loop which may provide a growth advantage to such tumors (Ma et al., 1998). Another ligand, amphiregulin, is also coexpressed (with EGF) in breast cancer cell lines and tumors (Plowman et al., 1990).
The EGFR plays an extremely important role in tumor progression and maintenance, and also EGFR and its ligands have been shown to be potentially involved in the growth of breast cancers in vivo. On an average, 45% of the breast cancers investigated by 40 different study groups were shown to be EGFR positive (Klijn et al., 1992). EGFR overexpression has been shown to have a positive correlation with poor prognosis and a high risk of relapse (Ma et al., 1998).

EGFR expression is an important target of inhibition in many cancers because it is presumed to be one means by which cells acquire a selective growth advantage (Chandler et al., 1998). EGFR expression has been correlated with metastatic potential and capacity of human blood-borne uveal melanoma cells to localize in the liver. EGFR renders these cells resistant to the cytolytic activity of TNF-α. This is also true of colon carcinomas; colon carcinomas form progressive liver metastases in nude mice because TGF-α and HGF produced in the liver stimulate the proliferation of tumor cells by interacting with EGFR expressed in a wide variety of normal and malignant cells. EGFR expression is also correlated with the growth and metastases of hepatic, renal, laryngeal, esophageal and lung cancers (Ma and Niederkom, 1998).

Blocking EGFR with a neutralizing antibody increased the susceptibility of uveal melanoma cells to transforming growth factor-α (TNFα) mediated cytolytic effects (Ma and Niederkom, 1998). Also, it has been shown that growth factor deprivation by the use of anti-EGFR monoclonal antibody (MAb) 225 in DiFi colorectal carcinoma cells,
induces apoptosis (Mandal et al., 1998). In a pilot study, where breast fine needle aspirations were performed on 224 high-risk and 30 low-risk women, it was found that overexpression of EGFR occurred in 37% of high risk and 3% of low risk subjects; the percentage of subjects with EGFR overexpression was higher than any other parameter measured including p53, ER (estrogen receptor) and HER-2/neu overexpression in both high and low risk patients (Fabian et al., 1997).

3.1.2 Approaches to inhibition of the EGFR

PTK inhibitors are useful tools to study the mechanism of action of various oncogenes exhibiting tyrosine kinase activity and perhaps also in the suppression of neoplastic diseases (Onoda et al., 1989). Tyrosine kinase inhibitors have been used to dissect various intracellular effects of receptor and non-receptor kinases in a variety of in vivo and in vitro systems. It has been reported that inhibition of the EGFR kinase blocks EGF-dependent cell proliferation (Gazit et al., 1989). A class of PTK inhibitors, the tyrphostins are antiproliferative because they block the EGF-induced EGFR phosphorylation and subsequent PLC phosphorylation and Ca\(^{2+}\) release in living cells. Both of these EGF induced signaling steps lead to a cascade of molecular events ultimately leading to DNA synthesis and growth (Margolis et al., 1989).
3.1.3 EGFR phosphorylation and cell viability

Growth factor-stimulated EGFR kinase activity has been studied extensively. A correlation between cell survival and EGFR tyrosine kinase activity in growth factor dependent signaling pathways has been reported (Ertel et al., 1998). Overexpression of the EGFR and elevated tyrosine kinase activity is associated with tumors of various types (Ward et al., 1994). Downregulation of the receptor kinase activity by anti-EGFR MAb 225 induces apoptosis in human colorectal carcinoma DiFi and FER cells (Mandal et al., 1998). DiFi cells, which also overexpress the EGFR, were found to be sensitive to inhibition of EGFR kinase activity which eventually resulted in a loss of cell viability. EGFR blockade in EGFR overexpressing tumors induces cell death by apoptosis (Modjtahedi et al., 1998). It is also well established that ligand deprivation leading to inhibition of the EGFR tyrosine kinase activity causes cell death by apoptosis (Moyer et al., 1997).

In hematopoietic cells apoptosis is prevented by the inhibition of protein tyrosine kinase phosphorylation in response to a variety of stimuli (Hagar et al., 1997). In contrast, in thymocytes (Otani et al., 1993) and Molt-4 cells (Falcieri et al., 1993) inhibition of PTK activity enhances apoptotic mode of cell injury in thymocytes. In ovarian tumour cells, taxol (an antineoplastic agent)-induced apoptosis can be prevented by inhibition of PTK activity (Liu et al., 1994). From these studies it can be seen clearly that tyrosine phosphorylation is linked to apoptotic cell death.
3.1.4 Objectives

The basis of experiments described in this chapter deals with the modulation of EGF-stimulated EGFR tyrosine kinase activity in MDA 468 cells. To this end, we used different PTK inhibitors to achieve inhibition of autophosphorylation of the EGFR. Initially these inhibitors were screened for their ability to inhibit EGFR autophosphorylation in isolated membrane preparations from serum-starved MDA 468 cells. Among the inhibitors screened, the PTK inhibitor lavendustin A (LA) effectively inhibited the EGF-stimulated EGFR autophosphorylation in a time and concentration dependent manner. Since we intended to use this inhibitor for physiological studies, we also tested this inhibitor for any effects on cell viability in a time and concentration dependent manner. The results of this chapter lay the foundation for further experiments where the effects of blocking EGFR phosphorylation were investigated in MDA 468 cells.

3.2 RESULTS

3.2.1 Identification of the 170 kD EGFR

EGF receptor phosphorylation is the initial event in downstream signaling events following EGF binding. In the initial experiments we confirmed the identity of the 170 kD EGFR protein by immunoprecipitating the EGFR with a mouse monoclonal antibody specific to the protein. Figure 3.1 (lane 1) represents the immunoprecipitated 170 kD EGFR band in response to EGF treatment. EGF greatly increased the phosphotyrosine
Figure 3.1 Identification of the 170 kD EGFR band in MDA 468 cells.

Cells were serum-starved for 48 h following which they were treated with $10^{-8}$ M EGF for 10 min. Immunoprecipitated EGFR from EGF-treated cells and cell lysates of control (untreated) and EGF-treated cells were run on a 7.5% polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane, probed with antiphosphotyrosine-specific PY20, and detected by enhanced chemiluminescence (Section 2.2.2, Chapter 2). This Western blot depicts the phosphotyrosine content of the immunoprecipitated EGFR and of EGFR from cell lysates of unstimulated control and EGF-treated cells. The blot was probed with PY20 as described in the Materials and Methods (Section 2.2.2, Chapter 2).

Lane 1-immunoprecipitated EGFR from cells stimulated with EGF.
Lane 2-phosphotyrosine content of EGFR of cell lysate from unstimulated control cells.
Lane 3-phosphotyrosine content of EGFR of cell lysate from cells stimulated with EGF.
content of the EGF-stimulated receptor (lane 3) with respect to the non-stimulated control (lane 2).

3.2.2 Effect of PTK Inhibitors on the autophosphorylation of the EGF-stimulated receptor

In order to test the most effective PTK inhibitor capable of inhibiting the EGF-stimulated EGFR autophosphorylation, we used the inhibitors genistein, baicalein (an analogue of genistein), methyl 2,5-dihydroxycinnamate (MHC), lavendustin B (LB), and lavendustin A (LA) in crude membrane preparations of serum-starved as well as whole MDA 468 cells. Examination of the chemical structures showed that (Figure 3.2) LA and LB possess three benzene rings, A, B, C with a 2,5-dihydroxybenzene moiety in ring A. LB (LB is an analogue of LA) has a similar structure with the exception of a hydroxyl (–OH) group at position 5 in ring A. MHC has a 2,5-dihydroxybenzene moiety and in genistein the hydroxyl groups are in the 3 and 5 positions of the benzene ring. In the following experiments we wanted to test whether the structural similarities and the dissimilarities among these PTKs would have an effect on the EGF-stimulated EGFR autophosphorylation.

Cognizant of potential difficulties in obtaining sufficient drug concentration intracellularly, we tested them initially in crude membrane preparations of serum-starved MDA 468 cells. Genistein inhibited receptor autophosphorylation (Figure 3.3, lane 4)
Figure 3.2 Chemical structures of PTK inhibitors.

Benzene ring A is present in all the 4 inhibitors. LA, LB, genistein and MHC possess a hydroxybenzene moiety (denoted by a square).
LAVENDUSTIN B

LAVENDUSTIN A

METHYL 2,5-DIHYDROXYCINNAMATE

GENISTEIN
Figure 3.3 Effect of PTK inhibitors on the autophosphorylation of EGFR in crude membrane preparations of serum-starved MDA 468 cells.

Cells were serum-starved for 48 h following which crude membrane preparations were prepared as described in Materials and Methods (Section 2.2.2, Chapter 2). Serum-starved cells were exposed to PTK inhibitors listed below and then treated with $10^{-8}$ M EGF for 10 min to test for their ability to inhibit the EGF-stimulated EGFR autophosphorylation. Cell lysates of control (untreated), EGF-treated, and inhibitor+EGF-treated cells were run on a 7.5% polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane, probed with antiphosphotyrosine-specific PY20, and detected by enhanced chemiluminesence (described in Section 2.2.2, Chapter 2). This Western blot depicts changes (inhibition or no response) in EGFR autophosphorylation in response to genistein, MHC, LA, and LB.

Lane 1-control,
lane 2-$10^{-8}$ M EGF,
lane 3-1% DMSO+$10^{-8}$ M EGF,
lane 4-250 μM genistein+$10^{-8}$ M EGF,
lane 5-250 μM baicalein+$10^{-8}$ M EGF,
lane 6-530 μM MHC+$10^{-8}$ M EGF,
lane 7-1 μM LA+$10^{-8}$ M EGF,
lane 8-1 μM LB+$10^{-8}$ M EGF.
at a concentration of 250 μM. MHC showed no activity (lane 6). Both LA and LB effectively inhibited the receptor phosphorylation (lanes 7, 8). This differential sensitivity to these PTK inhibitors was also evident in serum-starved whole MDA 468 cells. Genistein was ineffective even at a concentration of 350 μM (10 h) (Figure 3.4, lane 3). Cells exposed to as much as 530 μM (2 h) MHC (lane 5) did not show any reduction in receptor autophosphorylation. LB inhibited the receptor autophosphorylation in isolated membrane preparations, but failed to inhibit receptor autophosphorylation at 1 μM after 6 h of exposure (lane 6).

3.2.3 Time-dependent inhibition of EGF-stimulated EGFR autophosphorylation by LA

In the following experiments, we tested the ability of LA to inhibit the EGF-stimulated receptor autophosphorylation in serum-starved MDA 468 cells. Although LB was effective in membrane preparations (Figure 3.3) but did not show any effect even after 6 h of exposure in whole cells (Figure 3.4), even after 10 h exposure (not shown), LB has been reported to have an IC<sub>50</sub> value of 1.3 μM (Onoda <i>et al.</i>, 1989) of inhibition of receptor phosphorylation. As seen from Figure 3.5, LA induced inhibition of the EGF-stimulated receptor autophosphorylation in a time-dependent manner; this inhibition was not pronounced before 2 h of exposure (lanes 6-7). Inhibition appeared complete at 6 h (Figure 3.5, lane 4). Therefore, in later experiments a period of 6 h incubation was
**Figure 3.4 Lack of effect of some PTK inhibitors on EGF-stimulated EGFR autophosphorylation in serum-starved MDA 468 cells.**

Serum-starved cells were exposed to PTK inhibitors listed below and then treated with 10⁻⁸ M EGF for 10 min to test for their ability to inhibit the EGF-stimulated EGFR autophosphorylation. Cell lysates of control (untreated), EGF-treated, and inhibitor+EGF-treated cells were run on a 7.5% polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane, probed with antiphosphotyrosine-specific PY20, and detected by enhanced chemiluminescence (described in Section 2.2.2, Chapter 2). This Western blot depicts changes in EGF-stimulated EGFR autophosphorylation in response to the PTK inhibitors tested. Arrows to the left of figure indicate molecular weights of protein standards.

Lane 1-control,
lane 2-10⁻⁸ M EGF,
lane 3-350 μM genistein (10 h)+10⁻⁸ M EGF,
lane 4-350 μM baicalein (10 h)+10⁻⁸ M EGF,
lane 5-530 μM MHC(2 h)+10⁻⁸ M EGF,
lane 6-1 μM LB (6 h)+10⁻⁸ M EGF.
Figure 3.5 LA inhibits the EGF-stimulated autophosphorylation of EGFR in a time-dependent manner in MDA 468 cells.

Serum-starved cells were exposed to 1 \( \mu M \) LA from 15 min to 10 h and then treated with \( 10^{-8} M \) EGF for 10 min to test for its ability to inhibit the EGF-stimulated EGFR autophosphorylation. Cell lysates of control (untreated), EGF-treated, and inhibitor+EGF-treated cells were run on a 7.5% polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane, probed with antiphosphotyrosine-specific PY20, and detected by enhanced chemiluminescence (described in Section 2.2.2, Chapter 2). Arrows in the figure indicate molecular weights of protein standards.

Lane 1-control,

lane 2-\( 10^{-8} M \) EGF,

lane 3-1 \( \mu M \) LA (10 h)+\( 10^{-8} M \) EGF,

lane 4-1 \( \mu M \) LA (6 h)+\( 10^{-8} M \) EGF,

lane 5-1 \( \mu M \) LA (4 h)+\( 10^{-8} M \) EGF,

lane 6-1 \( \mu M \) LA (2 h)+\( 10^{-8} M \) EGF,

lane 7-1 \( \mu M \) LA (15 min)+\( 10^{-8} M \) EGF.
chosen as a time period during which LA effectively inhibited the EGF-stimulated EGFR autophosphorylation. Interestingly, LA-induced inhibition of receptor autophosphorylation could be completely reversed 4 h after removal of the drug from the medium (Figure 3.6, lane 5). Taken together, these results showed that LA effectively inhibited EGF-stimulated EGFR autophosphorylation in a reversible and a time-dependent manner in MDA 468 cells.

3.2.4 Dose-dependent inhibition of receptor autophosphorylation by LA

LA was found to effectively inhibit receptor autophosphorylation in a dose-dependent manner (Figure 3.7A). LA inhibited the EGF-stimulated EGFR autophosphorylation in MDA 468 cells with an IC$_{50}$ value of 0.8 μM (800 nM) as predicted from a sigmoidal dose response curve (Figure 3.7B). The IC$_{50}$ of inhibition of autophosphorylation of EGFR was much higher in MDA 468 cells in contrast to the reported IC$_{50}$ value of 20 nM for the inhibition of EGFR phosphorylation in A431 cells (Onoda et al., 1989).

3.2.5 Time and dose-dependent effect of LA on cellular morphology and DNA fragmentation

MDA 468 cells treated with 1 μM LA were examined by light microscopy and
Figure 3.6 LA induces reversal of inhibition of EGF-stimulated autophosphorylation of the EGFR.

Cells were treated with 1 μM LA for a period of 6 h, and then the drug containing medium was removed at specified intervals. At the end of these intervals, cells were stimulated with $10^{-8}$ M EGF for 10 min and protein extraction was done. Cell lysates of control (untreated), EGF-stimulated, and LA+EGF-treated cells were loaded on a 7.5% gel (Section 2.2.2, Chapter 2).

Lane 1-control,
lane 2-$10^{-8}$ M EGF,
lane 3-1 μM LA+$10^{-8}$ M EGF,
lane 4-1 μM LA 2 h after removal of LA,
lane 5-1 μM LA 4 h after removal of LA.
Figure 3.7 LA inhibits the EGF-stimulated EGFR autophosphorylation in a dose-dependent manner in MDA 468 cells.

Cells were serum-starved for 48 h were exposed to various concentrations of LA for 6 h and stimulated with $10^{-8}$ M EGF for 10 min.

3.7A A Western blot showing the concentration-dependent effects of LA on the EGF-stimulated phosphotyrosine content of the EGFR.

Lane 1-control,

lane 2-$10^{-8}$ M EGF,

lane 3-0.5 % DMSO+$10^{-8}$ M EGF,

lane 4-1.0 μM LA+$10^{-8}$ M EGF,

lane 5-0.5 μM LA+$10^{-8}$ M EGF,

lane 6-0.25 μM LA+$10^{-8}$ M EGF,

lane 7-0.125 μM LA+$10^{-8}$ M EGF,

lane 8-0.01 μM LA+$10^{-8}$ M EGF.

3.7B A dose-response curve was constructed from densitometric analysis of blots from 5 separate experiments. Results were normalized for each experiment using unstimulated controls as zero and EGF-treated group as 100%. A sigmoidal dose-response curve was fitted to the data from which an IC$_{50}$ value of 0.8 μM was determined for the inhibition of EGFR phosphorylation.
quantitation showed that beyond 10 h, a large population of cells were surrounded by apoptotic bodies (not shown). This was confirmed by electron microscopy (EM). Cellular morphology was examined at various times (up to 24 h) after LA treatment (Figure 3.8). Cells exhibited membrane blebbing and nuclear condensation (14 h). At 14 h 0.5 and 1 μM LA-treated cells exhibited classical ultrastructural features of apoptosis (Konstantinov et al., 1998) such as chromatin condensation, cell shrinkage, vacuolization, and membrane blebbing (Figure 3.9). Since morphological evidence indicated that LA might be causing cell death by apoptosis, we examined the DNA fragmentation pattern by DNA gel electrophoresis. Figure 3.10 shows that at higher concentrations, LA produced DNA smearing (Lanes 3 and 4) similar to the positive control, cisplatin (20 μg/ml)-treated cells (Lane 7). Cisplatin is a known cytotoxic agent with DNA binding capability (Coste et al., 1999). We did not see a DNA laddering pattern which is produced as a result of smaller DNA fragments (180 bp), as is commonly seen in apoptotic cell death (Moyer et al., 1997).

3.2.6 Effect of LA on cell cycle

Morphological evidence (Figures 3.8 and 3.9) is consistent with LA-induced apoptotic cell death in MDA 468 cells exposed beyond 10 h, as compared to cisplatin-treated cells. We next examined if treatment of cells with LA produced any perturbations in the distribution of cells in various phases of the cell cycle (Figure 3.11 and Table 3.1).
Figure 3.8 LA (1 µM) induces changes in cellular morphology of MDA 468 cells beyond 10 h exposure time.

Cells were seeded in 60 mm dishes. The next day, cells were treated with 1 µM LA for different periods of time in order to identify a time period when cells lost viability. Cells were trypsinized, fixed, and the cell pellets were resuspended in 0.1 M sodium cacodylate buffer, pH 7.4. EM specimens were processed as described in Materials and Methods (Section 2.2.4, Chapter 2) and viewed under a JOEL 1200 EX electron microscope at 3000X and photographed.

1-control
2-1 µM LA-treated cells (8 h)
3-1 µM LA-treated cells (10 h)
4-1 µM LA-treated cells (14 h)
5-1 µM LA-treated cells (24 h)
6-20 µg/ml cisplatin (a positive control)-treated cells (8 h)

B denotes blebbing (of membrane)
Figure 3.9 LA induces changes in cellular morphology of MDA 468 cells in a dose-dependent manner at 14 h.

Cells were seeded in 60 mm dishes. The next day, cells were treated with different doses of LA for a period of 14 h. Cells were trypsinized, fixed, and the cell pellets were resuspended in 0.1 M sodium cacodylate buffer, pH 7.4. EM specimens were processed as described in Materials and Methods (Section 2.2.4, Chapter 2) and viewed under a JOEL 1200 EX electron microscope at 3000X and photographed.

A-control untreated cells
B-1 µM LA-treated cells
C-0.5 µM LA-treated cells
D-0.25 µM LA-treated cells
E-0.125 µM LA-treated cells

In the figures, B denotes membrane blebbing; V-vacuoles; RER-rough endoplasmic reticulum; M-mitochondria.
**Figure 3.10** Dose-dependent effect of LA on DNA fragmentation at 14 h.

Cells were seeded in 10 cm dishes. The next day LA was applied in various concentrations and cells were exposed to the drug for a period of 14 h. At the end of this period, cells were harvested by trypsinization and DNA was extracted as described in Materials and Methods (Section 2.2.5, Chapter 2).

Lane 1-1 kb DNA marker

Lane 2-untreated control

Lane 3-1 μM LA-treated cells

Lane 4-0.5 μM LA-treated cells

Lane 5-0.25 μM LA-treated cells

Lane 6-0.125 μM LA-treated cells

Lane 7-cisplatin (20 μg/ml)-treated cells
Figure 3.11 LA produces concentration-dependent effects in cell cycle distribution at 14 h in MDA 468 cells.

Representative FACS generated DNA histograms of MDA 468 cells treated with LA for 14 h. The histograms are a representation of propidium iodide (PI) fluorescence (FL2-A) versus cell number. The PI fluorescence is directly proportional to the amount of DNA in the cell and the relative cell number is given by counts. DNA was analyzed using PI fluorescence. Approximately 10,000 cells were analyzed per sample as described in the Materials and Methods (Section 2.2.6, Chapter 2). A0 denotes a subdiploid peak.

1-untreated control
2-1 μM LA-treated cells
3-0.5 μM LA-treated cells
4-0.25 μM LA-treated cells
5-0.125 μM LA-treated cells

0.5 μM (3) and 1 μM (2) LA caused a decrease in the surviving cell population (represented in the G₁, S and G₂M peaks respectively), compared to 4 (0.25 μM) and 5 (0.125 μM) respectively. In 2 and 3 respectively, a prominent subdiploid peak (A0) is present.
Table 3.1 Percentage distribution of cells in various phases of the cell cycle in response to different concentrations of LA after 14 h incubation.

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<th>G\textsubscript{1}</th>
<th>S</th>
<th>G\textsubscript{2M}</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.3 ± 0.95</td>
<td>33.9 ± 0.52</td>
<td>14.9 ± 1.60</td>
</tr>
<tr>
<td>1 μM LA</td>
<td>41.1 ± 0.74</td>
<td>21.0 ± 0.54</td>
<td>37.9 ± 1.21*</td>
</tr>
<tr>
<td>0.5 μM LA</td>
<td>36.3 ± 0.50</td>
<td>18.5 ± 2.60</td>
<td>47.8 ± 2.60*</td>
</tr>
<tr>
<td>0.25 μM LA</td>
<td>42.8 ± 1.41</td>
<td>17.8 ± 1.30</td>
<td>39.4 ± 2.10*</td>
</tr>
<tr>
<td>0.125 μM LA</td>
<td>61.8 ± 1.62</td>
<td>24.6 ± 1.80</td>
<td>13.6 ± 1.83</td>
</tr>
</tbody>
</table>

Data are represented as mean ± S. E. M. of four experiments. *Values are significantly higher than control (p<0.005).
Flow cytometry showed the appearance of a subdiploid peak (A₀), at concentrations of 0.5 and 1 μM after cells were exposed for 14 h. LA treatment caused a distinct G₂M arrest at concentrations between 0.25-1 μM with a concomitant decrease of cells in the G₁ and S phases respectively (Figure 3.11 and Table 3.1).

3.2.7 Toxicity of LA in MDA 468 cells

EM, DNA fragmentation, and cell cycle analysis data are consistent with the idea that LA might induce cell death by apoptosis. From Figure 3.7 we found that when cells were exposed to 1 μM LA, there was an inhibition in the EGF-stimulated phosphorylation. However, it was evident that beyond 10 h exposure, cells lost their viability. Thus, 1 μM LA was lethal to the cells at longer exposures. We therefore tested the cytotoxic activity of LA using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in MDA 468 cells (Cole, 1986; Alley et al., 1988) with 1 μM as the highest concentration. To predict an TC₅₀ value for the cytotoxicity of LA, we exposed the cells for a period of 24 h to various concentrations of LA. A 24 h exposure time is a more sensitive assessment of the cytotoxicity (or cell killing) activity of a cell cycle specific drug (Matsushima et al., 1985). Cells were found to be growth arrested at 14 h, and therefore, the MTT was used to assess cell viability following a 24 h period of exposure to LA. Cells were exposed to doubling dilutions up to 1 μM, the highest concentration used earlier. Figure 3.12 shows that LA was also highly cytotoxic at
Figure 3.12 Toxicity of LA in MDA 468 cells at 24 h.

Cells were grown in 96-well plates. The next day LA (at a starting concentration of 1 µM) was applied in doubling dilutions in five wells for each concentration and the cells were exposed to drug for a period of 24 h. After 24 h cells were washed with 1 x PBS and fresh media was applied and the cells were incubated for a further 24 h period. The next day 0.5 mg/ml MTT (in L15) was added to the cells for 4 h. At the end of 4 h, MTT was removed and replaced by DMSO and the resulting absorbance was read at 540 nm (Section 2.2.7, Chapter 2). Each concentration is represented as a mean ± S. E. M. of five data points.
concentrations which caused maximal inhibition of EGFR autophosphorylation. In MDA 468 cells the TC\textsubscript{50} of LA was determined to be 0.4 \( \mu \text{M} \) from the fitted sigmoidal dose response curve (Figure 3.12). At a lesser exposure time (6 h) of cells to LA in the same concentrations, we found that LA caused inhibition of the EGFR autophosphorylation, but inhibited the biochemical effect with a much higher 50\% value (IC\textsubscript{50} was 0.8 \( \mu \text{M} \); Figure 3.7b).

MTT is reduced by mitochondrial dehydrogenases (succinate-tetrazolium reductase system of the mitochondrial respiratory chain) to form an insoluble formazan salt (Bradshaw et al., 1998), which absorbs maximally at 540 nm. The MTT is cleaved by metabolically active cells with an active respiratory chain, and therefore, this assay detects only viable cells (Cole, 1986; Alley et al., 1988), which would reduce the MTT to form insoluble formazan crystals. Following exposure of drug to the cells, cells were allowed a further 24 h recovery period. From this dose-response curve we were able to predict the effective non-toxic concentration range (and exposure time) of LA to use for further studies.

### 3.3 DISCUSSION

Receptor autophosphorylation is a central event in growth factor signaling. The tyrosine kinase domain of the EGFR, intracellular to the ligand binding region, is not only responsible for the receptor-triggered stimuli, but also non-specific stimuli like cell-cell
contact or chemical insult. Thus this domain is regarded as an attractive target for various PTK inhibitors developed in an attempt to inhibit uncontrolled proliferation in the study of malignant diseases. Our aim was to find a drug that would effectively block EGF-stimulated EGFR tyrosine kinase activity in whole MDA 468 cells.

Our experiments with a selection of drugs showed that EGF-stimulated EGFR autophosphorylation in MDA 468 cells could only be inhibited by the PTK inhibitor LA. LA was reported to be a potent inhibitor of EGFR autophosphorylation in A431 cells (Onoda et al., 1989). LA, first isolated from the butyl acetate extract of Streptomyces griseolavendus culture filtrate, is a competitive inhibitor of intracellular ATP (Onoda et al., 1989). However, there have been reports that LA is a “mixed-type” inhibitor i.e. it is competitive with respect to ATP binding as well as the ligand EGF with the receptor (Hsu et al., 1991). As a potent PTK inhibitor, LA blocked the autophosphorylation of the EGFR in MDA 468 cells. LA matched the criteria for successful inhibitors like stability in biological media, selectivity, cell permeability, and formulability respectively (Levitzki and Gazit, 1995; Traxler, 1997).

MDA 468 cells exhibited a differential sensitivity towards the PTK inhibitors tested (Mandal et al., 1998). From results in this chapter, we can hypothesize a systematic structural relationship between the PTK inhibitors used and correlate it with the response of the MDA 468 cells to the inhibitors. Of the inhibitors with a 2,5-dihydroxybenzene moiety, namely LA, and MHC (Figure 3.2), only LA effectively
inhibited EGFR autophosphorylation in crude membrane preparations and in whole cells. LA has an additional -OH group as compared to LB and genistein has two -OH groups in positions 3 and 5 of the benzene ring. LB was effective only in crude membrane preparations. Therefore it can assumed that having the 2,5-dihydroxyl benzene moiety with the other structures in LA must provide the essential criteria necessary for cell permeability and effectiveness in inhibiting autophosphorylation in MDA 468 cells. Interestingly, this moiety present in LA is also present in another PTK inhibitor, erbstatin (we did not test this compound), which is a competitive inhibitor of substrate binding to the receptor (Burke, 1992). It has been shown from structure-activity studies that this simplified structure of LA may contain the key elements necessary for PTK inhibition (Onoda et al., 1989). Changing the positions of -OH groups (3 and 5 of A in genistein), the absence of the additional hydroxyl group (at position 5 of A in LB), or the presence of the 2,5-hydroxylbenzene moiety alone (as in MHC) were probably inadequate to meet the structural requirements for effective inhibition of EGFR autophosphorylation in MDA 468 cells.

The ineffectiveness of the other PTK inhibitors with the exception of LA may also be due to factors other than their structural characteristics. Pharmacological properties such as stability in biological media, cell permeability, or metabolism of the inhibitor are some of the factors which also determine the potency of a particular drug (Levitzski and Gazit, 1995; Traxler, 1997). Several erbstatin derivatives were synthesized with
improved stability, but the result was poor cell permeability (Traxler, 1997). MHC was synthesized as an erbstatin derivative with improved stability and cell permeability, and it, like genistein, was found to be very effective in inhibiting EGFR autophosphorylation in A431 cells (Umezawa et al., 1990). Since MHC was not effective either in MDA 468 membrane preparations (Figure 3.2) or in whole cells (Figure 3.3), it may be indicative of the instability of the compound under the conditions used (in membrane preps.), as well as perhaps poor penetration (in whole cells), or both (this is particularly true in case of whole cells). The required effective concentration of some PTK inhibitors may be higher than that of monoclonal antibodies (MAb) because larger number of inhibitor molecules are also required to prevent phosphorylation of the five tyrosines on the EGFR molecule compared to one MAb which can block activation of two receptors (Modjtaheidi et al., 1998). In some cases, PTK inhibitors (those that are competitive with ATP binding to the intracellular membranes) that are potent \textit{in vitro} are ineffective in whole cells unless the intracellular concentration of ATP is reduced artificially (Levitzski and Gazit, 1995). We have no data of the energy content of the MDA 468 cells after serum starvation. Genistein, a natural isoflavone, isolated from the fermentation broth of \textit{Pseudomonas sp.}, is a highly specific inhibitor of tyrosine kinases. It was found to effectively inhibit the EGF-stimulated tyrosine phosphorylation of the EGFR overexpressing cell line, A431 (Akiyama et al., 1987), but was ineffective in MDA 468 cells, which also overexpress the EGFR (Figure 3.3). It is difficult to say, but inhibitors
used, other than LA, were perhaps ineffective in inhibiting the receptor kinase activity due to a high intracellular ATP content which actually blunted the effect of the inhibitors.

In this study, LA can produce an inhibition of receptor tyrosine kinase activity in a time and concentration dependent manner at short incubation times (6 h) without cytotoxicity. Ultrastructural changes in morphology of MDA 468 cells treated with 1 μM LA at various times show that this concentration is lethal to the cells when exposed for a period beyond 10 h (Figure 3.8). Cells start to show changes in morphology such as membrane blebbing, nuclear condensation, and vacuolization at 14 h (Figures 3.8 and 3.9). In addition, the appearance of a pronounced subdiploid peak in cells treated with 0.5-1 μM LA (14 h) from flow cytometric analysis is also consistent with apoptotic cell death (Figure 3.11). From these data we concluded that at longer incubation times, LA at higher concentrations produces a loss of cell viability at concentrations which inhibited EGFR kinase activity maximally. However, we observed DNA smearing (Figure 3.11) rather than a fragmentation pattern commonly observed in apoptosis (Moyer et al., 1997) in response to LA or cisplatin treatment. Internucleosomal DNA degradation results in DNA ladder formation, although the absence of DNA laddering is not unprecedented in apoptotic cell death. Apoptosis without DNA ladder formation has been reported (Tomei et al., 1993; Gulli et al., 1996; Oridate et al., 1996). It has also been shown that DNA can be cut into larger (30 kbp) rather than smaller (180 bp) fragments, resulting in a DNA smear instead of a DNA ladder on gel electrophoresis
It is also possible that internucleosomal DNA fragmentation as a result of LA treatment may be occurring at a later time point beyond 14 h. Probably this is the reason we saw an absence of DNA laddering; we observed smearing in response to higher doses of LA and cisplatin-treated cells.

Apoptosis or programmed cell death (PCD) occurs in response to both physiological and pathological stimuli in a variety of tissues. This mode of cell death may be triggered by specific inducers, such as cytotoxic agents, irradiation, growth factor deprivation, hormones, or by blockers of G1, S, or M phases of the cell cycle (Alison and Saraf, 1992; Columbel et al., 1992; Ryan et al., 1993; Amstrong et al., 1994; Yamamoto et al., 1999). Such is the case in squamous cell carcinomas overexpressing the EGFR where inhibition of the receptor by anti-EGFR MAbs or PTK inhibitor causes the cells to undergo apoptosis (Modjtahedi et al., 1998). In MDA 468 cells too we speculate that LA may be producing cell death by blocking EGFR tyrosine kinase activity which may be thus playing a functional role in cell death progression.

EGFR overexpression is an important factor in the progression and maintenance of a wide variety of cancers. Besides extensive laboratory research of the EGFR signaling pathway in different cell lines and xenographic studies, the approach of interception of the EGFR kinase activity has advanced to phase II clinical trials. Thus, the concept of inhibition of growth factor receptor-mediated signal transduction via inhibition of its PTK is a novel concept which is and will be in future greatly utilized in
clinical studies in attempts to stop unwanted proliferation of malignant cells. Using this estrogen receptor-negative human breast cancer cell line model, we have been able to interfere with EGF-stimulated EGFR kinase pathway to further investigate the proliferative outcome of MDA 468 cells.
CHAPTER IV

INHIBITION OF EGF RECEPTOR TYROSINE KINASE ACTIVITY BY LAVENDUSTIN A: EFFECTS ON PI TURNOVER IN MDA 468 CELLS
4.1 INTRODUCTION

4.1.1 Background

Stimulation of MDA 468 cells by the epidermal growth factor (EGF) leads to the autophosphorylation of EGF receptor (EGFR). From the results of chapter 3, it became clear that this growth factor stimulated effect was inhibited by the protein tyrosine kinase (PTK) inhibitor lavendustin A (LA) after a 6 h exposure. Longer incubation with LA however, became toxic to the cells. In this chapter we discuss the effect of inhibition of EGF-mediated EGFR autophosphorylation on an early signaling event following receptor autophosphorylation, namely phosphatidylinositol (PI) turnover.

Activated EGFR associates with a number of signaling molecules. It is known that EGF stimulation leads to an increase in PI turnover because of EGFR interaction with phospholipase C-γ (PLC-γ). This comes about due to a high-affinity interaction between autophosphorylated tyrosine 992 in the EGFR molecule and PLC-γ (Rotin et al., 1993) through its SH2 domain (Koch et al., 1991). Earlier work from this laboratory (Church et al., 1992) demonstrated an increase in PI turnover in response to EGF. On closer examination it was found that MDA 468 cells exhibit an atypical PI turnover profile, unlike that generated by PLC-γ (Church et al., unpublished observations). Here we show that changes in EGFR phosphorylation, either by EGF stimulation or by inhibition with the PTK inhibitor LA, correspond with changes in an acid labile PI metabolite, which we identify as myo-inositol 1,2-(cyclic) monophosphate (cIP) using
electrospray ionization mass spectrometry (ESI MS/MS). The implications of the formation of cIP in these cells as a result of changes in the status of EGFR autophosphorylation are discussed.

4.1.2 PI turnover and cIP

Binding of EGF in a wide variety of cells (Fantl et al., 1993) expressing the 170 kD epidermal growth factor receptor (EGFR) leads to receptor dimerization and rapid phosphorylation (Margolis et al., 1989) leading to the downstream phosphorylation and activation of substrates including PLC-γ. The tyrosyl phosphorylation of PLC stimulates the increased hydrolysis of the membrane phosphatidylinositols (PIs), namely phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to the generation of inositol phosphates and diacylglycerol (DAG) (Rhee et al., 1989; Ross et al., 1991).

When PLC cleaves PI (direct cleavage) or when PIP_2 is cleaved (indirect cleavage) both cyclic and non-cyclic inositol phosphates are generated (Ryu et al., 1987). The enzymatic removal of phosphates at positions 4 and 5 of the inositol ring results in the production of myo-inositol 1,2-(cyclic) monophosphate (cIP) (Connolly et al., 1986; Kim et al., 1989). In most mammalian systems the proportion of cyclic and non-cyclic inositol phosphates generated depends upon which of the various isoforms of PLC interact with the activated receptor. All three isoforms of PLC (namely β, δ, γ)
catalyze the hydrolysis of the PI (Ryu et al., 1987). The ratio of production of cyclic to non-cyclic phosphates decreases in the order of PLC-β>PLC-δ>PLC-γ (Connolly et al., 1986). Only 5-10% of the PLC-γ hydrolysis products are cyclic in contrast to 90-95% of non-cyclic PI products comprising of DAG, IP (inositol monophosphate), IP₂ (inositol bisphosphate) and IP₃ (inositol trisphosphate) (Berridge and Irvine, 1989; Rhee and Choi, 1992). Accumulation of cyclic PI metabolites in response to agonist stimulation has been observed in mouse pancreas (Dixon and Hokin, 1985; Sekar et al., 1987; Dixon and Hokin, 1987), in kidneys of mouse, rat, guinea pig and ox (Dawson and Clarke, 1973), human platelets (Tarver et al., 1987), Limulus photoreceptor cells (Wilson et al., 1985) and SV40 transformed STU-51A/232B mouse cells (Koch and Diringer, 1974). In some species, such as the intracellular bacterial pathogen L. monocytogenes, a specific PLC produces only cIP and DAG from PI breakdown (Camilli et al., 1991). In Morris hepatomas and in cell lines derived from these tumors, high levels of cIP (100 μM) have been observed (Graham et al., 1987) compared to non-detectable levels in normal liver tissues (Ross et al., 1991).

Evidence suggests that cIP and its polyphosphates have physiological functions in cells. They have been shown to act as second messengers (Putney and Bird, 1993) correlated with cell growth (Ross et al., 1991). Thus, if cIPs are physiologically important, a mechanism for their breakdown must be present (Sekar et al., 1997). Dawson and Clarke (1972) showed the existence of an enzyme known as cyclic inositol
phosphohydrolase (cIPH) which had a high degree of specificity because it cleaved the cyclic bond of cIP between the phosphates at positions 1 and 2 only, but not the higher cyclic inositol polyphosphates (Ross and Majerus, 1986). This 1:2 cyclic bond in cIP is also sensitive to acid hydrolysis (Dean and Moyer, 1987). The activity of this enzyme has been found to change in disease. cIPH released per day was found to be several hundred-fold higher in urine of patients with various pathological conditions, such as diabetes, hypertension, multiple myeloma, renal transplants and stroke compared to the relatively low activity in normal, healthy volunteers (Sekar et al., 1997). Thus, cyclic inositol phosphates are potentially important components of the PI metabolic pathway and changes in their metabolism may be indicative of disease.

4.1.3 Analysis and identification of PI metabolites in MDA 468 cells

Prelabeling cells with radiolabeled inositol enables the investigation of components of PI turnover after their extraction and separation from the cells (Dean and Moyer, 1987). To avoid dilution of the radiolabel leading to inadequate labeling of the inositol pools, we used an inositol-free media and labeled the inositol pools for a period of 48 h. Lithium chloride (10 mM) was added to the media before adding EGF, because Li⁺ inhibits several of the inositol phosphomonoesterases and causes increased accumulation of inositol phosphates (Dixon et al., 1992; Lee et al., 1992; Dixon and Hokin, 1994); without Li⁺ the increase in levels of the IPs is substantially reduced.
because of the rapid turnover of these compounds in tissues. The use of Li⁺ is dependent on the objective of the experiment. For example, if an increase in IP₃ is to be related to an increase in [Ca²⁺], then Li⁺ should be avoided. But if a measurement of the extent of receptor activation is done, the paccumulation of inositol phosphates in the presence of Li⁺ is a more accurate measure of the state of activation of the cell (Dean and Beaven, 1989). We extracted the inositol phosphates under neutral conditions to avoid breakdown of any acid-labile PI metabolites (Dean and Beaven, 1989).

High pressure liquid chromatography (HPLC) has been the method of choice for separating the PI metabolites, both cyclic and non-cyclic. Most HPLC procedures employ anion-exchange column (example, Partisil SAX 10) and an aqueous mobile phase of ammonium formate, acetate, or phosphate to elute the water-soluble inositol phosphates. Stepped gradients are employed now, although original procedures used linear gradients. In our experiments, we have used ammonium phosphate gradient and a Partisil SAX 10 anion-exchange column with stepwise increments in concentrations of ammonium phosphate to allow adequate time for the separation of PI metabolites (Dean and Beaven, 1989). This aqueous mobile phase gradient gave us a good separation. The elution profile of non-cyclic IPs were compared with the available tritiated IP standards (described in Chapter 2, Section 2.2.8.2).

To confirm the identity of the acid labile PI metabolite, we used the technique of
low energy collision tandem mass spectrometry (ESI CAD MS/MS) using electrospray ionization mass spectrometry. Mass Spectrometry using electrospray ionization (ESI MS) was selected as a method of choice for the purpose of identification of the PI metabolite since this ionization method did not require derivatization of the analytes. ESI MS allows rapid, accurate and sensitive analysis of a wide range of analytes of a broad spectrum of natural products from the low molecular weight polar compounds (100 Daltons) to biopolymers larger than 200 kDa. The ESI MS technique usually yields the intact protonated or deprotonated molecular ion. By using ESI MS/MS identification of unknown substances can be achieved by their characteristic ion fragmentation pattern (Gysler et al., 1999). Dissociation may be induced or activated by collision. In this process part of the kinetic energy of the ions is converted to internal energy by colliding with a neutral gas phase species, usually in the pressurized collision cell of the tandem mass spectrometer (MS/MS) instrument. Ions that have undergone this collisional excitation process may subsequently fragment. Thus, collision activated dissociation (CAD) MS/MS and in particular low energy CAD MS/MS is a valuable method for generating structural information (Busch et al., 1988).

4.1.4 Objectives

From the results of Chapter 3, it is clear that stimulation of MDA 468 cells with growth inhibitory concentrations of EGF (10^{-8} M), caused autophosphorylation of the
EGFR. This effect could be inhibited by the PTK inhibitor, LA. It was demonstrated by Church et al. (1992) that 10^8 M EGF caused an overall increase in the total PI turnover in the MDA 468 cells. In the course of further experiments, it became clear that a considerable portion of the PI turnover constituents consisted of a metabolite which was acid-labile. Our aim was to identify this metabolite and to study the effect of both the stimulation and inhibition of EGFR phosphorylation on the PI turnover profile in this cell line. We further demonstrated that EGFR autophosphorylation-mediated increase in PI turnover was largely due to increase in cIP production, and that this increase was inhibited by the PTK inhibitor, LA in MDA 468 cells.

4.2 RESULTS

4.2.1 Separation of IPs and their HPLC profiles in EGF and LA treated cells

Since increased PI turnover has been linked to EGFR autophosphorylation, we examined the PI turnover profiles in both EGF and LA+EGF treated cells (6 h). Figure 4.1A shows the HPLC profile of individual [³H]-inositol phosphate standards. Figures 4.1B, 4.1C, and 4.1D show the representative profiles of [³H]-inositol phosphates of control, EGF and LA treated, serum-starved MDA 468 cells, respectively. Compared to the standard profile, we found that cell extracts from all three groups (B, C, and D respectively) showed a peak eluting between fractions 9-14 (peak 3). As can be seen
Figure 4.1 Effect of EGF and LA on the profiles of inositol phosphates in MDA 468 cells.

Cells were labelled with $[^3]$H]-myo-inositol for 48 h under serum-free, inositol-free conditions at 5% CO$_2$ at 37°C followed by the addition of 10 mM LiCl for 30 min. Cells were treated with 1 µM LA for 6 h and stimulated with 10$^{-6}$ M EGF for 20 min and inositol phosphates were extracted under neutral conditions as described under Materials and Methods (Section 2.2.8.1, Chapter 2). Samples were analyzed by HPLC and inositol and its charged metabolites were separated by HPLC using a Partisil SAX 10 column (Section 2.2.8.2, Chapter 2). Representative HPLC profiles generated by EGF and LA treatment are shown: standard inositol phosphates (A), untreated control (B), EGF-treated (C) and LA-treated (D) MDA 468 cells.

Peak “3” is the component which corresponded to the changes as a result of various changes in B, C, and D as mentioned above.
from Figure 4.1B, the untreated control cells showed that peak 3 was a major (60%) constituent of the PI metabolites in the HPLC profile. Interestingly, peak 3 showed a large increase upon $10^{-8}$ M EGF treatment (Figure 4.1C) while 1 μM LA prevented the EGF-mediated increase (Figures 4.1D).

4.2.2 Effect of acid treatment on peak 3

Peak 3 elutes immediately after the $[^{3}H]$-myo-inositol peak comparing that with similar data from published literature (Dean and Moyer, 1987). We therefore suspected that this peak could be cIP. If this was the case, peak 3 eluting between fractions 9-14, might be acid labile. Therefore, we separated the IPs from a control, untreated cell lysate and collected fractions from it (Figure 4.2A). We then subjected the fractions harboring this peak (peak 3) to acid hydrolysis for 3 min with 0.1 M HCl at 80°C. As can be seen from Figure 4.2B, as a result the peak was abolished and 90% of the radioactivity was recovered as the inositol 1-phosphate (I-1P) and inositol 2-phosphate (I-2P) peak. From this experiment we obtained biochemical evidence that peak 3 was acid-labile and that subjecting it to acid hydrolysis caused its breakdown to IP.

4.2.3 Identification of peak 3 by ESI MS/MS analysis

To confirm the identity of this acid labile metabolite, we subjected peak 3 (Figure 4.1B) to mass spectrometric analysis. We analyzed a sample of available cIP standard
Figure 4.2 Effect of acid-treatment on peak 3

Cells were seeded and then after 48 h inositol phosphates were extracted under neutral conditions. The PI turnover metabolites as a result of different treatments are shown in Figure 4.1. A control, untreated sample before acid treatment (A); the same sample was treated with 0.1M HCl at 80°C for 3 min and then run on the column with the gradient. Acid hydrolysis of the sample was done to test if peak 3 was acid-labile or not. Counts in peak 3 disappeared on acid treatment and the counts were recovered as the I-1P peak (B).
by negative ion electrospray mass spectrometry (Figure 4.3A) at m/z 241. The ESI MS showed the presence of the deprotonated molecule [M-H]⁻ at m/z 241 and the deprotonated dimer [2M-H]⁻ at m/z 483 and no fragmentations were observed. Low energy collision activated dissociation (CAD MS/MS) analysis was conducted to enhance the fragmentations observed in [M-H]⁻ at m/z 241 in the conventional electrospray mass spectrum of ciP. The product ion spectrum (CAD MS/MS) arising from the fragmentation of precursor ion at m/z 241 in the RF-only hexapole collision cell is presented in Figure 4.3B. The connectivity between fragment ions was established using MS/MS analysis of precursor and fragment ions. The CAD tandem mass spectrum of deprotonated ciP molecule shown in Figure 4.3B suggested that the series of product ions originated from ciP precursor deprotonated molecule. Figure 4.3C shows the CAD tandem mass spectrum of the precursor ciP deprotonated molecule (obtained from peak 3, Figure 4.1B).

4.2.4 Effect of EGF and LA on the overall PI turnover and the proportion of cyclic and non-cyclic IPs

Cells were labelled with [³⁵H]-myo-inositol for 48 h and then treated with 10⁻⁶ M EGF and 1 µM LA (6 h) and the cellular content of total inositol phosphates were determined as described in Materials and Methods (Chapter 2; Section 2.2.8). Figure 4.4 shows the changes in the overall PI turnover and the change in the proportion of
Figure 4.3 ESI MS and CAD MS/MS of standard ciP and peak 3.

Neutrally extracted samples from HPLC fractions (9-14) were collected, freeze-dried and analyzed using ESI MS/MS and CAD MS/MS on a Micromass VG Quattro quadrupole-hexapole-quadrupole mass spectrometer. Fractions harboring peak 3 (Figure 4.1B, C and D respectively) were compared to the standard ciP for the specific fragmentation pattern. A) is the electrospray mass spectrum of deprotonated standard ciP molecule [M-H]− at m/z 241. B) is the product ion spectrum (CAD MS/MS) of product ion arising from the fragmentation of m/z 241, and C) is the CAD MS/MS spectrum of the precursor deprotonated molecule derived from the HPLC fraction (peak 3, 4.1B) (described in Section 2.2.9, Chapter 2).
Figure 4.4 Effect of EGF and LA on the total PI turnover and the relative proportions of cyclic and non-cyclic PI metabolites in MDA 468 cells.

Neutral extracts from serum-starved cells treated with EGF (10^{-8} M) and 1 \mu M LA+ 10^{-8} M EGF respectively were analyzed by HPLC and the overall PI turnover and the change in the proportion of cyclic and non-cyclic phosphates were quantitated from the area under the curves. Results are expressed as mean ± S. E. M. of four experiments each performed in duplicate. *Total PI turnover significantly higher control (p<0.05); **total PI turnover significantly lower compared to control (p<0.005).
cyclic and non-cyclic phosphates in response to EGF (10⁻⁸ M) and LA (1 μM)+10⁻⁶ M EGF respectively. As seen from Figure 4.4, EGF causes a significant increase (p<0.05) in the overall PI turnover, and LA causes a significant decrease (p<0.005) when compared to the control. Untreated control MDA 468 cells showed a very high proportion of cIP which was about 60% of the total inositol phosphates. EGF stimulation more than doubled the cIP fraction, accounting for 80% of the total IP. In comparison, LA treatment resulted in more than a 90% decrease in cIP production. Clearly, the fraction responsible for the overall change in PI turnover in these cells was cIP, and thus changes in receptor phosphorylation status are reflected through the changes in the proportion of cIP alone in MDA 468 cells.

4.3 DISCUSSION

Cyclic inositol phosphates have been reported to have cellular functions. It is known that cyclic inositol phosphates produced as a result of PI turnover accumulate and persist longer as a result of agonist stimulation (Putney and Bird, 1993). [³H]-myo-inositol prelabelled pancreatic minilobules incubated with carbamylcholine for 30 min, showed increases in cIP, cIP₂, and cIP₃ which preceded increases in inositol mono, bis and trisphosphates, respectively (Sekar et al., 1987), suggesting a second messenger role for the cyclic metabolites. Furthermore, the metabolite cIP₃ was found to be five times more potent than IP₃ in increasing Ca²⁺ conductance in Limulus photoreceptor
cells (Wilson et al., 1985). The accumulation of cIP$_3$ (Dixon and Hokin, 1987) and the formation of cIP upon agonist stimulation in mouse pancreatic minilobules (Dixon and Hokin, 1985) all suggest a second messenger function for cIP.

PLC-γ is a substrate of the EGF and platelet derived growth factor (PDGF) receptor kinases. This phosphorylation is thought to be responsible for the increase in PLC-γ activity in cell types where these growth factors stimulate PI turnover (Meisenhelder et al., 1989). Certainly, both EGF and PDGF stimulate PI turnover in 3T3, A431 (Meisenhelder et al., 1989; Wahl et al., 1987), MDA 468 cells and R1hER cells (Church et al., 1992). While it is not clear as to what determines the ratio of cyclic and non-cyclic PIs produced (Majerus, 1992), in the PLCγ-dependent PI metabolic pathway, less than 10% of the total inositol phosphates produced are cyclic phosphates of inositol (Berridge and Irvine, 1989; Koch and Diringer, 1974; Sekar et al., 1993). In MDA 468 cells the early signal transduction events beginning with the autophosphorylation of the EGFR lead to the association of PLC-γ with the receptor and activation of it by tyrosine phosphorylation leading to an increase in the total PI turnover (Church et al., 1992). Such increases in PI turnover have been demonstrated in cell lines such as A431, NA, HSC-1, HSC-2 and CA-922, all of which overexpress the EGFR. However, the increases were largely due to changes in the levels of non-cyclic phosphates, even in MDA 468 cells (Wahl et al., 1987). In our experiments in MDA 468 cells, the percentage cIP of total phosphatidylinositol generated increased
to 80% as a result of EGF stimulation and decreased to 90% following LA treatment (Figure 4.4). While such differences in response to PI turnover in EGF treated MDA 468 may be due to differences in cell culture conditions or other factors (Bjorge and Kudlow, 1987), suffice it to say that receptor overexpression may not always accurately reflect the nature of PI metabolites generated. However, receptor phosphorylation seems to be a requirement for increases in PI turnover, whatever metabolites result. Our findings suggest that production of the cyclic PI metabolite is associated with EGF-stimulated receptor autophosphorylation, since changes in phosphorylation is related to the changes in the production of this metabolite. Conversely, when LA inhibits the effects of EGF on EGFR phosphorylation status, cIP levels go below those in control, untreated MDA 468 cells. Thus the only component of PI turnover in MDA 468 cells whose proportion changes concomitant to changes in receptor phosphorylation is cIP.

In general, while the signal transductional control seems similar, the mechanism by which PI turnover occurs is unlike that generated by EGFR phosphorylated PLC-γ. It is possible that the activated EGFR may associate with an unidentified PLC isoform and cause the agonist mediated changes in the levels of cIP in these cells. An activated EGFR-complex has been shown to be coupled to a G protein and this step has been shown to be mandatory in producing EGF-mediated responses (Church and Buick, 1988). It is also known that the β isoform of PLC, acting through a G protein, causes PI hydrolysis upon agonist stimulation (Cockcroft and Thomas, 1992). In fact, PI-PLCs
producing ciP and DAG have been demonstrated in many bacterial species. PI-PLC from *B. thuringiensis* was found to catalyze the formation of DAG and ciP (Lewis *et al.*, 1993). In *S. antibioticus* two distinct types of PI-PLCs were present. Of the two, PLC1, a Ca\(^{2+}\)-dependent enzyme, had a short stretch of sequence similarity with the eukaryotic PLC enzyme and was found to generate both non-cyclic and the cyclic inositol phosphates simultaneously from PI (Iwasaki *et al.*, 1998). The *B. cereus* PI-PLC enzyme was found to possess both phosphotransferase and cyclic phosphodiesterase activities (Volwerk *et al.*, 1990). A cDNA encoding the *B. cereus* PI-PLC transfected in NIH 3T3, Swiss mouse 3T3, CV-1, and Cos-7 cells resulted in increased levels of ciP (Ross *et al.*, 1992). Thus it may be possible that a non-PLC-\(\gamma\) may be interacting with the EGFR to produce changes in the proportion of ciP in response to stimulation or inhibition of receptor phosphorylation. On the other hand, synthesis of ciP from glucose 6-phosphate is unlikely because this pathway constitutes the agonist-insensitive inositol phosphate metabolism (Berridge and Irvine, 1989).

The aberrant production and accumulation of cyclic inositol phosphates may be due to defects either in their production and/or their degradation. The very fact that ciP levels only change may actually be the result of alterations in the degradation pathway of cyclic metabolites. Our observation that even untreated MDA 468 cells contain about 60% of its IPs as ciP may be suggestive of a slow turnover of this metabolite. And, indeed, high levels of cyclic PI metabolites may contribute to the maintenance of a
transformed phenotype. In a Morris hepatoma cell line which also has a very high level of ciP, it has been suggested that there is a metabolic defect in the phosphatidylinositol pathway in the fast growing tumor, facilitating inositol accumulation (Graham et al., 1987). Such slow metabolism of ciPs may be a factor for sustaining uncontrolled cellular growth; ciP involvement was demonstrated in 3T3 cells, where decreased ciP levels correlated with a lower cell density at confluence (Ross et al., 1991).

Accumulation of ciP in an EGFR overexpressing human breast cancer cell line such as MDA 468 cells raises some interesting questions as to its relationship to cell proliferation and transformation. It is possible that a class of breast cancers may produce high amounts of ciP and that this may help in the maintenance of a transformed phenotype. Thus a thorough knowledge is required about the possible synthesis and degradation components in the signaling pathways generating this metabolite. Our work demonstrates that using a PTK inhibitor, any sustained effects on cellular proliferation by ciP in these cells can be modulated starting from the initial signaling event of ligand mediated receptor phosphorylation. This study adds to a growing body of evidence about the possible role of this constituent or its higher phosphorylated forms, in EGF receptor mediated signal transduction pathways in human breast cancer cells.
CHAPTER V

INHIBITION OF EGF RECEPTOR TYROSINE KINASE ACTIVITY BY LAVENDUSTIN A: A PHOSPHORYLATION-DEPENDENT EFFECT ON CELL PROLIFERATION AND ITS RELATION TO CELL CYCLE AND REGULATORS IN MDA 468 CELLS
5.1 INTRODUCTION

5.1.1 Background

From the results of Chapter 3 and 4, it is clear that the protein tyrosine kinase (PTK) inhibitor lavendustin A (LA) can inhibit the epidermal growth factor receptor (EGFR) autophosphorylation and Pi turnover in MDA 468 cells during agonist (EGF)-stimulation. We postulated in Chapter 4 that any effects on cell proliferation by cyclic inositol phosphate (cIP) in the longer term can be modulated through receptor phosphorylation, the initial signaling event in EGF-mediated signaling. In this study, we intended to investigate any sustained effects of modulation of receptor phosphorylation (in the presence of EGF and LA) on cell proliferation.

5.1.2 EGFR and cell proliferation

MDA 468 cells undergo growth arrest in response to pharmacological concentrations of EGF (Filmus et al., 1987; Armstrong et al., 1994). MDA 468 cells overexpress the EGFR. An EGF concentration of $10^{-8}$ M, though stimulatory to cell proliferation in many cell types (Carpenter and Cohen, 1979), including mammary epithelium (Taketani, and Oka, 1983), is responsible for causing growth inhibition in MDA 468 cells (Filmus et al., 1985). It has also been reported (Yaish et al., 1988) that PTK inhibitors can cause growth inhibition leading to growth arrest in cell lines overexpressing the EGFR. In fact the PTK inhibitor LA has been reported to inhibit
growth of A431 cells, NIH 3T3 cells, RSV-NIH 3T3 cells, P388 leukemia cells and L1212 leukemia cells (Onoda et al., 1989) and MDA 468 cells (this thesis).

Extensive research is under way using various approaches, such as monoclonal antibodies (Modjtahedi et al., 1998) or PTK inhibitors (Chang et al., 1992; DeWitte et al., 1993; Posner et al., 1993; Fry et al., 1994; Traxler et al., 1997), to interfere with or block EGFR-mediated growth stimulation as a means to control proliferation. In fact, the approach of blocking the EGFR by using monoclonal antibodies is now being used in xenograft studies (van Gog et al., 1998; Perrotta et al., 1999) and in clinical trials to control proliferation of various tumors which overexpress the EGFR (Bier et al., 1995; Grandis et al., 1998).

Previous studies in this lab have demonstrated a link between EGF-mediated G₁ cell cycle arrest and alteration in the cellular localization of the growth regulatory protein, p53 in MDA 468 cells (Prasad and Church, 1997). Perturbations in p53 levels induce changes in normal cell proliferation. Wild-type p53 functions in growth arrest, while mutant p53 which accumulates in transformed cells, has been shown to enhance malignant transformation (Shaulsky et al., 1991). It has also been observed that p53 undergoes alteration of subcellular localization with the cell cycle. This protein accumulates in the cytoplasm during the G₁ phase, enters the nucleus at the early S phase and remains there for a short period. Modulations in the subcellular distribution suggest that p53 is regulated during the normal cell cycle (Shaulsky et al., 1990). MDA
468 cells possess p53$^{273\text{His}}$ mutant of p53; this mutant has a wild-type epitope map which reacts with the wild-type specific antibody PAb 1620 and is PAb 240 negative (this antibody recognizes wild-type p53) (Slingerland et al., 1993). p53$^{273\text{His}}$ has reduced non-specific DNA binding and it also fails to bind some sequences that are recognized by wild-type p53, but it retains the ability to transactivate through a consensus sequence identified by Funk et al. (1993) (Bargonetti et al., 1991; Kern et al., 1991). Though wild-type p53 is a negative regulator of cell proliferation, p53 mutants such as p53$^{273\text{His}}$ are active in inducing proliferation. Therefore, there exists a definite possibility that if the PTK inhibitor LA has any negative effect on cell proliferation, then in MDA 468 cells, p53$^{273\text{His}}$ would actively function in this growth inhibition and that it may also trigger other checkpoint regulators that participate in regulating the cell cycle.

5.1.3 Estimation of DNA synthesis

The rate of cell proliferation can be assessed by measuring DNA synthesis. Incorporation of $[^3]$H-thymidine is a common parameter used to measure accumulated DNA synthesis in high density cultures (Freshney, 1994a).

5.1.4 Analysis of a cell population by flow cytometry

Flow cytometry allows the detailed analysis of cell population and discriminates between viable and non-viable cell (apoptotic and necrotic) populations (Barbiero et al.,
1995). This method allows the estimation of cell populations in each phase of the cell cycle from the overall DNA content (as determined from the DNA per cell) in a quantitative and reproducible way; one stain extensively used for this purpose is propidium iodide to quantitate DNA fluorescence. Since fluorescence is directly proportional to the amount of DNA in the cell, the trace gives a distribution analysis of the cell population by the DNA content, thus depicting the cell population in the various phases of the cell cycle, namely the G₀-G₁, S and G₂M phases respectively (Freshney, 1994b).

5.1.5 Objectives

From the results of Chapter 3 we see that LA caused an inhibition of EGF-stimulated EGFR autophosphorylation (Figure 3.7). On one hand, we can enhance receptor phosphorylation (by EGF), and on the other, we can an inhibit it (by LA). We know from the results of Chapter 4 that differences in the phosphotyrosine content of the receptor correlate with differences in PI turnover, an EGF-dependent early signaling event.

MDA 468 cells exhibit a biphasic growth response when treated with EGF, and EGF receptor phosphorylation increases with increasing concentrations of EGF (Gulli et al., 1996). EGF receptor phosphorylation and growth show a positive linear relationship at picomolar concentrations of EGF. However, at nanomolar
concentrations, although the receptor kinase activity and phosphotyrosine content increase, there is growth inhibition (Filmus et al., 1987). In general however, inhibition of cell growth also results from the blocking of EGFR kinase activity (Yaish et al., 1988). In this study, our objective was to examine the effects of modulating receptor tyrosine kinase activity and observe the effect on cell proliferation in MDA 468 cells. We attempted to investigate the changes in the status of EGFR phosphorylation and its relationship with growth, in the presence of exogenous ligand, and secondly, by inhibiting the intrinsic receptor tyrosine kinase activity of EGFR. We therefore used EGF and LA alone and in combination for this purpose. Since growth responses in the cell are related to the progression of cells through the cell cycle, our objective was also to relate growth responses to the role of cell cycle checkpoint regulators.

5.2 RESULTS

5.2.1 Effect of EGF, LA, and EGF+LA on cell number and [³H]-thymidine uptake

In initial experiments, we found that at 0.2 μM, LA effectively inhibited the proliferation of MDA 468 cells, and this inhibition was enhanced in the presence of EGF at 72 h (Figure 5.1). At this concentration there was growth inhibition and cells were viable during this period. In the experiments that followed, we examined the effect of EGF and LA alone and in combination on cell proliferation and DNA synthesis after 24 and 72 h respectively. Figures 5.2A and B display the effects of 0.2 μM LA, 10⁻⁶ M EGF
Figure 5.1 Effect of LA in presence and absence of EGF on the proliferation of MDA 468 cells.

24 h after seeding, cells were exposed to different concentrations of LA in presence and absence of $10^{-8}$ M EGF for a period of 72 h. At the end of the exposure period, cells were harvested by trypsinization and cell numbers were counted using a haemocytometer. Data are shown as a percentage of control. Values are mean±S.E.M. from four experiments, each performed in duplicate.
Figure 5.2 Effect of EGF, LA, and EGF+LA on cell proliferation and DNA synthesis of MDA 468 cells.

After seeding, the next day cells were exposed to $10^{-8}$ M EGF or 0.2 μM LA, or $10^{-8}$ M EGF+0.2 μM LA for a period of 24, 72, and 96 h (24 h after EGF or LA removal) respectively. At the end of the indicated intervals, the cell number or [³H]-thymidine uptake were determined (Section 2.2.10 in Chapter 2).

Figure 5.2A represents the effect of LA, EGF, and LA+EGF on cell proliferation at the indicated intervals. Data are shown as a percentage of control. Values are mean±S.E.M. from four individual experiments, each performed in duplicate.

Figure 5.2B represents the effect of LA, EGF, and LA+EGF on DNA synthesis at the indicated intervals. Cells were labelled with [³H]-thymidine (3.0 μCi/ml) and incubated for 24 h prior to harvesting. At the end of labelling, cells were harvested by trypsinization and the TCA-precipitable counts were determined. The data are shown as a percentage of control. Values are mean ± S.E.M. from four individual experiments, each performed in triplicate.

Arrows in Figures 5.2 A and B indicate time of drug removal from media.
**Figure A**

- □ EGF
- • 0.2 μM LA
- ○ 0.2 μM LA+EGF

**Figure B**

- □ EGF
- • 0.2 μM LA
- ○ 0.2 μM LA+EGF

Cell Number (% of control)

3H-thymidine uptake (% of control)
and LA+EGF on cell number and DNA synthesis (as measured by $[^3]$H-thymidine uptake) respectively. Both LA and EGF caused a significant reduction in cell proliferation (35-40%) and $[^3]$H-thymidine uptake at 72 h (25-35%), and in the case of both LA+EGF, the effect was augmented as early as after 24 h incubation. The effect of LA alone was reversed 24 h after its removal from the media, compared to that of untreated control cells. In contrast, the EGF-treated cells, however, continued to show a steady decline in both cell number and $[^3]$H-thymidine uptake, 24 h after its removal; the growth of cells treated with EGF+LA was partially reversed 24 h after their removal. It is clear that the growth inhibitory effect of EGF is not as easily reversed as that of LA. Though a combination of EGF and LA produced a more pronounced growth inhibition (as measured by cell number and $[^3]$H-thymidine incorporation), this growth inhibition was more readily reversed than the inhibitory effect of EGF alone.

### 5.2.2 Flow cytometric analysis of cell cycle distribution

To investigate the effect of EGF, LA and EGF+LA on cell cycle progression and the distribution of cells in various phases of the cell cycle, the DNA content was measured by flow cytometry using propidium iodide-dependent DNA fluorescence (described in Chapter 2, Section 2.6). Flow cytometry revealed perturbations in the distribution of cell populations in the various phases of the cell cycle in response to LA, EGF and LA+EGF. Figure 5.3 shows the representative DNA histograms with cell
Figure 5.3 Effect of EGF, LA, and EGF+LA on the cell cycle at 72 h.

Representative FACS generated DNA histograms of MDA 468 cells of control, untreated cells (1), cells treated with $10^{-8}$ M EGF (2), cells treated with 0.2 μM LA (3), and cells treated with $10^{-8}$ M EGF+0.2 μM LA (4) for 72 h. The histograms are a representation of propidium iodide (PI) fluorescence (FL2-A) versus counts. The PI fluorescence is directly proportional to the amount of DNA in the cell and the relative cell number is given by counts. DNA was analyzed using PI fluorescence. Approximately 10,000 cells were analyzed per sample as described in the Materials and Methods (Section 2.6, Chapter 2).
distribution in cells treated with LA or EGF alone, or both for 72 h. EGF (Figure 5.3-2), LA (Figure 5.3-3), and EGF+LA (Figure 5.3-4) caused a significant \((p<0.005)\) accumulation of cells in G\(_1\) phase of the cell cycle (Table 5.1) compared to control after 72 h, concomitant with a decrease in the number of cells in S phase. Table 5.1 also shows a numerical summary of the percentage distribution of cells in various phases at 72 h and 24 h after removal EGF or LA. G\(_1\) arrest produced by EGF was more persistent. Though LA alone and in combination with EGF produced a pronounced G\(_1\) arrest, this arrest was more readily reversed.

5.2.3 IMMUNOFLUORESCENCE EXPERIMENTS

5.2.3.1 Effect of EGF, LA, and EGF+LA on the subcellular localization of p53

The p53 protein acts in the nucleus of the cell. MDA 468 cells contain a particular mutant, p53\(^{273.\text{His}}\), which has been shown to have a differential reactivity with conformation-specific antibodies, corresponding to alterations in the cell cycle (Shaulsky et al., 1990; Prasad and Church, 1997). Since we have demonstrated in this study that both EGF and LA produce a pronounced G\(_1\) arrest in MDA 468 cells, we examined how p53\(^{273.\text{His}}\) might be involved in producing changes in its conformation in response to G\(_1\) arrest. We now examined if there are any changes in p53 localization at the subcellular level in response to EGF and LA, alone and in combination. To this end, we made use of three conformation-specific monoclonal antibodies to p53. PAb
Table 5.1 Effect of EGF, LA and EGF+LA on the percentage distribution of cells in the different phases of the cell cycle at 72 h and 24 h after removal respectively.

<table>
<thead>
<tr>
<th></th>
<th>G&lt;sub&gt;1&lt;/sub&gt;</th>
<th>S</th>
<th>G&lt;sub&gt;2&lt;/sub&gt;M</th>
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</thead>
<tbody>
<tr>
<td>72 h (A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>47.6 ± 1.80</td>
<td>37.7 ± 0.71</td>
<td>14.7 ± 2.12</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt; M EGF</td>
<td>59.9 ± 1.15&lt;sup&gt;*&lt;/sup&gt;</td>
<td>24.2 ± 0.80&lt;sup&gt;**&lt;/sup&gt;</td>
<td>16.4 ± 0.71</td>
</tr>
<tr>
<td>0.2 μM LA</td>
<td>57.9 ± 2.13&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25.1 ± 1.01&lt;sup&gt;**&lt;/sup&gt;</td>
<td>16.3 ± 1.00</td>
</tr>
<tr>
<td>0.2 μM LA +10&lt;sup&gt;-8&lt;/sup&gt; M EGF</td>
<td>62.0 ± 1.60&lt;sup&gt;*&lt;/sup&gt;</td>
<td>21.9 ± 0.80&lt;sup&gt;**&lt;/sup&gt;</td>
<td>16.1 ± 1.10</td>
</tr>
<tr>
<td>24 h after removal (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>48.9 ± 1.90</td>
<td>38.4 ± 1.90</td>
<td>14.6 ± 1.10</td>
</tr>
<tr>
<td>10&lt;sup&gt;-8&lt;/sup&gt; M EGF</td>
<td>60.4 ± 0.50&lt;sup&gt;*&lt;/sup&gt;</td>
<td>22.1 ± 2.40</td>
<td>17.5 ± 1.91</td>
</tr>
<tr>
<td>0.2 μM LA</td>
<td>52.0 ± 0.61</td>
<td>34.0 ± 0.30</td>
<td>14.0 ± 0.34</td>
</tr>
<tr>
<td>0.2 μM LA +10&lt;sup&gt;-8&lt;/sup&gt; M EGF</td>
<td>53.0 ± 1.41</td>
<td>32.4 ± 1.80</td>
<td>14.6 ± 2.40</td>
</tr>
</tbody>
</table>

Data are represented as mean ± S. E. M. of four experiments. *Values significantly higher than control (p<0.005); **values significantly higher than control (p<0.0001).
PAb 240 specifically reacts with mutant p53 (Milner et al., 1987; Halazonetis et al., 1993). A human-specific PAb 1801 (pan-specific p53) which reactive with both wild-type and mutant p53 (Banks et al., 1986) was used. These antibodies react with p53 in MDA 468 cells, both in immunoprecipitation and in immunofluorescence reactions (Milner et al., 1987; Bartek et al., 1990; Prasad and Church, 1997). PAb 240 reliably detects a wide variety of p53 mutations (Bartek et al., 1990). We used PAb 1620, known to recognize wild-type p53 (Milner et al., 1987) to detect wild-type p53.

The results of immunofluorescence experiments from time points as early as 24 h after treatment indicated changes in the subcellular localization of the wild-type and mutant forms of p53. Control untreated cells displayed a very strong nuclear and cytoplasmic staining at 24 h with mutant-specific PAb 240 (Figure 5.4, panel A) and pan-specific PAb 1801 (Figure 5.4, panel C). This pattern of staining was altered when cells were treated with EGF, LA alone or EGF+LA. In more than 95% of cells treated with LA and EGF, an absence of nuclear staining with mutant p53-specific PAb 240 was observed (Figure 5.4, panel A). In contrast EGF and EGF+LA treated cells (95%) had a very strong nuclear with both pan p53-specific PAb 1801 (Figure 5.4, panel C, 2 and 4) and wild-type p53-specific PAb 1620 (Figure 5.4, panel B, 1).

Nuclear staining with PAb 240 at 72 h of EGF, LA, and EGF+LA treatment (Figure 5.5, panel A, 2, 3, and 4) was absent. When EGF, LA, or EGF+LA were removed at 72 h, EGF-treated cells continued to show a lack of nuclear staining with
Figure 5.4 Effect of EGF, LA, and EGF+LA on the subcellular localization of p53 at 24 h.

Cells were grown in 8-chambered glass slides. The next day, cells were treated with EGF, LA, and EGF+LA for a period of 24 h. At the end of this period, cells were fixed in methanol and acetone, blocked with 3% BSA, and incubated with different antip53 antibodies. Cells were then stained with FITC-conjugated antimouse IgG (1:50). The p53 localization was detected by fluorescence microscope and presented at a final magnification of 600X (Section 2.2.11, Chapter 2).

Primary anti-p53 used in Panel A-PAb 240 (mutant-specific),
primary anti-p53 used in Panel B-PAb 1620 (wild-type-specific),
primary anti-p53 used in Panel C-PAb 1801 (pan-specific).
Figure 5.5 Effect of EGF, LA, and EGF+LA on the subcellular localization of mutant p53 at 72 h and 24 h after drug removal respectively.

Cells were grown in 8-chambered glass slides. The next day, cells were treated with EGF, LA, and EGF+LA for a period of 72 h. In another set of experiments cells were treated with the respective drugs for a period of 72 h, at the end of which the drugs were removed and the cells were washed with 1 x PBS and fresh media (with no drugs) was added to the cells for a further period of 24 h. At the end of the incubation periods (72 h or 24 h after removal), cells were washed with 1 x PBS, fixed in methanol and acetone, blocked with 3% BSA, and incubated with PAb 240 (mutant-specific) antip53 antibody. Cells were then stained with FITC-conjugated antimouse IgG (1:50). The mutant p53 localization was detected by fluorescence microscope and images are presented at a final magnification of 600X.

Panel A-duration of treatment of LA and EGF was 72 h,

Panel B-24 h after removal of drugs.
PAb 240 (Figure 5.5, panel B, 2), in contrast to cells treated with both EGF and LA respectively, in which case about 75% of cells showed a reappearance of nuclear signal with mutant p53-specific PAb 240. 24 h after removal of EGF, LA, or EGF+LA, only cells treated with EGF showed a lack of p53 reactivity with mutant p53-specific PAb 240 (Figure 5.5, panel B, 3). Thus, 24 h after EGF or LA (or both) removal, only cells treated with EGF alone continued to show a loss of reactivity with mutant p53-specific PAb 240, evident from the absence of mutant p53 staining in the nucleus.

Taken together, our results show that EGF or LA alone or in combination produce an alteration in the subcellular localization of mutant and wild-type p53. However, only EGF alone, in contrast to LA or EGF+LA continues to show an absence of mutant p53 in the nucleus 24 h after removal of EGF from the media. These data are consistent with DNA synthesis (Figure 5.2B) and flow cytometry (Table 5.1, Figure 5.3) results which show that the effects of EGF on growth are persistent in comparison to LA or EGF+LA respectively. Our immunofluorescence results also indicate that the loss of reactivity of p53 to mutant p53-specific PAb 240 and p53 reactivity to wild-type p53-specific PAb 1620 as early as 24 h is consistent with a possible involvement of wild-type p53 in G1 growth arrest in MDA 468 cells.
5.2.3.2 Effect of EGF, LA, and EGF+LA on the subcellular localization of p21\textsuperscript{WAF1/CIP1} and cdk2

The CDKs play important roles in the regulation of the cell cycle, since their activation and subsequent deactivation have been shown to regulate the progression of cells from one stage to the other (Sherr, 1993). In many mammalian cell lines, transcription of the p21\textsuperscript{WAF1/CIP1} gene is directly promoted by wild-type p53. The cdk inhibitory protein p21\textsuperscript{WAF1/CIP1} exerts its growth inhibitory effect during the G\textsubscript{1} phase, by binding to multiple CDKs (Ding et al., 1999). This protein has a wide range of actions in cell cycle control, including induction of G\textsubscript{1} arrest (Petrocelli et al., 1996; Sherr, 1996; Waldman et al., 1996; Bunz et al., 1998; Kramer et al., 1999; Saito et al., 1999). After 24 h incubation, EGF (Figure 5.6, panel A, 1) and EGF+LA- (Figure 5.6, panel A, 4) treated cells showed nuclear staining for p21\textsuperscript{WAF1/CIP1}. Nuclear staining of p21\textsuperscript{WAF1/CIP1} was not evident with cells treated with LA at 24 h (Figure 5.6, panel A, 3). LA, after 72 h, induced nuclear staining (Figure 5.6, panel B, 3), while EGF (Figure 5.6, panel B, 2) and EGF+LA (Figure 5.6, panel B, 4) continued to show strong nuclear staining at 72 h. As early as 24 h, EGF, LA and EGF+LA treated cells displayed an absence of nuclear localization of cyclin dependent kinase 2 (cdk2), known to regulate the G\textsubscript{1} to S transition (Harper et al., 1993; Xiong et al., 1993) in the cell cycle (Figure 5.7).
Figure 5.6 Effect of EGF, LA, and EGF+LA on the subcellular localization of p21$^{\text{WAF1/CIP1}}$ at 24 h and 72 h respectively. 

Cells were grown in 8-chambered glass slides. The next day, cells were treated with EGF, LA, and EGF+LA for 24 h or 72 h periods respectively. At the end of the incubation periods, cells were fixed in methanol and acetone, blocked with 3% BSA, and incubated with antip21$^{\text{WAF1/CIP1}}$. Cells were then stained with FITC-conjugated antimouse IgG (1:50). Subcellular localization of p21$^{\text{WAF1/CIP1}}$ was detected by fluorescence microscope; the images are presented at a final magnification of 600X.

Panel A-24 h after treatment with LA or EGF

Panel B-72 h after treatment with LA or EGF.
Figure 5.7 Effect of EGF, LA, and EGF+LA on the subcellular localization of cdk2 at 24 h.

Cells were grown in 8-chambered glass slides. The next day, cells were treated with EGF, LA, and EGF+LA for a period of 24 h. At the end of this period, cells were fixed in methanol and acetone, blocked with 3% BSA, and incubated with rabbit polyclonal anticdk2. Cells were then stained with FITC-conjugated antirabbit IgG (1:100). The cdk2 localization was detected by fluorescence microscope; the images are presented at a final magnification of 600X.
5.3 DISCUSSION

It is known that receptor phosphorylation and growth have a positive correlation at picomolar concentration of EGF. This correlation does not hold at nanomolar EGF concentrations in cell lines overexpressing the EGFR. In A431 cells there is a 15-fold increase in EGFR autophosphorylation at EGF concentrations of 1 nM, leading to inhibition of cell proliferation (Gulli et al., 1996). DiFi cells, derived from human colorectal carcinoma, overexpressing EGFR, are also growth inhibited by EGF concentrations above 1 nM (Gross et al., 1991). Thus, mitogenic responses mediated by growth factors in EGFR overexpressing cell lines do not correlate with increased EGF binding capacity (Gulli et al., 1996). In fact EGF-stimulated receptor tyrosine kinase activation and growth of cells overexpressing the EGFR at nanomolar concentrations are inversely related (Gulli et al., 1996). EGF causes a biphasic response to growth in A431 cells; cells are growth-inhibited at high (nanomolar) EGF concentration and growth-stimulated at low (picomolar) EGF concentration.

It was shown in Chapter 3 that at 10^{-8} M (10 nM), EGF caused an increase in the phosphotyrosine content of the EGFR, while LA effectively inhibited this EGF-stimulated increase. From Figure 3.7 (Chapter 3) it is evident that at 0.25 μM LA significantly inhibits EGFR phosphorylation. From initial trial experiments, we observed that at 0.2 μM, LA effectively caused growth inhibition without compromising viability of cells exposed for a period of 72 h (Figure 5.1). At 72 h, EGF, in nanomolar concentration
caused growth inhibition; this is in agreement with published literature (Kawamoto et al., 1983; Gulli et al., 1996; Prasad and Church, 1997). EGF or LA alone, caused a significant reduction in cell number and DNA synthesis which, though not evident at 24 h, was clearly seen by 72 h. EGF and 0.2 μM LA in combination produced a greater reduction in cell number and DNA synthesis (Figure 5.2 A and B).

From our results too we found growth inhibition in the presence of nanomolar EGF concentrations in MDA 468 cells. Nevertheless, this increased EGF concentration led to increased receptor phosphorylation (Figure 3.7, lane 2). EGFR overexpressing cell lines such as A431 exhibit increased receptor phosphorylation (as a result of EGF treatment) and growth inhibition. Our results are in agreement with this observation. Studies with antibodies antagonistic to EGF binding and tyrosine kinase activity of the receptor have demonstrated the relationship between proliferative responses of EGF and the receptor kinase activity of the (at picomolar and nanomolar concentrations) receptor. These antibodies block EGF binding to EGFRs. Reduction in available EGFRs permits growth stimulation. In A431 cells low EGF concentration (20 pM) stimulated proliferation whereas high concentrations (2-200 nM) inhibited proliferation (Kawamoto et al., 1984). In the presence of 1 nM IgG there was maximal growth stimulation, while there was optimal stimulation at 2 nM EGF even when the concentration of IgG was increased from 10-100 nM. But at a very high EGF concentration (200 nM), there was strong growth inhibition even in the presence of very
high IgG concentration of 1 µM. Thus, EGF concentration seems to be the determinant factor for effects on growth. With increasing concentrations of EGF (2-200 nM), the concentration of antibody has to be increased in order to attain a reversal of the growth inhibitory effect of EGF to growth stimulation (Kawamoto et al. 1984). Since MDA 468 cells are also growth inhibited at nanomolar concentrations, we started these growth experiments by hypothesizing that LA, by causing a decrease in receptor phosphorylation (Figure 3.7, Chapter 3) would reverse the growth inhibitory effect of EGF; this in turn would lead to the reversal of growth inhibition and uncover the growth-stimulatory effect of EGF (Figure 5.8). We thus set out to test this hypothesis to examine cell proliferation in presence of both EGF and LA.

Reversion to EGF-mediated growth inhibition in A431 clones 29, 4, and 18 was associated with increased receptor concentration in each case. In certain clones, a reduction in EGF receptor number was associated with a loss of growth inhibition and acquisition of EGF-stimulated growth (Kawamoto et al., 1984). This evidence indicates that increased kinase activity and growth inhibition are associated with each other in an inverse manner. The fact that reduction in receptor phosphorylation was associated with growth inhibition by LA (in the presence of EGF) proves that there may not be a direct correlation existing between receptor kinase activity and growth. However, LA caused both, a reduction in receptor phosphorylation and cell proliferation. This is due to the fact that LA is a protein tyrosine kinase inhibitor and that the inhibition of the
Figure 5.8 Biphasic response of EGF on cell proliferation

This hypothetical bell-shaped curve depicts the biphasic response to EGF with increasing concentrations. There is growth stimulation at low concentrations of EGF and growth inhibition at high concentrations. However, we hypothesized a rightward shift of this curve in presence of LA (dotted line curve). The bar in the X-axis represents the concentration range of EGF which is inhibitory (in control), but as we hypothesized, EGF becomes growth stimulatory in the presence of LA.
EGFR tyrosine kinase activity is the cause for growth inhibition in MDA 468 cells. In fact there is ample evidence that tyrosine kinase inhibitors (Seynaeve et al., 1993; Moyer et al., 1997), monoclonal antibodies (Wu et al., 1995; Mendelsohn, 1997; Ma et al., 1998), or EGFR antisense RNA (Hong et al., 1998) all, by inhibiting the EGFR tyrosine kinase activity cause growth inhibition in many cells, including those that overexpress the EGFR.

We find that the EGF-induced growth inhibition was persistent compared to that of LA-induced growth inhibition; also the EGF-induced growth inhibition was not reversed 24 h after its removal, as compared to LA. It seems likely that a constant presence of intracellular LA is required to produce a sustained inhibitory response. Removal of drug from the media probably releases cells of inhibition of receptor kinase and thus we see a reversal of the drug effect. On the other hand, a prolonged EGF-mediated EGFR phosphorylation may be a reason for a sustained effect on growth. It has been shown in A431 cells that the prolonged effect of growth arrest by EGF may be due to a more sustained phosphorylation of EGFR which may cause a higher expression of growth regulators, such as p21^{WAF1/CIP1} (Skarpen et al., 1998).

EGF and LA in combination produced a greater reduction in cell number and DNA synthesis in comparison to EGF or LA-treated cells alone (Figure 5.2A and B). This indicated that LA enhanced the growth inhibitory effects of EGF (making us unable to correlate growth inhibition with changes in receptor phosphorylation), although this
is contrary to the assumption that inhibition of EGFR kinase activity by LA would relieve the cells of the growth inhibitory effects of EGF (even when EGF is present in nanomolar concentrations). Kawamoto et al. (1983) showed that A431 cells were growth stimulated at picomolar concentration of EGF, but not at nanomolar concentration of EGF, in the presence of monoclonal antibody, which blocks EGF binding to EGFR. They attributed this growth stimulation (at picomolar EGF concentration) to a population of high affinity receptors which could bind EGF with a much higher affinity compared to the low affinity receptors. However, at nanomolar concentration of EGF, they suggested that EGF became growth inhibitory in the presence of antagonizing antibody, because EGF could now compete effectively for access to the low affinity receptors. Like Kawamoto et al. (1983), MDA 468 cells also underwent EGF-mediated growth inhibition at nanomolar EGF concentrations in presence of LA although the cause may be different. We know that LA is a competitive inhibitor of ATP binding to the receptor kinase, but is non-competitive with respect to substrate (EGF) binding at the cell surface (Hsu et al., 1991). Therefore we assume that EGF at nanomolar concentrations has a chance to bind to all available receptors and we thus see the inhibitory effect of EGF. In the similar situation LA also inhibits the kinase activity of the receptors. Thus we have a situation where there is excess EGF binding as well as inhibition of EGFR kinase activity. The result is growth inhibition. We might speculate that binding of LA to the receptor may cause a conformational change in the receptor, thereby facilitating the
EGF (in nanomolar concentrations) binding to EGF receptors causing growth inhibition. It is also possible that LA may be interfering with some post-receptor mechanisms which lead to growth inhibition in presence of EGF. In fact 24 h after removal of LA, there is only a partial reversal of growth. Whatever the mechanism, MDA 468 cells fail to demonstrate a stimulation of growth as a result of EGF treatment in the presence of LA.

Growth inhibition leading to G₁ growth arrest as a result of EGF and LA treatment led us to investigate some of the regulators of cell cycle, participating in this phase of the cell cycle. Previously published results by Prasad and Church (1997) showed that EGF-induced G₁ arrest was accompanied by an absence of nuclear localization of PAb 240 positive mutant p53. EGF as well as LA both produced a G₁ arrest in MDA 468 cells (Figure 5.3). Therefore we speculate that LA-treated cells may undergo alterations in the subcellular localization of p53 in cells as a result of G₁ arrest. If this is the case, then LA may also produce alteration in the subcellular localization of p53. To test this we examined alterations in the subcellular localization of p53 as well as p21WAF1/CIP1 and cdk2, associated with the G₁ phase of the cell cycle.

The interplay between checkpoint regulators of the cell cycle determines the final balance between mitogenesis or inhibition of mitogenesis. G₁ to S transition is a major checkpoint in cell cycle progression, and deregulation of the G₁/S checkpoint is considered to be an important step towards carcinogenesis (Ding et al., 1999). p53,
a tumor suppressor gene, activates the transcription of the cyclin-dependent kinase inhibitor p21^{WAF1/CIP1}, which, in turn, prevents cdk2 activation and cell cycle progression, thus delaying tumorigenesis and progression of the cell cycle (López-Sáez et al., 1998), ultimately leading to G₁ arrest. In this part of the study we demonstrate that MDA 468 cells were growth arrested in the G₁ phase of the cell cycle in response to the growth factor EGF or by the PTK inhibitor LA, alone, or in combination. Our studies thus focused on the localization of the cell cycle regulatory proteins controlling G₁/S phase of cell cycle. Previous studies were consistent with the hypothesis that in its “pseudo wild-type” conformation, p53 plays an active role in EGF-mediated G₁ arrest (Prasad and Church, 1997) in MDA 468 cells. Consistent with these previous findings, we also found that EGF induced changes in the subcellular localization of p53, p21^{WAF1/CIP1} and cdk2, the G₁ checkpoint regulators. In addition, we found that the PTK inhibitor LA also altered the subcellular localization of G₁ checkpoint regulators. These results are consistent with the fact that EGF/LA-mediated G₁ arrest causes an induction of p21^{WAF1/CIP1} in a p53-dependent manner.

Mutations of p53 have been implicated in the etiology of breast cancer because it is required continuously to maintain the transformed phenotype (Avila et al., 1994). The wild-type p53 protein can negatively regulate cell proliferation (Martinez et al., 1991) and act as a suppressor of transformation (Finlay et al., 1989) and tumorigenesis (Chen et al., 1990). EGF (Prasad and Church, 1997), PTK inhibitors (Moyer et al.,
1997), or monoclonal antibodies (Mendelsohn, 1997) activate wild-type p53. In some breast cancers sequestration of wild-type p53 protein in the cytoplasm, away from its site of action in the cell nucleus, may also inactivate p53 function without mutation (Moll et al., 1992). Besides mutations of p53 found in many types of breast tumors, some cancer cells can also inactivate the tumor-suppressor activity of the wild-type protein by means other than mutations (Moll et al., 1992). These evidence are in favor of the role of wild-type p53 as a tumor suppressor.

MDA 468 cells possess a single allele for p53 with a point mutation at codon 273 (p53<sup>273·His</sup>) resulting in the substitution of arginine by histidine (Nigro et al., 1989; Bartek et al., 1990); this mutant is frequently present in human tumors (Hollstein et al., 1991). p53<sup>273·His</sup> has a wild-type epitope map, which is PAb 240 negative and PAb 1620 positive (Slingerland et al., 1993). Consistent with this notion is the fact that p53<sup>273·His</sup> exhibits a tumor suppressor or a wild-type function, which, in turn, is consistent with its translocation to its active site, the nucleus. Our immunofluorescence data agrees with this. We have observed that cells showing positive nuclear p53 staining with PAb 1620 (PAb 1620 stains untreated control cells very weakly) may have p53<sup>273·His</sup> in the wild-type conformation (PAb 1620 positive) (Figure 5.4; panel B) during G<sub>1</sub> arrest as a result of EGF, LA or EGF+LA treatment (Bartek et al., 1990). Conversely, the absence of nuclear staining with mutant p53-specific PAb 240 (this means that nuclear p53 is non-reactive with PAb 240) (Figure 5.4; panel A) shows that cells arrested in G<sub>1</sub>
due to both EGF and LA, by some mechanism exhibit a translocation of mutant p53 population from nucleus to the cytoplasm within 24 h of treatment. Cytoplasmic staining with pan-specific p53 was more pronounced in LA-treated cells in contrast to the EGF and EGF+LA-treated cells (which showed less intense staining). This may be indicative of lesser quantities of PAb 1620 positive p53 in the nucleus (Figure 5.4, panel C). In MDA 468 cells undergoing arrest at G1 phase, PAb 1620 positive p53 appears to play an active role as a tumor suppressor protein; this is evident from a shift in its localization to its active site, the nucleus.

Our data indicates that the subcellular localization of p53^{273.His} has relevance to the growth suppressor function of p53 that is a characteristic of wild-type p53. We see a change in the PAb 240 positive p53 translocation from nucleus to the cytoplasm in response to EGF and LA. It appears that in MDA 468 cells G1 arrest alters the PAb 240 positive p53 population subcellularly. Changes in p53 immunoreactivity are evident from changes in subcellular localization as a result of cell cycle arrest. The wild-type function of p53^{273.His} is evident from literature (Chen et al., 1990; Slingerland et al., 1993; Prasad and Church, 1997).

The p53^{273.His} allele in cooperation with H-ras had a weak transforming potential in rat embryo fibroblasts even when it was highly expressed from the strong human β-actin promoter (Slingerland et al., 1993). This mutant allele lacked ability to interfere with wild-type p53 protein in a dominant negative manner. When single copies of wild-
type human p53 and p53\textsuperscript{273 His} alleles were coexpressed in SAOS-2, an osteosarcoma cell line lacking endogenous p53 protein expression, the wild-type p53 phenotype function slowed growth, decreased saturation density, and reduced tumorigenicity (Chen et al., 1990). In the absence of wild-type p53, the mutant promoted cell growth to a higher saturation density than that of parental cells (Chen et al., 1990). In rat embryo fibroblast transformation assays, the weakly transforming p53\textsuperscript{273 His} became strongly transforming when the intact nuclear localization signal site was replaced by the mutated nuclear localization signal site (Slingerland et al., 1993). Bartek et al. (1990) called the p53 molecules with mutations in the primary sequence as “pseudo-wild type.” On careful examination of cell lines with mutant p53, Bartek et al. (1990) also found a second population of molecules which they called “overtly mutant.” These molecules are present in human cell lines because both PAb 240 positive (overtly mutant) and PAb 1620 positive (pseudo-wild type) are derived from the same mRNA (Bartek et al., 1990). Halazonetis et al. (1993) have shown that wild-type p53 can adopt a “mutant” conformation and these changes are associated with sequence-specific DNA binding. They showed that under certain conditions (such as during cell division) wild-type p53 can transiently adopt the “mutant” conformation (Halazonetis et al., 1993). Thus p53 (mutant or wild-type) is associated with changes in its conformation under certain conditions and also in a majority of tumors which is manifest in its altered conformation. Our results taken together conform to the notion of change of p53 conformation as a
result of changes in the cellular environment (EGF or LA); the overall effect is in favor of growth arrest.

Change in the subcellular localization of mutant and wild-type 53 in MDA 468 cells led us to examine another cell cycle regulatory protein, p21\textsuperscript{WAF1/CIP1}. p53 transcriptionally activates the production of p21\textsuperscript{WAF1/CIP1}, a potent inhibitor of cyclin-dependent kinases (Harper et al., 1993). Also, induction of p21\textsuperscript{WAF1/CIP1} has been reported to occur in cells undergoing p53-mediated growth arrest (El-Deiry et al., 1994). We wanted to see if localization of wild-type 53 in the nucleus as a result of EGF or LA treatment had any influence on the localization of p21\textsuperscript{WAF1/CIP1}. This protein exerts its growth inhibitory effect (El-Deiry et al., 1993; Kagawa et al., 1997) primarily during G\textsubscript{1} to S phase transition in the cell cycle (Ji et al., 1997; Ding et al., 1999). Data from our immunofluorescence experiments indicate that the G\textsubscript{1} phase arrest is accompanied by a shift in the subcellular localization of p53 and is concomitant with the nuclear localization of p21\textsuperscript{WAF1/CIP1}. EGF, LA alone and in combination produces a nuclear staining of p21\textsuperscript{WAF1/CIP1} but in varying intensities. At 24 h, LA-treated cells showed a moderate increase in the intensity of nuclear staining. In a mixed population, not all cells exhibited staining with p21\textsuperscript{WAF1/CIP1}, in contrast to the very strong staining induced by EGF or EGF+LA treated cells at 24 h (Figure 5.4; panel A 2, 3, 4). At 72 h, LA induced a relatively stronger nuclear staining of p21\textsuperscript{WAF1/CIP1}. We may speculate that induction of p21\textsuperscript{WAF1/CIP1} by LA treatment may be a more delayed and a weaker response than
that of EGF and EGF+LA. Correlation between increased expression of p21\textsuperscript{WAF1/CIP1} and growth suppression at high concentrations of EGF has been demonstrated in A431 cells (Fan \textit{et al.}, 1995; Chin \textit{et al.}, 1996), while increased p21\textsuperscript{WAF1/CIP1} levels was not observed at growth stimulatory concentrations of EGF (Jakus \textit{et al.}, 1996). Our results also show that both EGF or LA, alone or in combination, produced an absence of nuclear localization of cdk2 which is possibly an outcome of p53\textsuperscript{273.Hif1α} -induced (in the wild-type conformation) upregulation of p21\textsuperscript{WAF1/CIP1} in these cells 24 h after treatment (Figure 5.6; panel A). This is consistent with the fact that p21\textsuperscript{WAF1/CIP1} is a potent inhibitor of cdk2 controlling the G\textsubscript{1} to S phase transition (Harper \textit{et al.}, 1993; Xiong \textit{et al.}, 1993).

We initiated this part of the study with the aim of understanding the relationship between EGF-mediated cell proliferation and changes in cellular levels of \textit{myo}-inositol 1,2-(cyclic) monophosphate (cIP) (in response to EGF/LA). Our studies have shown that EGF and LA both influence the production of cIP in the short term; but irrespective of changes in cIP production in the short term (Chapter 4), growth inhibition occurs in response to EGF and LA (this Chapter). Therefore alterations in cIP production and cell proliferation do not seem to correlate with each other in MDA 468 cells.

Taken together, these data suggest that MDA 468 cells undergoing arrest in G\textsubscript{1} phase exhibit alterations in subcellular localization of various proteins which regulate the G\textsubscript{1} phase of the cell cycle; it is also suggestive that alterations in the subcellular localization of G\textsubscript{1} regulators which accompany this arrest is consistent with their
involvement in G₁ arrest in MDA 468 cells. In other words, MDA 468 cells exhibit a correlation between the subcellular localization of G₁ regulators and arrest in G₁ phase of the cell cycle.
CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS
6.1 SUMMARY OF RESULTS

Cell signaling events mediated by the epidermal growth factor receptor (EGFR) regulate survival, proliferation, and differentiation of many cell types (Haugh et al., 1999). Our study was initiated with the aim of understanding the influence of the EGFR in EGF-mediated growth inhibition in the human breast cancer cell line, MDA 468. Our specific aim was to examine the influence of changes in EGFR autophosphorylation on PI turnover and cell proliferation. To address this issue, we used the strategy of inhibiting phosphorylation of the receptor in response to growth factor stimulation. By inhibiting the receptor kinase activity in a time and concentration dependent manner, we were able to delineate the nature of PI turnover, as well as the various cell cycle regulators associated with cell proliferation in MDA 468 cells. Our results with the PTK inhibitor, lavendustin A (LA) show that the drug inhibits EGFR autophosphorylation and causes perturbations in the cell cycle in a time and concentration dependent manner.

In the initial part of the study, our aim was to interfere with the EGF-mediated EGF receptor (EGFR) phosphorylation. To this end we tested some of the well-recognized PTK inhibitors. In the course of our studies, we found that these inhibitors were ineffective in inhibiting receptor phosphorylation in MDA 468 cells. Of the inhibitors used in this study, only LA effectively inhibited the EGF-stimulated EGFR phosphorylation in these cells. In addition, we observed that cells exposed to 1 μM LA beyond 10 h, lost viability. Since LA is a potent PTK inhibitor which exerts its inhibitory
action on the EGF receptor tyrosine kinase, we speculated that a inhibition of receptor kinase activity may have effects on cell viability as well as cell proliferation. Evidence from EM and FACS analyses are consistent with the fact that LA appears to cause cell death by apoptosis in the absence of internucleosomal DNA fragmentation. At higher concentrations LA caused a G2M arrest leading to cell death after 14 h of exposure. Thus, biochemical and pharmacological effects of LA are manifest by dependence on concentration and duration of incubation in these cells, and that, inhibition of receptor kinase activity is the mechanism for the action of the drug. In the later experiments, we used this inhibitor to study the effects of inhibition of receptor phosphorylation on PI turnover and cell proliferation.

This part of our study was initiated after it was observed by Church et al. (1992) that EGF stimulated an increase in PI turnover. We examined the nature of PI turnover and found that a major portion of the metabolites comprised a constituent distinct from the major PLC-γ metabolites; this metabolite was later found to be acid-labile. Using the technique of electrospray ionization mass spectrometry, we identified this metabolite to be cyclic 1,2-inositol monophosphate (cIP). Changes in EGFR phosphorylation resulted almost entirely in changes in the proportion of this metabolite alone. This kind of a PI profile is unlike that of a PLC-γ generated PI profile. We speculate that this kind of aberrant production of the cyclic metabolite could either involve an unknown PLC producing it or that this cell line may have a defect in
breakdown in cIP.

LA inhibited EGFR phosphorylation and PI turnover in the short term. This inhibitor was cytocidal at higher concentrations on longer exposure; however, at lower concentrations it was cytostatic. Both EGF and LA inhibited cell proliferation as a result of G₁ arrest, alone and in combination. Irrespective of changes in receptor phosphorylation, by LA (decrease) or EGF (increase), growth arrest in the G₁ phase occurred. LA failed to inhibit the EGF-mediated growth inhibition and uncover the growth-stimulatory effect of EGF. The resultant effect was growth inhibition.

6.2 DISCUSSION

In this study, we have used an inhibitor which fulfills the relevant criterion of a "true" signal transduction inhibitor. Such an inhibitor would, at the cellular level, cause a selective inhibition of early signal transduction events such as (auto)phosphorylation, and inhibition of proliferation in cellular systems of study (Traxler, 1997). LA effectively inhibited the EGF-stimulated EGFR (auto)phosphorylation and PI turnover and caused an inhibition of proliferation in the system that we used, namely MDA 468 cells. With the help of this inhibitor we were able to further study the influence of the disruption on EGFR signaling and cellular checkpoint regulators in cell growth and inhibition of cell growth.

LA is a competitive inhibitor of ATP binding to the EGF receptor kinase (Onoda
et al., 1989). Interference with EGFR kinase activity influences the EGF-mediated signaling events. In some cases though, a correlation between the EGF-mediated short-term and long-term effects is not evident. LA inhibited the EGF-stimulated PI turnover resulting in a decrease in cIP levels (Chapter 4). Thus, increases (by EGF) or decreases (by LA) in receptor phosphorylation reflect changes in cIP production in the short term. However, changes in cIP levels do not correlate with growth inhibition, because inhibition occurs in the longer term regardless of changes in cIP. It is not unprecedented that enhanced PI turnover that results from EGFR phosphorylation is not an indicator of proliferative outcome. In NIH 3T3 cells treated with PDGF, DNA synthesis was not enhanced; this was also true in cells in which PLC-γ was overexpressed and these cells demonstrated PDGF-induced IP₃ production (Margolis et al., 1990). Swiss 3T3 cells in which PLC-β is activated immediately after IGF-1 (insulin-like growth factor) stimulation (and enhanced PI turnover) and whose activity returns to normal after 30 mins., it seems unlikely that the enzyme plays important roles during the S phase (Hughes et al., 1988). Another evidence comes from the subcellular localization of PLCs. PLC-γ and δ are mostly located in the cytosol and whose expression do not change during the cell cycle make them unlikely candidates for participating in the S phase (Hughes et al., 1988). In MDA 468 cells PLC-γ has been shown to be associated with activated EGFR resulting in enhanced PI turnover irrespective of EGF concentration (physiological or pharmacological) (Church et al.,
It has been unequivocally demonstrated that the cellular decision concerning the proliferative response of EGF rests at some considerable distance from EGF-mediated early signaling events which are identical irrespective of the proliferative outcome in MDA 468 cells (Church et al., 1992). Our results of EGF-dependent PI turnover and growth conform to this notion in MDA 468 cells.

p53 mutations provide cells with a selective growth advantage; with such mutations cells suffer a significant checkpoint deficit, as a result they cannot respond normally to DNA-damaging agents, and therefore they enter mitosis and subsequently replicate their genomes in spite of DNA damage. These checkpoint defects may be exploited by specific targeting to treat many cancers with abnormalities of p53 function (Bunz et al., 1998). MDA 468 cells possess a single allele for p53 with a point mutation at codon 273. Thus p53<sup>273</sup>·His has substitution of arginine by histidine (Nigro et al., 1989; Bartek et al., 1990). We provide evidence which suggests that p53<sup>273</sup>·His is undergoing a change in its conformation from mutant to the wild-type. Exposure of MDA 468 cells to the PTK inhibitor LA in a time and concentration-dependent manner influences cells to be growth arrested or to die. We see a change in the conformation of p53<sup>273</sup>·His from mutant to wild-type at 24 h after 0.2 μM LA treatment, prior to the manifestation of growth arrest at 72 h (in response to EGF or LA). We do not have evidence of a correlation between change in EGFR kinase phosphorylation and the altered conformation of p53. We know from Chapter 3 that in the shorter term (6 h), LA
decreases the receptor phosphorylation at 0.25 μM (Figure 3.7, lane 6), and we also see that there is an increase in the phosphorylation of EGFR in response to EGF (Figure 3.7, lane 3). Therefore, we conclude two things: G₁ arrest is independent of changes in the phosphorylation of EGFR kinase, and secondly, irrespective of the phosphorylation status of EGFR kinase, MDA 468 cells exhibit changes in p53 conformation, an effect which accompanies G₁ arrest.

In these studies the antiproliferative effect of both EGF and LA in MDA 468 cells is evident. They exert their antiproliferative effect by causing an accumulation of cells in the G₁ phase. Fluorescence microscopy data are consistent with the fact that this cell cycle arrest may be responsible for the nuclear localization of p53^{273,His} in the wild-type conformation, which, in turn, induces the nuclear localization of p21^{WAF1/CIP1}, a cdk inhibitor. At 24 h, EGF-treated cells showed a pronounced nuclear localization of p21^{WAF1/CIP1} (compared to LA) along with an absence of nuclear localization of cdk2. We suggest a possible involvement of p53 in EGF/LA-mediated G₁ block. A functional role for p53^{273,His} was deduced by Prasad and Church (1997) in EGF-mediated G₁ growth arrest. Our studies confirm the role of p53^{273,His} in G₁ growth arrest by both the PTK inhibitor LA as well as EGF. Irrespective of the status of EGFR phosphorylation, alterations in the subcellular localization of p53^{273,His} and other cell cycle regulatory proteins accompany G₁ arrest.

The PTK inhibitor LA has a dual mode of action, because it exerts both cytotoxic
and cytostatic effects in a time and concentration-dependent manner. Therefore, its antiproliferative effects are manifest through growth arrest or cell killing. Its effect on the cell cycle is differential. It causes inhibition of cellular proliferation leading to G₁ arrest (cytostatic effect) and G₂M arrest leading to apoptosis.

6.3 FUTURE DIRECTIONS

EGF-stimulated receptor phosphorylation is a crucial step in the EGF-mediated signaling pathway which ultimately influences a cells' mitogenic behavior. Our results have provided evidence of some atypical signaling events in a breast cancer cell line with EGFR overexpression. We discuss in the ensuing paragraphs some possible future directions in understanding the influence of EGFR in regulating signaling responses.

According to Levitzski and Gazit (1995) many PTK inhibitors which are effective in inhibiting receptor phosphorylation in membrane preparations may not be effective in whole cells. We tested PTK inhibitors in this study as a strategy aimed at inhibiting the EGFR phosphorylation. Our work shows that only LA (but not lavendustin B, genistein, or 2,5-dihydroxymethylcinnamate) effectively inhibited the EGFR phosphorylation in whole cells, although others were effective in membrane preparations. We do not have any evidence of the intracellular ATP content of MDA 468 cells. It is important to measure the [ATP]ᵢ (intracellular ATP) content in these cells and
titrate it with each of these inhibitors. In this way the effective concentration of the inhibitor required to produce a biochemical effect such as inhibition of receptor phosphorylation could be predicted. The effectiveness of these inhibitors could also be different due to differences in the binding of inhibitors to EGFR and/or ATP. The binding of inhibitors with EGFR and/or ATP could be studied using techniques like X-Ray crystallography or by mass spectroscopy. No crystal structure of EGFR has yet appeared (Traxler et al., 1999). Thus future studies will lead to elucidation of crystal structures of EGFR/ATP/inhibitor complexes and this, in turn, would enable one to study the differences in the effectiveness of the inhibitors.

The aberrant production of cIP in a breast cancer cell line, such as MDA 468 cells is an interesting observation and warrants further work because the profile of inositol phosphates was unlike that generated by PLC-γ. Untreated control cells have a high percentage of cIP production and this malignant phenotype shows a PAb 240 (reactive with mutant p53) positive p53 staining. It is possible that these cells may require this metabolite for maintenance of the transformed phenotype as well as viability. It is intriguing to see that cells are still susceptible to growth inhibition in presence of cIP increases to a certain range (10⁻⁸ M EGF produces a 20% increase in cIP production). Therefore, there appears to be no relationship between the long term EGF-mediated growth inhibition and cIP production in the short term. Decreases in cIP (we see ~30% decrease in cIP production on treatment with LA) levels probably play
some as yet unidentified role in cooperating with LA in the loss of cell viability in the longer term (exposure of cells to LA for 14 h causes loss of viability). If the level of intracellular ciP could be altered by underor overexpressing cyclic hydrolase, we could study the effective levels of ciP required for cells to prevent growth arrest (or vice versa) or lose cell viability.

An in depth investigation is required to find out what might be a possible reason for the high intracellular levels of ciP. We have extensively discussed the different possibilities in Chapter 4. We speculate that aberrant production of ciP could very well be the effect of PLC-β, a PLC isoform known to generate the most cyclic PI metabolites, interacting with EGFR via an intermediate G protein. Changes in the PLC-β protein as a result of EGF stimulation using PLC-β-specific antibody can be studied in order to assess if the activity of this PLC isoform changes with EGFR autophosphorylation (both EGFR autophosphorylation and PI turnover results from EGF-stimulated EGFR phosphorylation). It is also possible that an unidentified PLC may be responsible for the generation of ciP. Studies of growth factor signal transduction have often taken advantage of the fact that intracellular substrates form a tight association with activated receptors through the interaction of phosphotyrosine and SH₂ domains (Koch et al., 1991; Cohen et al., 1995; Pawson et al., 1995). Using available monoclonal antibodies directed at EGFR, the activated receptor immunoprecipitate complex can be incubated with PI (PI, PIP or PIP₂) in vitro and the products can be
measured by HPLC following extraction under neutral conditions to obtain profiles of PI metabolites. From such experiments we could show, firstly, which of the above PIs give a profile that is similar to that as we obtain from our experimental results. Secondly, we eventually would be able to show an SH2 domain-tyrosylphosphorylation-dependent interaction of the receptor with a distinct PLC isoform. If this isoform is isolated, we could eventually characterize this protein.

Since aberrant production of cIP may possibly be due to the low expression of the enzyme responsible for its breakdown (cyclic hydrolase), investigation to delineate the possible involvement of this enzyme is necessary in MDA 468 cells. Changes in the production of cIP in proliferating cells treated with sense or antisense constructs to this enzyme would allow one to elucidate the role of this metabolite in proliferation, cell viability or even in the maintenance of the transformed phenotype of these cells. Low expression may allow the cells to have a growth advantage, and on the other hand, overexpression may favor a decrease in proliferation by triggering the activation of cellular regulators which favor decreased proliferative response. In other words, this would lead us to elucidate if cyclic hydrolase could act as a possible antioncogene and even reverse the transformed phenotype.

Results in Chapter 5 show that EGF and LA in combination produced a growth inhibitory response greater than the inhibition produced by EGF and LA alone. Our initial hypothesis was that LA would block the EGF-mediated growth inhibition because
it inhibited EGFR phosphorylation (Figure 3.7; Chapter 3). A431 cells are growth stimulated at picomolar EGF concentration and growth inhibited at nanomolar concentration of EGF, and this is also true for MDA 468 cells (Filmus et al., 1985; Prasad and Church, 1991; Gulli et al., 1996). Kawamoto et al. (1983) showed that even in the presence of an antagonizing antibody (which blocks EGF binding to EGFR), EGF was growth stimulatory at picomolar concentrations and growth inhibitory at nanomolar concentrations. They suggested that this difference in growth response was due to the presence of a population of high affinity receptors which could bind EGF with a much higher affinity compared to the low affinity receptors. From $^{125}$I-EGF binding, they predicted that 0.1-0.2% of the total receptors were high affinity receptors with an apparent $K_d$ of $7 \times 10^{-11}$ M (7.0 pM), which bound EGF with very high affinity. At high EGF concentration cells were growth inhibited even in presence of saturating concentrations of the antagonizing antibody because EGF could now compete effectively for access to the low affinity receptors. We assume that EGF becomes growth stimulatory at picomolar concentrations (ascending portion of the bell-shaped curve; Figure 5.8) due to binding of EGF by a population of high affinity receptors. With increasing concentrations of EGF, all populations of receptors are saturated and growth inhibition occurs (descending portion of the curve; Figure 5.8). In MDA 468 cells too, we found that EGF ($10^{-8}$ M) caused growth inhibition in presence of LA. In this study we do not have evidence of the existence of low or high affinity receptors in MDA 468 cells.
We also faced problems with cell viability at high concentrations of the drug (Chapter 3). Therefore, we could not increase the concentration of the drug and expose cells for a longer length of time. Besides this, we also know that LA is a competitive inhibitor of ATP binding to the intracellular kinase domain of the EGFR. Therefore, we assumed that LA would not compete with EGF binding at the cell surface. We would like to further investigate how an antagonizing antibody competes with EGF binding at the cell surface in a concentration-dependent manner and what impact this might have on EGF-mediated growth responses. Through $^{125}$I-EGF/IgG binding assays, EGF/IgG binding of EGF or the antagonizing antibody to the EGF receptor can be estimated. With increasing concentrations of EGF in presence of more than saturating concentrations of antibody, we could predict from $^{125}$I-binding the existence of high and low affinity receptors as well as predict their dissociation constant ($K_d$) in MDA 468 cells.

In the present study we provide evidence whereby receptor tyrosine kinase activity can be modulated by a PTK inhibitor in a cell line which overexpresses the receptor. The PTK inhibitor LA exerts a dual action in a time and concentration-dependent manner. Acting as a cytostatic agent LA caused inhibition of proliferation via a $G_1$ arrest at low concentrations, while it induced a $G_2M$ arrest leading to apoptosis at higher concentrations. Thus, using LA, we have studied the effect of EGF-stimulated receptor phosphorylation in EGF-mediated signaling events.
REFERENCES


Catarsi, S., Ching, S., Merz, D. C., and Drapeau, P. (1995): Tyrosine phosphorylation during synapse formation between identified leech neurons: 485, 775-786.


Dixon, J.F. and Hokin, L.E. (1994): Lithium stimulates accumulation of second-
messenger inositol 1,4,5-trisphosphate and other inositol phosphates in mouse pancreatic minilobules without inositol supplementation: Biochem. J.: 304, 251-258.


Filmus, J., Benchimol, S., and Buick, R. N. (1987): Comparative analysis of the


benzylidenemalononitrile tyrphostins: Potent inhibitors of EGF receptor tyrosine kinase 


Gill, G. and Lazar, C. (1981): Increased phosphotyrosine contents and inhibition of 

Gill, G. N., Kawamoto, T., Cochet, C., Le, A., Sato, J. D., Masui, H., McLeod, C., and 
Mendelsohn, J. (1984): Monoclonal anti-epidermal factor receptor antibodies which are 
inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor 

and regulation of the epidermal growth factor: Cancer cells, Feramisco, J., Ozanne, B., 
and Stiles, C. (Ed) Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.: 3, pp11- 
18.


tyrosine kinase inhibitor or monoclonal antibody inhibits growth, directs terminal
differentiation and induces apoptosis in human squamous cell carcinoma HN5: Int. J.
Oncol.: 13, 335-342.


Moyer, J. D., Barbacci, E. G., Iwata, K. K., Arnold, L., Boman, B., Cunningham, A.,
DiOrio, C., Doty, J., Morin, M. J., Moyer, M. P., Neveu., Pollack, V. A., Pustilnik, L. R.,
and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor


Nasmyth, K. (1996): Viewpoint: Putting the cell cycle in order: Science: 274, 1643-
1645.

hexaphosphate (phytic acid) enhances Ca\textsuperscript{2+} influx and D-[3H] aspartate release in cultured cerebellar neurons: J. Neurochem.: 53, 1026-1030.


Sekar, M. C., Sambandam, V., and McDonald, J. M. (1993): Bombesin and muscarinic receptor activation in rat pancreas generate cyclic inositol monophosphate: Possible


Tomei, L.D., Shapiro, J. P. & Cape, F. O. (1993): Apoptosis in C3H/10T½ mouse embryonic cells: Evidence for internucleosomal DNA modification in the absence of


receptor tyrosine kinase by Lavendustin A and its analogue: J. Biol. Chem.: 266, 21106-21112.