The combination of pan-ErbB tyrosine kinase inhibitor CI-1033 and lovastatin: A potential novel therapeutic approach in squamous cell carcinoma of the head and neck

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ABSTRACT

The ErbB family of receptors are key regulators of growth, differentiation, migration and survival of epithelial cells. CI-1033 is an irreversible pan-ErbB tyrosine kinase inhibitor that has the ability to inhibit EGFR function but has shown limited therapeutic efficacy. Lovastatin targets the activity of HMG-CoA reductase, the rate-limiting step in the mevalonate pathway. In this study, the ability of lovastatin to potentiate the cytotoxic effects of CI-1033 was evaluated. The combination of lovastatin and CI-1033 exhibited some cooperative cytotoxic activity in a squamous cell carcinoma–derived cell line. This combination resulted in enhanced cell death by induction of a potent apoptotic response. Furthermore, this drug combination inhibited EGF-induced EGFR autophosphorylation and activation of the downstream signaling effectors, ERK and AKT. These findings suggest that combining lovastatin and tyrosine kinase inhibitors may represent a novel combinational therapeutic approach in squamous cell carcinoma of the head and neck.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
AKT	Protein Kinase B
AML	Acute myelogenous leukemia
ANOVA	Analysis of variance
ATF3	Activating transcription factor 3
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
ERK	MAP kinase
Ets	E-twenty six
FPP	Farnesyl pyrophosphate
FRS2α	Fibroblast growth factor receptor substrate-2 alpha
GDP	Guanine diphosphate
GGPP	Geranyl-geranyl pyrosphophate
GPCR	G-protein coupled receptor
Grb-2	Growth factor receptor bound protein-2
Grb-7	Growth factor receptor bound protein-7
GTP	Guanine triphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase

Id1	Inhibitor of differentiation 1
IGF1	Insulin growth factor 1
IRS1	Insulin receptor substrate-1
Jak2	Janus tyrosine kinase 2
JML	Juvenile myelogenous leukemia
LDL	Low density lipoprotein
MAb	Monoclonal antibody
MAPKK/MEK	Map kinase kinase
MMP	Matrix metalloprotease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nck	Non-catalytic region of tyrosine kinase adaptor protein 1
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NK	Natural killer
NSCLC	Non small cell lung cancer
PARP	Poly ADP ribose polymerase
PBS	Phosphate-buffered saline
PDK1	Pyruvate dehydrogenase kinase isozyme-1
PH	Pleckstrin homology
PI3K	Phophoinositide 3-kinase
PIP_2	Phosphatidylinositol(3,4)P_2
PIP_3	Phosphatidylinositol(3,4,5)P_3
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PTB	Phosphotyrosine binding
pTyr	Phosphorylated-tyrosine
RTK	Receptor tyrosine kinase
SH2	Src homology 2

Sos	Son of sevenless
STAT	Signal transducer and activator of transcription
TBS-T	Tris-buffered saline with Tween 20
TGFα	Transforming growth factor α
Thr	Threonine
TKI	Tyrosine kinase inhibitor
Tyr	Tyrosine
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1. INTRODUCTION

1.1 Receptor Tyrosine Kinases.

Cell communication is essential for the coordination and regulation of cellular processes such as proliferation, differentiation, migration, metabolism, apoptosis and survival (Gschwind et al., 2001). Receptor tyrosine kinases (RTK's) mediate these processes through coupling ligand binding to downstream signal transduction cascades (Mendelsohn and Baselga, 2000). Approximately 60 different RTK's have been characterized and are divided into approximately 20 subfamilies according to receptor and/or ligand specificity (Mendelsohn and Baselga, 2000; Weiss et al., 1997).

Generally, RTK's are characterized by an extracellular ligand binding domain that is usually glycosylated (Hunter, 1998), a lipophilic transmembrane segment that allows for anchorage to the cell and an intracellular cytosolic tail domain (Jorissen, 2003). With the exception of RTK's in the insulin receptor family, RTK's exist as monomers in the cell membrane (Schlessinger, 1988; Lemon and Schlessinger, 1994). Ligand binding to the extracellular domain of the RTK allows for receptor homo- or hetero-dimerization (van der Greer, 1994) and initiates trans-autophosphorylation of tyrosine (Tyr) residues on the intracellular catalytic domain, activating the receptor (Figure 1). This autophosphorylation involves transfer of the γ phosphate of adenosine triphosphate (ATP) to Tyr hydroxyl groups on downstream target proteins, initiating signaling cascades (Hunter, 1998). Only receptor

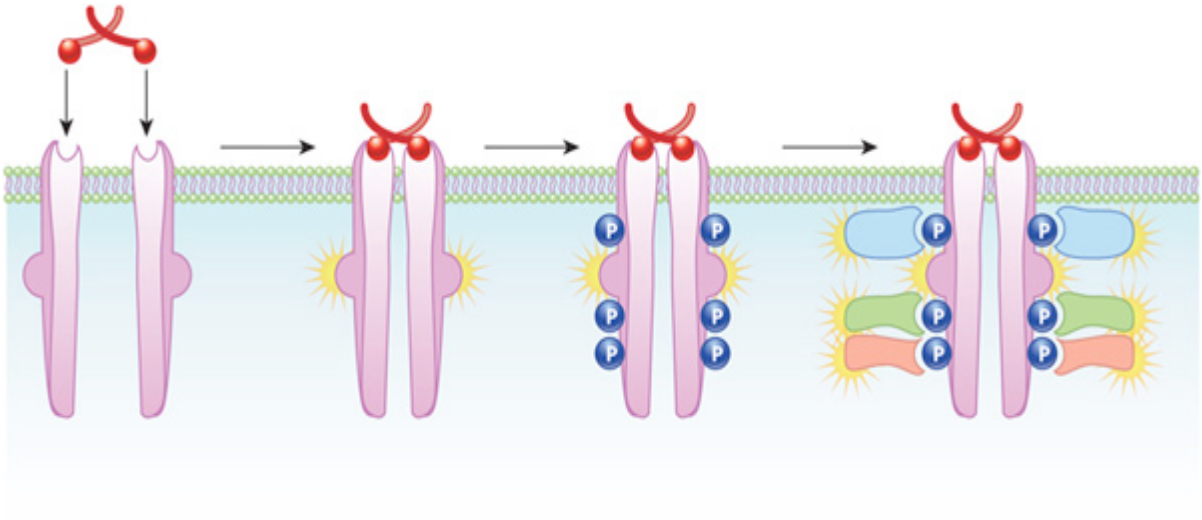

Figure 1: RTK Structure and Activation. RTK's are characterized by an extracellular ligand binding domain, a lipophilic transmembrane helical segment and an intracellular cytosolic tail domain. Following ligand binding, RTK's homo- or hetero-dimerize, resulting in autophosphorylation of Tyr residues on the cytosolic tail, subsequent RTK activation and recruitment of downstream signal proteins. (Copyright Nature Education, 2010)

dimers with specific extracellular and cytoplasmic domain configurations, however, result in receptor autophosphorylation and activation (Lemon and Schlessinger, 1994; Jiang and Hunter, 1999).

RTK autophosphorylation and activation is crucial for recruitment of downstream signaling proteins, for their subsequent activation and ultimately, activation of signaling cascades (Schlessinger, 2000). Downstream signaling proteins are recruited to docking sites on activated RTK intracellular domains that contain specific amino acid residue sequences (Normanno et al, 2005). These proteins include the adaptor proteins Shc, growth factor receptor bound protein 2 and 7 (Grb-2 and Grb-7), Crk, non-catalytic region of Tyr kinase adaptor protein-1 (Nck), phospholipase Cγ (PLCγ), the intracellular kinases Src and phophoinositide 3-kinase (PI3K), the protein tyrosine phosphatases SHP1 and SHP2 and E3 ubiquitin ligase Cbl (Yaffe, 2002; Marmor and Yarden, 2004). These signaling proteins are able to bind the RTK through specific protein domains (Normanno et al., 2005). Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains located on the signaling proteins bind to phosphorylated Tyr (pTyr's) on the RTK located in regions outside of the catalytic site of the receptor (Pawson and Schlessingert, 1993). Binding of SH2 protein domains to RTK intracellular sites, occurs 1 to 6 residues towards the C-terminal of the pTyr moiety (Songyang et al., 1993) and provides a platform for signaling complex protein recruitment and assembly (Pawson and Schlessingert, 1993). PTB domains usually bind 3 to 5 residues N-terminal to the pTyr moiety on the RTK but can also bind to nonphosphorylated sites (Margolis, 1995). Src homology 3 (SH3) domains bind to PXXP sequences, WW domains bind PXPX motifs whereas pleckstrin homology (PH) domains, which comprise a large family of domains, have various binding preferences (Kuriyan and Cowburn, 1997;

Ferguson et al., 1995; Lemmon et al., 1995; Lemmon et al., 1996; Czech, 2000). The FYVE family of domains recognize phosphatidylinositol-3-P sequences (Fruman et al., 1999) and the PDZ family of domains recognize hydrophobic residues located in the C-terminal of target proteins (Gomperts, 1996). Recruitment of specific signaling proteins leads to initiation of downstream signaling cascades such as the Ras/MAPK and PI3K/AKT pathways.

1.2 The Ras/Map Kinase Signaling Pathway.

Active RTK's recruit Grb2/Son of sevenless (Sos) complexes through the Grb2 SH2 domain, which binds to specific pTyr's on the receptor. This results in the translocation of Sos to the intracellular membrane where it can activate Ras through exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) (Schlessinger, 1994; Pawson, 1995; Bar-Sagi and Hall, 2000). Alternatively, Grb2/Sos complexes can also be recruited to the membrane by binding to the adaptor protein Shc (Margolis, 1999) or by binding to docking proteins in the membrane such as insulin receptor substrate-1 (IRS1) or fibroblast growth factor receptor substrate-2 alpha (FRS2α), which themselves are phosphorylated and activated via specific RTK activation (Sun, 1993; Kouhara, 1997). Once active, Ras interacts with PI3K and Raf where Raf can then activate MAP-kinase kinase (MAPKK or MEK) by phosphorylation of a specific serine (Ser) residue. Active MEK can then phosphorylate MAPK (ERK) on specific threonine (Thr) and Tyr residues, activating ERK. Activated ERK phosphorylates cytoplasmic and membrane linked substrates and is translocated to the nucleus where it can phosphorylate and activate a number of transcription factors (Karin and Hunter, 1995;

Hunter, 2000). The Ras/MAPK signaling cascade mediates metabolic processes, cell cycle, cell migration, cell shape, differentiation and proliferation (Davis, 2000) (Figure 2).

1.3 The PI3K/AKT Signaling Pathway.

The regulatory subunit, p85, of PI3K binds to pTyr's on activated RTK's inducing a conformational change in p85, which is transmitted to the catalytic subunit of PI3K, p110, leading to enhanced kinase activity of PI3K (Schlessinger, 2000). PI3K phosphorylates phosphatidylinositols (PI) in the plasma membrane, which leads to recruitment of AKT to the cell membrane where it is then activated (Hudis, 2007; Geyer et al., 2006; Hanahan and Weinberg, 2000; Luo et al., 2009). Below active AKT, the pathway branches off to influence a number of cellular processes including cell cycle progression, cell growth, metabolism, apoptosis resistance and protein synthesis (Knowles et al., 2009) (Figure 2).

1.4 Receptor Tyrosine Kinase- Mediated Protein Activation.

There are three mechanisms of downstream signaling protein activation by RTK's: activation by membrane translocation, activation following a conformational change, or activation by phosphorylation of Tyr (Schlessinger, 2000). Membrane translocation occurs when binding of PI3K to pTyr on the RTK leads to the generation of second messengers in the membrane such as $PI(3,4)P_2$ (PIP_2) and $PI(3,4,5)P_3$ (PIP_3). The generation of these proteins in the membrane is necessary in order to activate other downstream proteins such as pyruvate dehydrogenase kinase isozyme-1 (PDK1) and

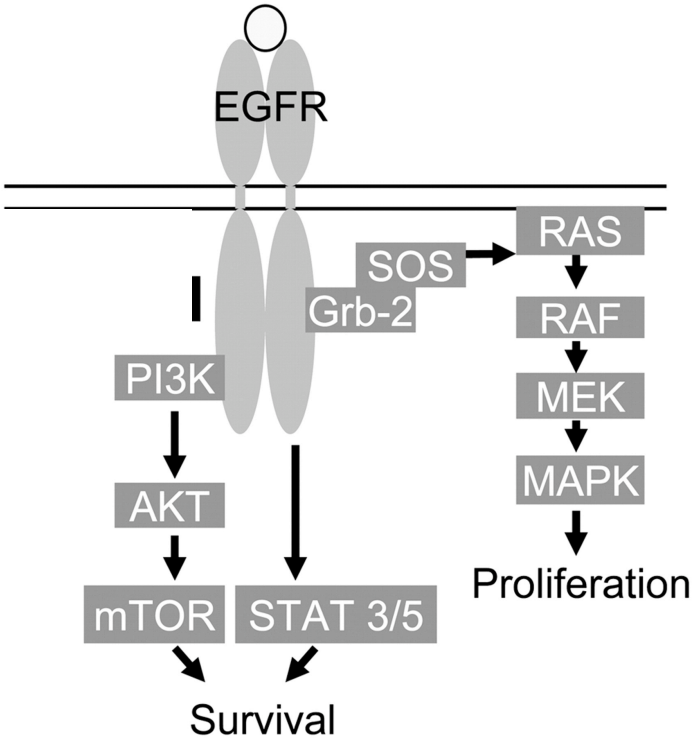

Figure 2. The Ras/MAPK and PI3K/AKT Signaling Pathways. Ras/MAPK: Active RTK's recruit Grb2/Sos complexes through Grb2 SH2 domain binding to specific pTyr's on the receptor, translocating Sos to the intracellular membrane where it can activate Ras through exchange of GDP for GTP. Ras interacts with Raf, which can then activate MEK. Active MEK phosphorylates ERK (MAPK), which is then translocated to the nucleus where it can activate transcription factors. PI3K/AKT: PI3K phosphorylates PI in the plasma membrane, recruiting AKT to the cell membrane where it is then activated. These pathways influence a number of cellular processes including angiogenesis, metastases, proliferation, apoptosis and differentiation. (Adapted from G.J. Riley et al. (2009) KRAS Mutations in Non-Small Cell Lung Cancer. Proc Am Thorac Soc. *6*, 201-205)

protein kinase B (AKT) activated by PIP_2 and PIP_3, respectively (Alessi, 1997; Anderson et al., 1998; Franke et al., 1995; Frech et al., 1997).

Many RTK targets are linked to the cellular membrane and thus membrane translocation of specific proteins is critical to facilitate downstream signaling cascades (Schlessinger, 2000).

Conformational changes necessary for protein activation occur following binding of SH2 domains to pTyr's on the RTK. This is evident when the conformational change in p85 following its binding to pTyr's on the intracellular domain of an RTK results in transmission of a conformational change in p110, leading to enhanced kinase activity of PI3K (Schlessinger, 2000).

Protein binding to pTyr's on the intracellular domain of the RTK allows RTK phosphorylation of that protein, activating it and allowing it to activate other downstream targets (Schlessinger, 2000). Thus, RTK dimerization allows for activation of the RTK, which further allows for the RTK to activate downstream proteins via one or more of these methods, which is ultimately needed for successful transduction of cellular signals.

1.5 Receptor Tyrosine Kinase Signal Attenuation/Termination.

RTK activity is tightly regulated in order to ensure normal function. Aberrant RTK expression or mutations can result in disease development, therefore several mechanisms are in place to terminate or attenuate RTK signaling activity (Schlessinger, 2000). Expression of endogenous membrane linked or soluble variants of the RTK that are deficient in protein kinase activity can lead to a dominant negative inhibition of functional RTK's (Jaye et al.,

1992). Activation of protein kinase-C (PKC), which phosphorylates RTK on Ser and Thr residues can inhibit RTK protein kinase activity and the binding of epidermal growth factor (EGF) to the extracellular portion of the receptor in a negative feedback manner (Cochet et al., 1984; Davis and Czech, 1985), which can result in signal attenuation. As well, regulation by protein Tyr phosphatases that dephosphorylate pTyr's on the catalytic site inhibit activity of the RTK and downstream proteins (Schlessinger, 2000). Lastly, RTK signal attenuation can occur through receptor endocytosis and degradation. Stimulation of the receptor by a growth factor leads to clustering of receptors in clathrin (Vieira et al., 1996) coated pits on the cell surface, and is followed by endocytosis, leading to either fusion with lysosomes resulting in degradation, or receptor recycling back up to the membrane for further usage (Schlessinger, 2000).

Along with this, several factors determine the specificity of signaling pathways (Schlessinger, 2000) and proper function of these factors is important for proper signal transduction. Protein cellular localization is important for proper cell signaling and mediation of proper biological function (Schlessinger, 2000). A number of proteins concentrate in cholesterol rich "membrane rafts" that function as sites of assembly for proteins involved in cell signaling including cell surface receptors, Src kinases and Ras proteins (Schlessinger, 2000).

1.6 The ErbB Family of Receptor Tyrosine Kinases.

The ErbB family of RTK's are key mediators of growth, differentiation, migration and survival of epithelial cells and epithelial cell derived tissues (Mendelsohn and Baselga, 2000;

Threadgill et al., 1995) and they also play an important role in normal development (Olayioye et al., 2000). The ErbB family consists of four members: ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. These receptors have distinct ligand binding specificities but are highly homologous structurally (Jorissen, 2003), containing a conserved Tyr kinase domain in the intracellular portion of the receptor (Guy et al., 1994).

Members of the ErbB family of RTK's bind growth factors from the epidermal growth factor (EGF) family of growth factors (Olayioye et al., 2000; Yarden and Sliwkowski, 2001). This family of growth factors contains intramolecular groups attached through disulfide bonds, which dictates their binding specificity and they also contain glycosylated sites, heparin-binding sites and immunoglobulin-like domains (Massague and Pandiella, 1993; Prenzel et al., 1999). These growth factors are normally synthesized as transmembrane precursors that are able to interact with receptors on adjacent cells, are processed by proteolytic cleavage via metalloproteases (Strachan et al., 2001; Riese and Stern, 1998; Massague and Pandiella, 1993) and are eventually shed as a soluble growth factors (Massague and Pandiella, 1993; Prenzel et al., 1999) but may be biologically active prior to protease cleavage (Brachmann et al., 1989; de Alava et al., 2007; Yotsumoto et al., 2008).

The epidermal growth factor receptor (EGFR) binds EGF as well as several ligands related to the EGF such as transforming growth factor α (TGFα), amphiregulin, betacellulin, HB-EGF, epigen and epiregulin (Olayioye et al., 2000; Yarden and Sliwkowski, 2001; Strachan et al., 2001; Riese and Stern, 1998; Massague and Pandiella, 1993). ErbB3 and ErbB4, bind to different isoforms of neuregulin differentiation factor (Sako et al., 2000) as well as epiregulin, HB-EGF and betacellulin (ErbB4 only) (Olayioye et al., 2000; Yarden and Sliwkowski, 2001; Brachmann et al., 1989; de Alava et al., 2007; Wong et al., 1989). The

ErbB2 receptor has no ligand, but is constitutively active allowing it to amplify signaling, increase binding affinity for respective ligands and broaden ligand-binding specificity when hetero-dimerized with EGFR, Erb3 or ErbB4 (Stern and Kamps, 1988; Fuller et al., 2008). ErbB3 has no catalytic activity as it contains substitutions of critical residues on the intracellular domain (Guy et al., 1994) but is thought to recruit additional ligands following phosphorylation by respective dimer partners (Carraway and Cantley, 1994) (Figure 3).

ErbB signaling is highly conserved among various species such as *Drosophila melongaster*, *Caenorhabditis elegans*, as well as in mammalians (Yarden and Sliwkowski, 2001; Freeman, 1998). The wide repertoire of ligands for the mammalian ErbB family members allows for greater specificity and induction of a wide range of biological responses (Yarden and Sliwkowski, 2001). ErbB RTK's are most often found in the basolateral membrane of epithelial cells and the majority of their ligands are found in the extracellular matrix suggesting a role for the ErbB family members in signal mediation between the stroma and epithelium (Troyer and Lee, 2001; Borg et al., 2000). Although normal cells do not express the EGFR, normal endothelial cells express ErbB2, ErbB3 and ErbB4 (Chandler et al., 1998; Amin, 2006).

Crystal structures of the extracellular domains of EGFR, ErbB2 and ErbB3 have provided further insight into the process of ligand-induced dimerization (Garrett et al., 2002; Garrett et al., 2003; Ogiso et al., 2002; Cho and Leahy, 2002; Cho et al., 2003). The extracellular domains of the ErbB receptors contain 4 subdomains (I-IV) (Garrett et al., 2002; Ogiso et al, 2002). Subdomains I and III are important for ligand binding and subdomain II is important for receptor dimerization (Garrett et al., 2002; Ogiso et al, 2002). The crystal

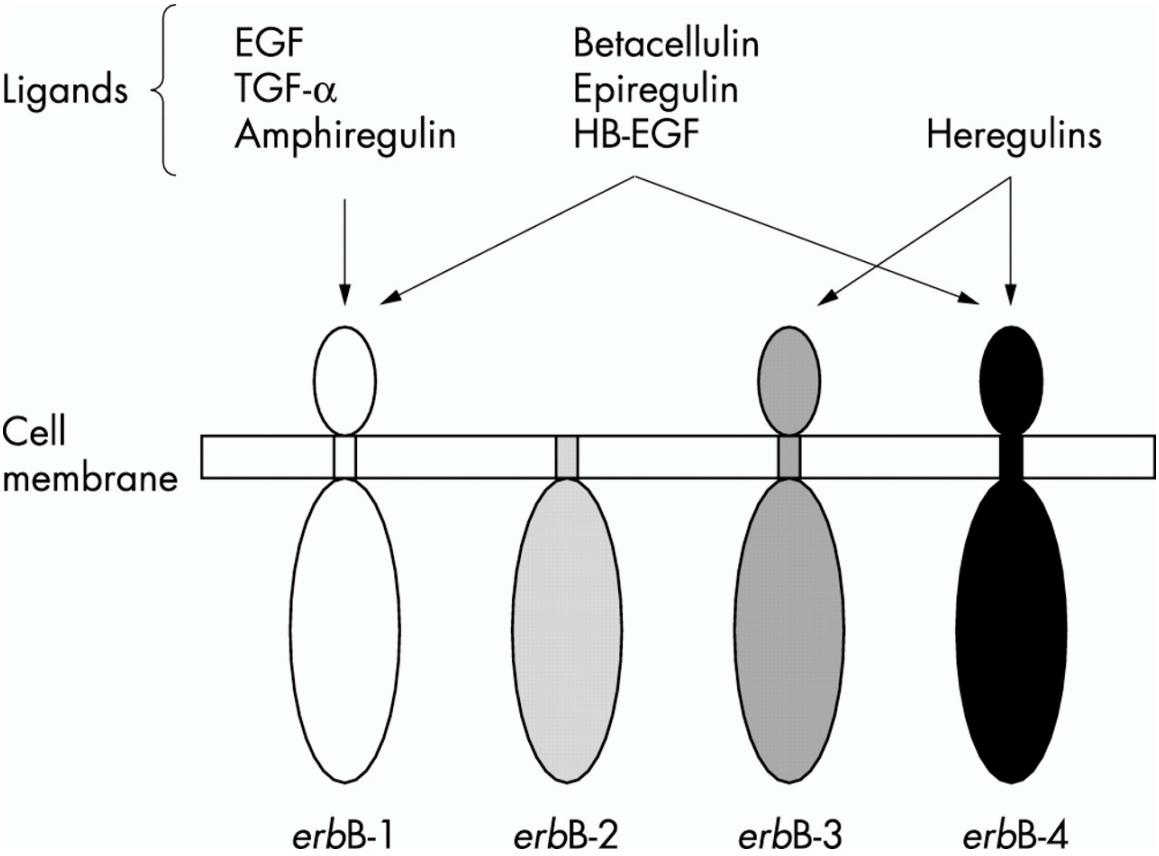

Figure 3. The ErbB Family of Receptors and their Ligands. The ErbB family consists of four members: EGFR, ErbB2, ErbB3 and ErbB4. The EGFR binds EGF, TGFα, amphiregulin, betacellulin, HB-EGF, epigen and epiregulin. The ErbB2 receptor has no ligand, but is constitutively active. ErbB3 and ErbB4, bind to different isoforms of neuregulin differentiation factors, epiregulin, HB-EGF and betacellulin (ErbB4 only). (Adapted from Colquhoun, A.J., and Mellon, J.K. (2002). Epidermal growth factor receptor and bladder cancer. Postgrad. Med. J. *78,* 584-589.)

structure of EGF bound to EGFR shows a protruding "dimerization loop" allowing for its interaction with another receptor (Garrett et al., 2002; Ogiso et al, 2002). The structure of ErbB2 is similar in the absence of a ligand; the dimerization loop is protruding, forming a structure favoring dimerization and explaining why ErbB2 is unable to bind a ligand (Garrett et al., 2003; Cho et al., 2003). This also explains the enhanced dimerization capacity of ErbB2 and its preference as a dimerization partner for the other ErbB receptors (Tzahar et al., 1996; Craus-Porta et al., 1997). ErbB2-ErbB3 dimers are the most potent signal transducers regarding cell proliferation and transformation. ErbB2-ErbB3 dimers couple to the MAPK pathway, driving cellular proliferation and the PI3K/AKT pathway, which drives cell survival and anti-apoptotic signals (Citri et al., 2003; Ben-Levy et al., 1994; Prigent and Gullick, 1994). These heterodimers can evade mechanisms of downregulation, have prolonged signaling due to enhanced recycling to the plasma membrane (Sorkin and Waters, 1993; Lenferink et al., 1998; Worthylake et al., 1999) and the presence of ErbB2 decreases the rate of ligand dissociation from ErbB2-ErbB3 heterodimers, increasing affinity of ligands for ErbB3 and widening the ligand spectrum for this heterodimeric complex (Sliwkowski et al., 1994; Alimandi et al., 1997; Pinkas-Kramarski et al., 1997).

The induction of specific signaling pathways is determined by a number of factors. The bivalent nature of EGF-like growth factors determines receptor dimeric complex formation (Beerli and Hynes, 1996; Riese et al., 1996). The affinity of the receptor for its ligand can also influence signal strength as well as duration (Tzahar et al., 1998). Receptor trafficking is also regulated in part by the pH stability of the ligand-receptor complex (French et al., 1995). Finally, signaling outcome is also dictated by the intracellular proteins recruited to active ErbB receptors (Yarden and Sliwkowski, 2001).

Following ligand binding and receptor activation, a number of signaling proteins are recruited to the ErbB receptors (Normanno et al., 2005). All of the ErbB receptors have the potential to bind to Shc and/or Grb2 and to stimulate the MAPK pathway or to bind p85 and activate PI3K/AKT signaling pathways (Citri et al., 2003; Jorissen et al., 2003; Carpenter, 2003; Soltoff and Cantley, 1996). The potency of PI3K signaling, however, differs among all four ErbB members (Normanno et al., 2005). EGFR and ErbB2 bind p85 indirectly whereas ErbB3 and some ErB4 isoforms contain p85 binding sites, with ErbB3 being the most efficient activator of the PI3K/AKT pathway (Prigent and Gullick, 1994; Fedi et al., 1994; Elenius et al., 1999).

All intracellular signaling pathways stimulated by the ErbB receptors converge in the nucleus leaving the biological outcomes in the hands of cell cycle regulators and transcription factors (Normanno et al., 2005). Cyclin D1 is upregulated following ErbB receptor activation through its phosphorylation by AKT, which promotes G1/S phase transition (Lee et al., 2000; Diehl et al., 1998). The ErbB receptors also activate a number of transcription factors such as c-fos, c-jun, c-myc, signal transducer and activator of transcription (STAT), nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), zinc finger transcription factor and E-twenty six (Ets) family members (Cutry et al., 1989; Quantin and Breathnach, 1988; Biswas et al., 2000; Olayioye et al., 1999; O'Hagan and Hassell, 1998).

1.7 Transactivation of ErbB Receptors.

The ErbB family of receptors can be transactivated by other membrane receptors (Normanno et al., 2005) and this transactivation is characterized by rapid phosphorylation and downstream signaling pathway activation (Normanno et al., 2005). ErbB receptors are either transactivated or phosphorylated by other kinases or autophosphorylated due to increased kinase activity (Normanno et al., 2005)). Other kinases such as Janus tyrosine kinase 2 (Jak2) or Src, can phosphorylate the cytoplasmic domains of ErbB family members following their activation by G-protein coupled receptors (GPCR's) leading to enhanced receptor signaling (Yamauchi et al., 1997; Yamauchi et al., 2000; Biscardi et al., 1999). Conversely, GPCR agonists can bind to the GPCR, inducing stimulation of metalloproteases from the A disintegrin and metalloproteinase (ADAM) family, further inducing cleavage of EGF-like ligand precursors, leading to increased ligand availability for the ErbB receptors (Prenzel et al., 1999; Gschwind et al., 2002; Riese and Stern, 1998).

1.8 ErbB Receptors and Cancer.

The World Health Organization (WHO) International Agency for Research on Cancer division has reported that cancer will soon become the leading cause of death worldwide (Nainggolan and Mulcahy, 2009). The EGFR autocrine pathway contributes to a variety of processes involved in the development and progression of cancer including cell proliferation, apoptotic inhibition, angiogenesis and metastasis (Perry et al., 1998; Noonberg and Benz, 2000; Woodburn, 1999). Activation of this pathway in cancer cells can occur via overexpressed ErbB receptors, increased ligand production, decreased phosphatase activity,

decreased turnover of the ErbB receptors, or from the presence of mutant receptors (Ciardiello and Tortora, 2001) and can lead to cell hyperproliferation, enhanced survival of cells and an increase in their metastatic potential (Mendelsohn and Baselga, 2000; Tan and Kim, 1999).

The growth factors that stimulate RTK activation and subsequent downstream signal cascades are the front line of regulation for cellular proliferation, differentiation and survival and as such, play a role in oncogenic transformation (Normanno et al., 2005). Transformed cells require reduced amounts of growth factors in order to maintain a high proliferation rate and can, in fact, produce their own tumor-derived growth factors that play a role in angiogenesis and metastasis of cancer cells (Normanno et al., 2005). Tumor cells can overexpress or constitutively activate their RTK's which can also decrease endogenous or tumor derived growth factor requirements in order to become activated (Normanno et al., 2005). There is a direct correlation between growth factors and cellular oncogenes (Aaronson, 1991; Goustin et al., 1986) as cellular oncogenes often code for growth factors, RTK's or proteins involved in RTK signal cascades (Normanno et al., 2005).

Signaling via the ErbB family of receptors is one of the regulatory pathways mediating normal vs. neoplastic cell proliferation (Schlessinger, 2000). Overexpression of the ErbB receptors and their ligands has been reported in the majority of human neoplasms (Salomon et al., 1995; Normanno et al., 2003; Yotsumoto et al., 2008).

Overexpression of EGFR can be due to gene amplification, mutant forms of the EGFR and either small in-frame deletions or point mutations (Salomon et al., 1995; Pedersen et al., 2001). The most common mutant form of EGFR found in human malignancies is EGFR

variant type III (EGFRvIII), which is a truncated form of the EGFR. The EGFRvIII mutant contains an extracellular segment that lacks subdomains I and II of the normal EGFR, which prevents binding of respective ligands (Moscatello et al., 1998; Voldborg et al., 1997). The EGFRvIII mutant occurs due to gene rearrangement and is often amplified resulting in its overexpression in malignant cells (Voldborg et al., 1997). EGFRvIII, however, is constitutively active rendering it able to stimulate signal transduction cascades independently of ligand binding (Ciardiello and Tortora, 2001). Other mutations/deletions in the extracellular domain can also cause ligand-independent constitutive receptor activation (Tang et al., 2000; Chu et al., 1997). EGFR is overexpressed in a number of cancers including non-small cell lung cancer (NSCLC), breast, head and neck (HNC), gastric, prostate, bladder, ovarian, colorectal, renal, pancreatic, cervical and glioblastoma (Woodburn, 1999; Sung et al., 2000; Porebska et al., 2000; Salomon et al., 1995). EGFR overexpression is often associated with advanced disease, poor patient prognosis and has been associated with resistance to hormonal therapy, cytotoxic agents and radiotherapy (Salomon et al., 1995; Nicholson et al., 1999; Akimoto et al., 1999; Chen et al., 2000). Some tumors such as HNC report expression of the EGFR in 100% of examined cases (Normanno et al., 2003).

Expression of ErbB2 in human primary carcinomas is not as common as expression of the EGFR. When overexpression of ErbB2 is observed it is mostly associated with gene amplification and ErbB2 is seldom mutated although some in-frame and missense mutations have been observed in NSCLC patients (Normanno et al., 2003; Stephens et al., 2004). Cleavage of ErbB2 by matrix metalloproteases (MMP's) can generate a constitutively active receptor denoted as $p95^{HER2}$ (Codony-Servat et al., 1999).

ErbB3 expression is comparable to that of the EGFR in human carcinomas and specific transcripts of ErbB4 or immunoreactive ErbB4 has been found in some breast, ovarian, squamous cell, esophageal, bladder and pancreatic tumors (Normanno et al., 2003).

1.9 The Prognostic Significance of ErbB Expression.

The prognostic significance of EGFR in cancer patients has been addressed in a number of studies (Normanno et al., 2005). Generally, the results are conflicting with one another. Some studies suggest that the EGFR is a strong prognostic factor (Veale et al., 1993) while others have observed no relationship between EGFR expression and patient outcome (Rusch et al., 1997) independent of cancer type, tumor stage and method of detection.

ErbB2 seems to have prognostic value in breast cancer patients that are node positive where ErbB2 protein overexpression and/or amplification is associated with poor patient outcome (Gullick, 1990; Ravdin and Chamness, 1995) and in lung cancer patients where high expression of ErbB2 is associated with poor prognosis (Tateishi et al., 1991).

Overexpression of more than one ErbB receptor, however, promotes a much poorer patient prognosis then in those overexpressing just one of the ErbB receptors as is the case for dual overexpression of EGFR and ErbB2 (Normanno et al., 2005). Expression or overexpression of one of more of the ErbB receptors has been associated with aggressive disease and poor patient prognosis (Scambia et al., 1995; Meden and Kuhn, 1997; Klijin et al., 1994; Bartlett et al., 1996; Hale et al., 1993; Maurizi et al., 1996; Wikstrand and Bigner, 1998; Grandis et al., 1998).

The prognostic role of ErbB3 is debatable, having been associated with a poor prognosis in some breast cancer patients and with a positive outcome in others (Pawlowski et al., 2000; Naidu et al., 1998; Knowlden et al., 1998). ErbB3 has been shown to play a role in progression of localized breast tumors to invasive cancers and ErbB3 expression may be increased in estrogen- and progesterone receptor – positive tumors (Allen et al., 2003).

ErbB4 expression in breast cancer is most often associated with a better patient outcome in breast cancer (Pawlowski et al., 2000; Knowlden et al., 1998).

1.10 The Transforming Ability of ErbB Receptors.

Many *in vitro* and *in vivo* studies have demonstrated the ability of the ErbB receptors and their ligands to induce oncogenic transformation, however, it is the cooperative activity between the receptors that is needed for *in vivo* transformation (Normanno et al., 2005; Kokai et al., 1989; Alimandi et al., 1995; Wallasch et al., 1995; Zhang et al., 1996; Cohen et al., 1996). Oncogenic transformation requires co-expression of at least two ErbB receptors along with an appropriate ligand (Normanno et al., 2005). Interestingly, heterodimers containing ErbB2 seem to have amplified signaling demonstrating an important role for ErbB2 in *in vivo* transformation (Normanno et al., 2005).

1.11 ErbB Receptor-Targeted Cancer Therapies.

The members of the ErbB family have been identified as relevant therapeutic targets in the literature. ErbB receptor targeted therapy is currently being evaluated and the rationale is

based on substantial research indicating an association between these receptors and their downstream signaling pathways with tumor formation, progression, invasion, and metastases (Slichenmyer et al., 2001). As previously mentioned, the overexpression of these receptors has been found in a number of tumor types including but not limited to breast, ovary, lung, prostate, gastrointestinal tract and brain (Davies and Chamberlin, 1996; Rajkumar and Gullick, 1994).

Current drug developments targeting the ErbB family receptors that are being evaluated clinically are monoclonal antibodies (MAb's) and small molecule tyrosine kinase inhibitors (TKI's) (Adams and Weiner, 2005; Shawver et al., 2002). These can be specific to one of the ErbB receptors or can act broadly on all four ErbB family members. Other therapeutic approaches being evaluated that target the EGFR specifically are recombinant proteins containing toxin-fused growth factors such as toxin-fused TGFα or toxin-fused EGF, EGF-genistein conjugates, EGFR-directed vaccine approaches and the use of antisense oligonucleotides (Woodburn, 1999; He et al., 1998; Mendelsohn and Baselga, 2000; Azemar and Schmidt, 2000; Anderson and Ahmad, 2002; Goldenberg, 1999).

MAb's target the extracellular ligand binding domain of the receptor and compete with the ligand for receptor binding, ultimately blocking ligand binding and receptor activation (Ciardiello and Tortora, 2001; Hynes and Lane, 2005; Li et al., 2005; Normanno et al., 2003). Those in clinical trials include trastuzumab and cetuximab (Grunwald and Hidalgo, 2002). Trastuzumab is a MAb directed against the extracellular domain of ErbB2 and has shown some promise clinically (Goldenberg, 1999; Cobleigh and Vogel, 1999; Shak, 1999; Pegram et al., 1998; Baselga et al., 1998; Cobleigh and Vogel, 1998) through its ability to reduce EGFR-ErbB2 complex formation (Cai et al., 2008). Cetuximab also targets the EGFR

and is currently approved showing an approximate 10% increased survival in patients (Amado et al., 2008).

Toxin-fused growth factors allow the selective targeting and inhibition of cells expressing the appropriate receptor (Chandler et al., 1998). These "mitotoxins" bind to target growth factor receptors via the growth factor portion and are internalized via receptor-mediated endocytosis (Chandler et al., 1998). Upon endocytosis, proteolysis separates the fused toxin from the growth factor, allowing the toxin to trigger cell death (Chandler et al., 1998). A number of toxins have been used in this manner, such as saporin (McDonald et al., 1996) or bacterial toxins from *Pseudomonas* (Stripe et al., 1992).

Genistein is an isoflavone found in soybeans that naturally acts as a broad spectrum TKI (Akiyama et al., 1987). When conjugated to EGF, it can trigger apoptotic cell death (Somlo et al., 1994), which has been seen in human breast cancer cells (Uckun et al., 1998).

ErbB-targeted vaccines employ the immune system of the host to raise an anti-tumorigenic response (Renard and Leach, 2007). A number of vaccine approaches have been investigated with the majority focused on inducing anti-tumor $CD8^+$ and $CD4^+$ T cell responses but some can also induce responses by natural killer (NK) cells (Renard and Leach, 2007; Jager and Knuth, 2005).

Antisense oligonucleotides target ErbB mRNA and can downregulate ErbB mRNA in cancer cells resulting in inhibition of proliferation (Roh et al., 2000). Antisense oligonucleotides bind mRNA through Watson-Crick base pairing and can activate degradation of the mRNA via RNase H, an endogenous nuclease (Green et al., 2000).

Hundreds of TKI's have been synthesized and evaluated for potential clinical application (Bridges et al., 1995; Levitski and Gazit, 1995; Shugar, 1995; Kohs et al., 1997; Traxler et al., 1997; Levitt and Koty, 1999; Levitski, 1999; Traxler and Furet, 1999). TKI's such as erlontinib (Herbst, 2002; Herbst, 2003), gefitinib (Grunwald and Hidalgo, 2002) and CI-1033 (Slichenmyer et al., 2001) target the intracellular catalytic domain of the receptor, preventing autophosphorylation of the intracellular tyrosine kinase domain (Ciardiello and Tortora, 2001; Widakowich et al., 2007; Herbst, 2002; Herbst, 2003). Modeling of compound binding in the ATP pocket of the intracellular kinase domain of the EGFR has been used to design selective EGFR-TKI's (Kris et al., 2007). These small molecules are generally reversible competitive inhibitors of ATP for binding to the intracellular catalytic domain on the EGFR (Ciardiello and Tortora, 2001). Erlontinib and gefitinib have shown efficacy in preclinical models (Wakeling et al., 2002) but are only selective for the EGFR, however, and have limited or no activity on the other members of the ErbB family (Grunwald and Hidalgo, 2002).

Due to constitutively active ErbB receptors lacking their extracellular domains MAb's, toxin-fused growth factors or genestein-growth factor conjugates may not always be the best option for treatment. As well, tumors may secrete soluble growth factors, which can interfere with the binding of these agents to the extracellular portion of the receptor (Brodowicz et al., 1997). Due to elevated levels of ErbB receptors other than the EGFR in various carcinomas, EGFR-specific therapeutic agents, including TKI's, may not be the best approach. Also, normally there is an excessive amount of growth factors within tumors (Uberall et al., 2008) and thus targeting the kinase activity of all of the ErbB receptors simultaneously may be more beneficial. Antisense oligonucleotide directed therapy is also flawed, as these agents

often have off target effects (Stein, 1995) and high concentrations of these oligonucleotides can be toxic (Roh et al., 2000). All currently approved anti-ErbB MAb cancer drug therapies result in some inherent or acquired resistance in those being treated (Huang et al., 2009). For example, the majority of patients acquired resistance to cetuximab (Ciardiello and Tortora, 2008) and at least half of ErbB2 overexpressing patients were resistant to trastuzimab (Hudis, 2007). Proposed mechanisms of resistance to anti-ErbB MAb's are inefficient blockade of ErbB heterodimerization or signaling from off-target ErbB's (Ritter et al., 2007; Wheeler et al., 2008), increased PI3K (Berns et al., 2007) or insulin-like growth factor 1 (IGF1) receptor signaling (Lu et al, 2001; Lu et al., 2004) and/or truncated constitutively active ErbB forms. Drug resistant splice variants of ErbB2 can also lead to patient acquired resistance (Castiglioni et al., 2006). Chemotherapy diminishes the anti-tumor response induced by anti-cancer vaccines over time and as such may work better as a combined therapeutic regimen (Perez et al., 2010).

Crosstalk between the ErbB family members with other ErbB members or with other RTK's can also lead to resistance to anti-ErbB therapies and seriously affects the efficacy of single-targeted therapies (Baselga et al., 1996; Cobleigh et al., 1999; Vogel et al., 2002; Engelman et al., 2007; Arpino et al., 2008; Sukocheva et al., 2006). Reduced or inhibited activity of one ErbB receptor can be compensated for by another ErbB receptor, which can reduce the efficacy of therapies directed at only one of the ErbB receptors (Sergina et al., 2007). Treatments directed at multiple ErbB receptors or all ErbB receptors simultaneously have proven better able to inhibit growth of tumors (Arpino et al., 2007), would overcome resistance acquired by receptor pathway intra and inter cross talk and is a useful strategy to explore in anti-cancer therapies (Huang et al., 2009).

1.12 The Pan-ErbB Tyrosine Kinase Inhibitor, CI-1033.

CI-1033 targets the EGFR, ErbB2 and ErbB4 simultaneously and has proven to be effective against a variety of human tumors (Karamouzis et al., 2007). In studies done by Pfizer, 90% of the solid tumors studied expressed at least one of the ErbB receptors (Slichenmyer et al., 2001). The tumorigenic ability of cells expressing ErbB receptors is increased when multiple members of the ErbB receptor are expressed (Olayioye et al., 2000). As is the case, drugs designed to target the EGFR alone may not suffice.

CI-1033 is a pan-ErbB Tyr kinase inhibitor that provides prolonged inhibition of the Tyr kinase activity of members of the ErbB family of receptors (Fry, 2000) and is highly specific to these members only (Slichenmyer et al., 2001). There is no cross reactivity with insulin receptor tyrosine kinases, fibroblast growth factor receptor or platelet derived growth factor receptor (Slichenmyer et al., 2001). CI-1033 is a 3-chloro, 4-fluoro, 4-anilinoquinazoline containing an acrylamide at position 6 on the molecule (Slichenmyer et al., 2001) (Figure 4). The positioning of the acrylamide on carbon 6 of the quinazoline brings the alkylating moiety into close proximity to cysteine-773 on the EGFR kinase domain (located in the ATP receptor pocket) and to similar cysteines on the other ErbB receptors (Fry et al., 1998). This proximal positioning allows covalent bond formation between CI-1033 and its target ErbB receptor for instantaneous inactivation and permanent binding of the ErbB target (Slichenmyer et al., 2001). Activity of CI-1033 is persistent until new ErbB receptors are generated (Tang et al., 2000).

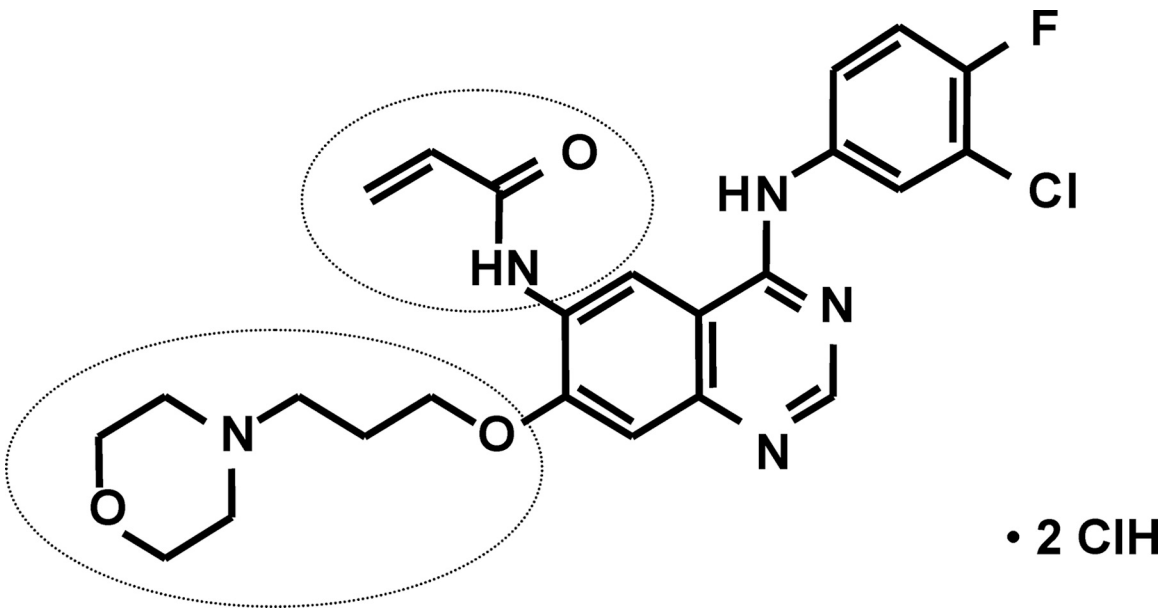

Figure 4. The chemical structure of CI-1033. The chemical groups circled represent the reactive groups that allow for inhibition of the ErbB receptors. The positioning of these groups brings the alkylating moiety into close proximity to cysteine residues on ErbB receptors allowing a covalent bond to form for instantaneous inactivation and permanent binding of the ErbB receptor target. (Adapted from Calvo, E., Tolcher, A.W., Hammond, L.A., Patnaik, A., de Bono, J.S., Eiseman, I.A., Olson, S.C., Lenehan, P.F., McCreery, H., Lorusso, P., and Rowinsky, E.K. (2004). Administration of CI-1033, an irreversible pan-erbB tyrosine kinase inhibitor, is feasible on a 7-day on, 7-day off schedule: a phase I pharmacokinetic and food effect study. Clin. Cancer Res. *10,* 7112-7120.)

In vitro studies using CI-1033 have shown its ability to suppress EGFR Tyr phosphorylation in A431 human epidermoid cancer cells and following heregulin treatment in MDA-MB-453 cells (Slichenmyer et al., 2001). CI-1033 was also able to inhibit expression of downstream proteins such as c-fos, in response to heregulin in MDA-MD-453 cells (Slichenmyer et al., 2001). CI-1033 activity was prolonged 8 hours after treatment in MDA-MB-453 cells demonstrating potentially enhanced antitumor capabilities over transient TKI's (Slichenmyer et al., 2001).

In vivo studies of CI-1033 were done on xenografts of human epidermal carcinoma, NSCLC and glioblastoma (SF-767) in nude mice (Slichenmyer et al., 2001), which represent tumor types expressing EGFR only, all four ErbB receptors and EGFRvIII, respectively. Antitumor activity was highly significant ($p < 0.01$) in all three tumor types following treatment of ≥ 40 mg/kg/day (Slichenmyer et al., 2001).

Animal studies done in monkeys and rats showed that CI-1033 was rapidly absorbed from the gastrointestinal tract, was distributed to all tissues except the brain, was highly protein bound and was cleared through the bile (Slichenmyer et al., 2001). Major side effects were diarrhea and some minor skin lesions (Slichenmyer et al., 2001).

A phase I clinical trial was conducted on patients with refractory cancer in order to determine safety, pharmacokinetics, and maximum tolerable dose, as well as to document any changes in ErbB receptor phosphorylation and to potentially identify antitumor biomarkers (Zinner et al., 2007). CI-1033 was given orally to patients and the maximum tolerable dose was found to be 650mg with diarrhea being the most common gastrointestinal side effect (Zinner et al., 2007). Downregulation of both EGFR and ErbB2 was noted in all patients as well as pEGFR

(Zinner et al., 2007) and a decrease in the cell proliferation marker Ki67 was observed along side an increase in the cell cycle inhibitor p27 (Zinner et al., 2007). There is some evidence, however, that CI-1033 along with other small molecule TKI's can bind to some off targets that contain ATP binding sites (Rowinsky et al., 2003).

A phase II study using CI-1033 as a single agent on pretreated metastatic breast cancer patients expressing >1 ErbB receptor resulted in a median progression free survival rate of two months (Rixe et al., 2009). These results were not clinically meaningful but suggested the potential of CI-1033 to enhance the activity of other agents (Rixe et al., 2009). Another phase II study was conducted using CI-1033 to treat platinum-refractory or recurrent advanced stage NSCLC (Janne et al., 2007). End-point was a 1 year survival rate and although the end point was never reached in this trial, the mean 1 year survival rate was 29% among all three treatment groups that correlated with overexpression of ErbB2 (Janne et al., 2007). A phase II trial was conducted also using CI-1033 on patients with platinum-refractory or recurrent ovarian cancer (Campos et al., 2005). In this study, the mean 1 year survival rate was 38% for the two groups and the best results were observed in patients with overexpression of ErbB3 and ErbB4 (Campos et al., 2005). In all three trials, the most common adverse side effect was the development of rash, which seemed to be directly related to disease stabilization. As well, doses above 50mg/day were slightly more effective but were not as tolerable as 50mg/day, which was determined to be the highest tolerable dose in all three studies. All of these studies have suggested the potential of these agents as part of a combined therapeutic approach.

1.13 Statins and The Mevalonate Pathway.

Although inhibitors of the ErbB receptors have shown some clinical promise in the treatment of cancer, questions remain on how to maximize their potential to treat cancer (Dimitroulakos et al., 2006). Preclinical studies looking at the combination of TKI's directed at the EGFR with inhibitors of the mevalonate pathway have shown some promise in enhancing their efficacy (Mantha et al., 2003; Mantha et al., 2005).

A potential option for the treatment and/or prevention of cancer is through the use of statins (Gonyeau et al., 2010). Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are widely prescribed to treat patients with elevated levels of cholesterol (Centers for Disease Control and Prevention). Statins block synthesis of cholesterol by the hepatic system resulting in reduction in the levels of low-density lipoproteins (LDL) (Knox et al., 2005). HMG-CoA catalyzes the conversion of HMG to mevalonate, the rate-limiting step in the mevalonate pathway (Friis et al., 2005; Stamm and Ornstein, 2005). Statins act as molecular mimics of the intermediate formed between HMG and mevalonate and bind to the active site of HMG-CoA reductase with a much higher affinity than the natural substrate intermediate (Corsini et al., 1995). This prevents the formation of downstream metabolites in the mevalonate pathway such as the isoprenoid intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), as well as dolichol, ubiquinone, cholesterol and retinoids (Friis et al., 2005; Stamm and Ornstein, 2005; Goldstein and Brown, 1990; Knox et al., 2005). Statins thus have pleiotropic effects on various cellular functions as each one of these metabolites plays a significant role in various cellular processes (Friis et al., 2005; Stamm and Ornstein, 2005). FPP and GGPP are important post-translational modification on 2-3% of all cellular proteins including

members of the Ras, Rac and Rho families (Hindler et al., 2006; Seabra et al., 2002; Pruitt and Der, 2001). Ras, Rac and Rho family proteins regulate cell proliferation, intracellular trafficking, cell motility, and their prenylation serves as the membrane anchor critical for their signaling activity (Seabra et al., 2002; Pruitt and Der, 2001). Dolichol is an N-linked protein carrier molecule involved in protein glycosylation in the endoplasmic reticulum (Goldstein and Brown, 1990) and plays a role in DNA synthesis (Wejde et al., 1998), ubiquinone is an important component of the electron transport chain, which generates ATP (Goldstein and Brown, 1990), cholesterol helps maintain cellular membrane structure and integrity (Goldstein and Brown, 1990) and retinoids induce cellular differentiation and growth inhibition (Knox et al., 2005) (Figure 5).

The relationship between statins and cancer has been studied extensively and the pleiotropic effects of statins seem to have positive effects on cancer by decreasing cancer incidence. This may be due to statin-induced tumor growth suppression, apoptotic induction or inhibition of angiogenesis (Gonyeau et al., 2010). Through decreasing dolichol production, statins have the potential to decrease DNA synthesis and to inhibit cell growth (Hindler et al., 2006; Wejde et al., 1998). Statins have also demonstrated the ability to down regulate cyclin dependent kinases (CDK's) and to upregulate the cell cycle inhibitors p21 and p27 (Okumadu and Dutta, 2003). Inhibition of tumor angiogenesis is accomplished by statins through decreasing proangiogenic factors including vascular endothelial growth factor (VEGF), inhibiting proliferation of endothelial cells and blocking cell adhesion to the extracellular matrix (Frich et al., 2003). Statins also have the potential to inhibit tumor metastases through their ability to decrease endothelial adhesion molecules including E-selectin and MMP9 thus inhibiting cell adhesion, migration and invasion (Wang et al., 2000)

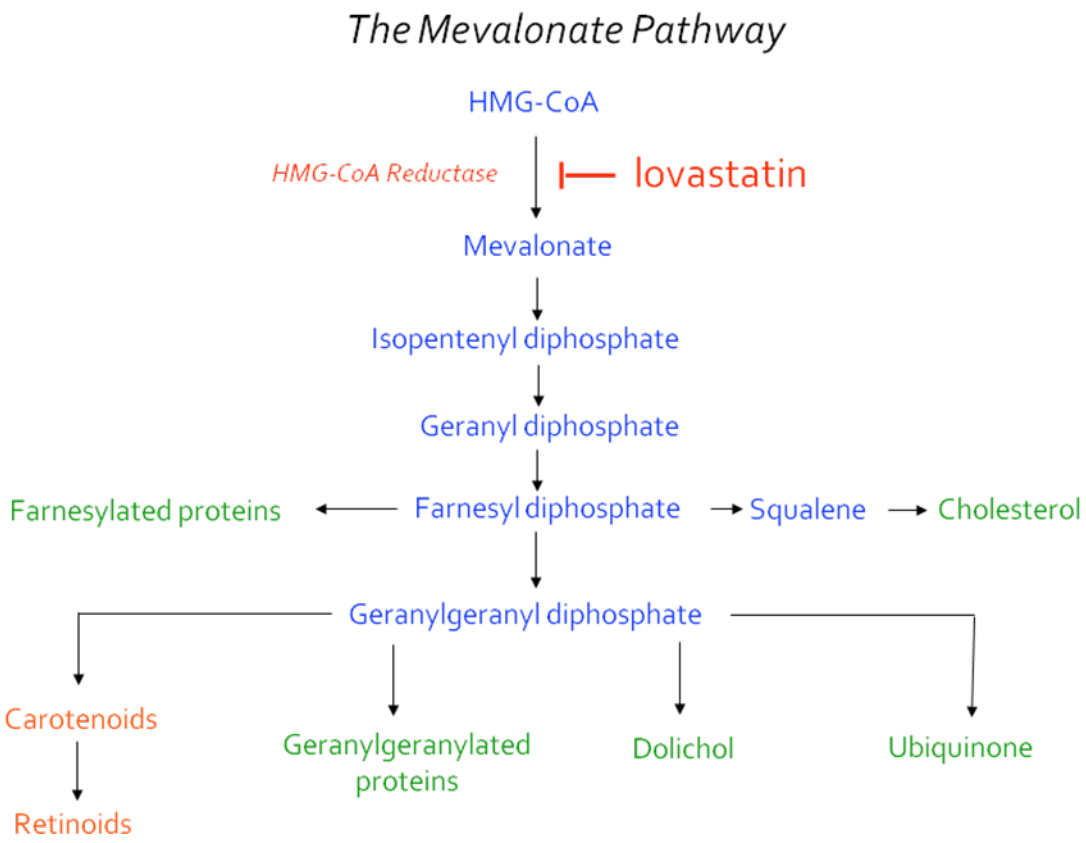

Figure 5. The Mevalonate Pathway. The mevalonate pathway involves the conversion of HMG to mevalonate, catalyzed by HMG-CoA reductase. Statins such as lovastatin, mimic the intermediate formed between HMG and mevalonate and bind to the active site of HMG-CoA reductase with high affinity. This prevents the formation of downstream metabolites such as cholesterol, ubiquinone, dolichol, as well as the generation of farnesylated and generanylgeranylated proteins.

and may also decrease epithelial growth factor-induced tumor cell invasion (Kusama et al., 2001). Statins also have the ability to induce apoptosis by inhibiting GGPP which is needed for Rho-mediated cell proliferation (Houten et al., 2003; Mo and Elson, 2004) and can up-regulate pro-apoptotic proteins such as Bax and Bim, down-regulate anti-apoptotic protein Bc12 and activate the cell death inducing caspase cascade (Cafforio et al., 2005). Research has indicated that some patients who were prescribed statins to lower their cholesterol levels had a somewhat decreased risk of cancer compared to non-statin users (Friis et al., 2005; Karp et al., 2008; Blais et al., 2000; Graaf et al., 2004; Farwell et al., 2008). Reduced risk of lung, prostate, breast and colorectal cancers (Farwell et al., 2008; Shannon et al., 2005; Cauley et al., 2006) has been observed although the role of statins and cancer risk is still being evaluated. Malignant cells, however, do seem to be dependent on the availability of the metabolites formed by the mevalonate pathway and deregulation of HMG-CoA reductase has been observed in a number of tumors (Chan et al., 2003). Studies have demonstrated the ability of statins to block tumor growth, invasion and metastases both *in vitro* and *in vivo* (Chan et al., 2003; Keyomarski et al., 1991; Wong et al., 2002).

1.14 Lovastatin.

Lovastatin is the most widely used and studied statin in advanced malignancies (Thibault et al., 1996). Lovastatin is a lipophilic statin as it is able to cross the cell membrane (Karp et al., 2008) and has no effect on other dividing cells such as bone marrow progenitors (Knox et al., 2005). A number of clinical trials have been conducted in which peak drug concentrations of 0.1 to 3.8μM were achieved, showing that lovastatin was well tolerated with rhabdomyolysis

(muscle wasting) being the most common side effect (Thibault et al., 1996; Kawata et al., 2001). Elevated and prolonged exposure of lovastatin (between 30 and 100μM) can induce apoptosis however since these concentrations are not achievable in animal models or in relevant phase I clinical trials (Keyomarsi et al., 1991; Wong et al., 2002; Dimitroulakos and Yeger, 1996), it was suggested that statins have potential as part of a combined therapy modality (Kawata et al., 2001). Our lab identified lovastatin as a mediator of the effects of retinoids, which are potent growth inhibitors and differentiation inducers of specific tumor types *in vitro* including pediatric malignancies, myeloid leukemias and squamous cell carcinomas (SCC; including HNC and cervical) (Dimitroulakos and Yeger, 1996). Retinoids repressed HMG-CoA reductase expression in various retinoid responsive cancers including acute myelogenous leukemia (AML), juvenile myelogenous leukemia (JML), neuroblastoma, pediatric solid malignancies, and SCC of the cervix and of the HNC, which were susceptible to lovastatin-induced apoptosis within therapeutically achievable levels (<5μM) (Dimitroulakos and Yeger, 1996; Dimitroulakos et al., 2001; Dimitroulakos et al., 1999). A phase I clinical trial was conducted in which a clinical evaluation of the cytotoxic effects of lovastatin on recurrent head and neck squamous cell carcinoma (HNSCC) and cervical carcinomas was undertaken (Knox et al., 2005). Disease stability for more than 3 months was observed in 23% of patients with rapidly progressive and resistant tumors, although tumor regressions were not observed (Knox et al., 2005). Lovastatin, however, appeared to be a potent pro-apoptotic agent in HNSCC and cervical SCC (Dimitroulakos et al., 2001). Together, these results further supported the feasibility of the use of lovastatin in cancer therapeutics.

1.15 Lovastatin and CI-1033 Targeting of the Epidermal Growth Factor Receptor.

ErbB and HMG-CoA reductase targeted therapies may be linked due to the ability of the mevalonate metabolites to affect functioning of the EGFR signaling pathway (Goldstein and Brown, 1990; Gschwind et al., 2001; Carlberg et al., 1996). Dolichol glycosylates RTK's including the EGFR allowing for the proper conformation of the receptor as well as ligand binding (Carlberg et al., 1996; Slieker et al., 1986). Membrane cholesterol composition is necessary for proper EGFR stimulation (Ringerike et al., 2002). The metabolites of the mevalonate pathway are also required for the function and localization of various signaling proteins downstream of the EGFR such as Ras, Rac and Rho family proteins that require FPP and GGPP modifications to properly transduce EGFR signals (Gibbs et al., 1994; Sebti and Hamilton, 1997). Our group has shown that treatment of HNSCC cells with lovastatin *in vitro*, inhibits ligand-induced EGFR activation (Mantha et al., 2005) and significantly affects AKT activation following induction with EGF (Mantha et al., 2005). More recently, we have demonstrated the ability of lovastatin to directly affect the EGFR through inhibiting receptor dimerization (Zhao et al., 2010). This is due to the effects of lovastatin on Rho A, namely disorganization of the actin cytoskeleton, preventing the proper formation of EGFR dimers (Zhao et al., 2010).

Upon recognizing the ability of lovastatin to affect EGFR functioning and downstream signaling our lab tested the effects of combining lovastatin with gefitinib (Dimitroulakos et al., 2006). This combination showed significant cooperative cytotoxicity *in vitro* on 16 SCC, NSCLC and colon carcinoma cell lines (Dimitroulakos et al., 2006). In some cell lines, namely SCC9, HCT116 and SIHA, the combination was synergistic in its cytotoxic abilities and was able to induce apoptosis (Dimitroulakos et al., 2006). The EGFR was assessed in

these cell lines and found to not contain ATP-binding site mutations, which increase sensitivity to gefitinib (Mantha et al., 2005). This combination showed a greater reduction in AKT activation in comparison to either of the two agents alone (Dimitroulakos et al., 2006). These results suggested that inhibitors of the EGFR and of HMG-CoA reductase may cooperatively target the EGFR, and allow for an attractive therapeutic approach (Dimitroulakos et al., 2006).

However, since various cancers also have aberrant expression of ErbB receptors other than the EGFR, this study aims to evaluate the ability of lovastatin to enhance the cytotoxicity of CI-1033.

Rationale:

Both lovastatin and CI-1033 target the EGFR; Lovastatin affects mevalonate pathway metabolites needed for proper EGFR function and activation and CI-1033 irreversibly inhibits the EGFR as well as other ErbB RTK's. Both drugs likely require being part of a combination therapy regimen in order to be effective. RTK's are elevated in a number of carcinomas.

Hypothesis:

Lovastatin will enhance the cytotoxicity of CI-1033 in ErbB expressing cell lines through dual targeting of ErbB receptors and their downstream signaling cascades.

Aims:

1) To evaluate the cytotoxic effects of lovastatin in combination with CI-1033 on malignant cell lines.

2) To determine the effects of this combination on ErbB receptor activity and downstream signaling cascades (P13K/AKT and MAPK pathways).

2. MATERIALS AND METHODS

2.1 Tissue Culture. The HNSCC cell line SCC25, the breast adenocarcinoma cell line MCF-7, the cervical carcinoma cell line HeLa, and the colon carcinoma cell line HCT116 were obtained from the American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained in Dulbecco's-MEM (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (Wisent Inc., St-Bruno, Quebec, Canada) and 100μg/ml penicillin-streptomycin (Invitrogen). Cells were exposed to solvent control or exposed to 0-50μmol/L lovastatin (generously provided by Apotex, Mississauga, Canada diluted from a 10mM stock in ethanol prepared as previously described by Dimitroulakos and Yeger, 1996). CI-1033 (generously provided by Pfizer, NY, USA) was diluted from a 10mM stock in dimethyl sulfoxide (DMSO) and human recombinant EGF (Sigma-Aldrich, St. Louis, USA) was diluted from a 50μg/mL stock in 10mM acetic acid/0.1% bovine serum albumin (Sigma-Aldrich).

2.2 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. In a 96-well flat-bottomed plate (Nunc, Naperville, USA) approximately 2,500 cells/75μL of cell suspension were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Cells were then treated with lovastatin in concentrations of 0-50μM alone for 72 hours or with CI-1033 in concentrations of 0-25μM alone for 48 hours or were pre-treated with either 1 or 5μM lovastatin for 24 hours followed by treatment with 0-10μM CI-1033 for 48 hours. Following treatment, 42μL of a 5mg/ml solution in phosphate buffered

saline (PBS) of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium substrate (Sigma-Aldrich) was added and incubated for up to 1 hour at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 82μL of a 0.01 mol/L HCl/10% SDS (Sigma-Aldrich) solution incubated at 37°C overnight. The plates were then analyzed (Dynex Technologies MRX plate reader, BioTek EL-808 plate reader, or BioTek Synergy MX plate reader; Dynex Technologies, Chantilly, USA and BioTek, Winooski, USA, respectively) at 570 nm to determine the absorbance of the samples.

2.3 Trypan Blue Exclusion Assay. MCF7 and SCC25 cells were plated at a density of 7.4 x 10^5 cells/mL in 10% DMEM in a 6-well flat bottomed plate (Nunc, Naperville, USA) and were pre-treated with either 1μM lovastatin, 5μM lovastatin or solvent control (100% Ethanol) after 24 hours of incubation at 37°C. Media was replaced following the 24 hour lovastatin pre-treatment and lovastatin was re-added. Cells were also treated with 1μM CI-1033, 5μM CI-1033 or solvent control (DMSO) and were allowed to incubate for 48 hours at 37°C. After incubation, cells were washed in 2mL PBS and harvested using 0.5μL trypsin. Samples were treated with 1.5ml 10% DMEM and 0.5μL aliquots were placed in cups on the haemocytometer (Beckman-Coulter Vi-Cell Automated Cell Viability Analyzer, Beckman-Coulter, Inc., USA). Total cell count and viable cell count of each sample was determined. The experiment was repeated twice in triplicate. Sample viable cell counts of the control cells (100% Ethanol and DMSO) were normalized to 1.0 and all subsequent samples were normalized to the control by the same variable.

2.4 Phase Contrast Microscopy. Phase contrast imagining was performed using the 10x objective on the Nikon Eclipse TE2000-U microscope (Nikon, USA). HCT116, HeLa, MCF7 and SCC25 cells were plated at a density of 5.0 x 10^5 cells/10cm dish (BD Biosciences, USA). Cells were treated with 5μM lovastatin for 24 hours followed by the combination of 5μM CI-1033 for 48 hours or were treated with the individual drugs alone (72 hour lovastatin treatment, 48 hour treatment). Control cells were treated either with solvent controls or were untreated.

2.5 Western Blot Analysis. Cellular protein was extracted using a RIPA lysis buffer that consisted of 50mM Tris-Cl pH 7.5, 0.25% sodium deoxycholate (Sigma-Aldrich), 0.1% sodium dodecyl sulphate (SDS, Sigma-Aldrich), 150mM NaCl, 1mM EDTA and 1% Triton X-100 in ddH_2O with or without 50mM sodium fluoride, 1mM sodium orthovanadate, and 5.4mM sodium pyrophosphate (phosphatase inhibitors added to prevent de-phosphorylation of phospho-proteins examined). Approximately 200μL of extraction buffer was used to treat SCC25 cells plated at 2.0 x 10^6 cells, MCF7 cells plated at 3.0 x 10^6 cells, HCT116 cells plated at 3.5 x 10^6 cells and HeLa cells plated at 2.0 x 10^6 cells as determined by optimal density experiments. Total protein was quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, USA) on the Biomate3 Spectrometer (Thermo Fisher Scientific, Waltham, USA). Protein extracts representing 45μg total protein for HeLa cells and 50μg total protein for HCT116, MCF7 and SCC25 cells. Protein was separated on a 10% SDS polyacrylamide gel and electrophoretically transferred onto PVDF membrane (Immobilon-P transfer membrane, Millipore, Billerica, USA). Membranes were blocked in 5% bovine serum albumin (BSA, Sigma-Aldrich) in 1 x Tris-buffered saline with Tween-20 (TBS-T) for 1 hour, shaking at

room temperature. Primary antibody, diluted in 5% BSA in 1 x TBS-T was incubated with the membrane shaking overnight at 4°C. The monoclonal antibodies used were specific for PY20 (1:1000, BD Biosciences, San Jose, CA), EGFR (1:1000), HER2/ErbB2 (1:750), HER3/ErbB3 (1:1000), HER4/ErbB4 (1:1000), PI3K (1:1000), ERK (1:1000) and phospho-ERK at site threonine 202/tyrosine 204 (1:750, all from Cell Signaling Technology, Beverly, USA). The polyclonal antibodies used were specific for AKT (1:1000), phospho-AKT at site serine 473 (1:750), PARP (1:1000), phospho-EGFR at site tyrosine 1045 (1:1000), phospho-EFGR at tyrosine 1048 (1:1000, all from Cell Signaling Technology), ATF3 (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and actin (1:10,000, Sigma-Aldrich). The horseradish peroxidase (HRP)-labeled secondary antibodies (Amersham, England) were applied at 1:2500 dilution for goat anti-rabbit HRP and 1:3000 for goat anti-mouse HRP in 5% BSA in 1 x TBS-T and incubated for one hour, shaking at room temperature. Washes following primary antibody incubations were 3 x 5 minutes in 1 X TBS-T and secondary antibody incubations were 2 x 5 minutes in 1 x TBS-T followed by 1 x 5 minutes in 1 x TBS. Chemiluminescent processing was done using chemiluminescent solutions from Thermoscientific or Bio-Rad and visualization of the protein bands was performed using the GeneSnap image acquisition system (Syngene, Frederick, USA).

2.6 Statistical Analysis. The probability of significant differences was determined by analysis of variance (one way ANOVA; multiple groups) and by the independent two sample t-test with either equal or unequal variance as determined by the F-test. For all analyses, significance was inferred at $P<0.05$. Analyses were performed using Microsoft® Excel data analysis software (Microsoft® Corporation, USA).

3. RESULTS

3.1 Lovastatin and CI-1033 have various effects on a panel of cell lines, *in vitro*. In this study, the ability of lovastatin to enhance the cytotoxicity of CI-1033 was evaluated. The effects of lovastatin and CI-1033 alone on representative cell lines, was first determined via the MTT cell viability assay. The HeLa, HCT116 and SCC25 cell lines were chosen as they have previously shown sensitivity to lovastatin in combination with gefitinib (Dimitroulakos et al., 2006). The breast adenocarcinoma cell line MCF7 was chosen to represent a cell line that was resistant to the combination of lovastatin and gefitinib (Dimitroulakos et al., 2006) and was expected to either provide a negative control or determine if using a pan-ErbB TKI would be effective on cell lines resistant to an inhibitor targeting the EGFR solely. In accordance to our previous studies, 72 hour treatment with lovastatin induced an 80% reduction in cell viability in SCC25 cells with the MCF7 cell line demonstrating a 10% reduction in cell viability following treatment with 5μM lovastatin alone. The HeLa and HCT116 cell lines responded with an approximate 15% and 30% reduction in cell viability, respectively. This 5μM dose is clinically relevant as these levels were achievable in previous clinical trials evaluating lovastatin in Phase I and II studies. Thus, SCC25 cells were the most responsive to treatment with 5μM lovastatin while MCF7 cells were the most resistant to its cytotoxic affect (Figure 6). In response to treatment with 5μM CI-1033 alone, the SCC25 cell line showed a 75% reduction in cell viability, MCF7 showed a 50% reduction where the HeLa cell line demonstrated a 20% reduction and the HCT116 a 40% reduction in cell viability. Similar to treatment with lovastatin alone, SCC25 cells were the most responsive to treatment with 5μM CI-1033 and MCF7 cells were among the least sensitive (Figure 7).

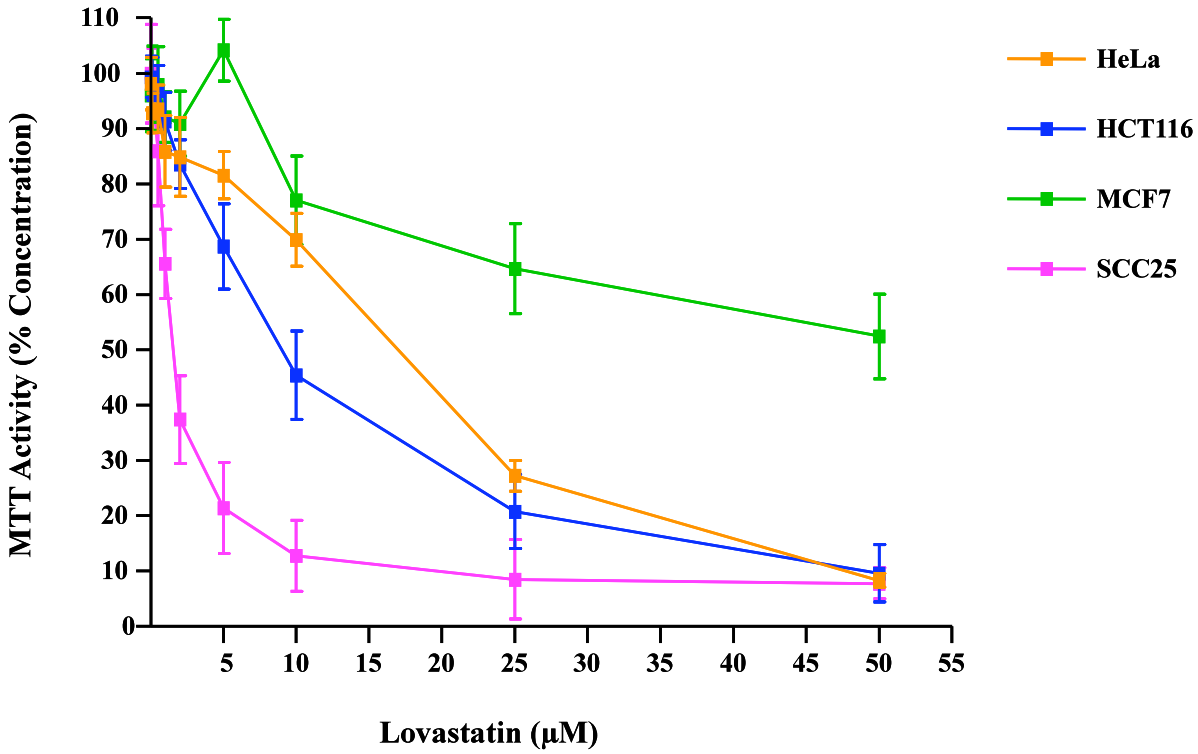

Figure 6. Cytotoxic effects of lovastatin on a panel of cell lines, *in vitro*. MTT viability assay comparing the responses of HCT116, HeLa, MCF7 and SCC25 cells treated with 0 - 50µmol/L (six replicates per dose) lovastatin for 72 hours. Each MTT was performed in triplicate. Graph represents pooled data.

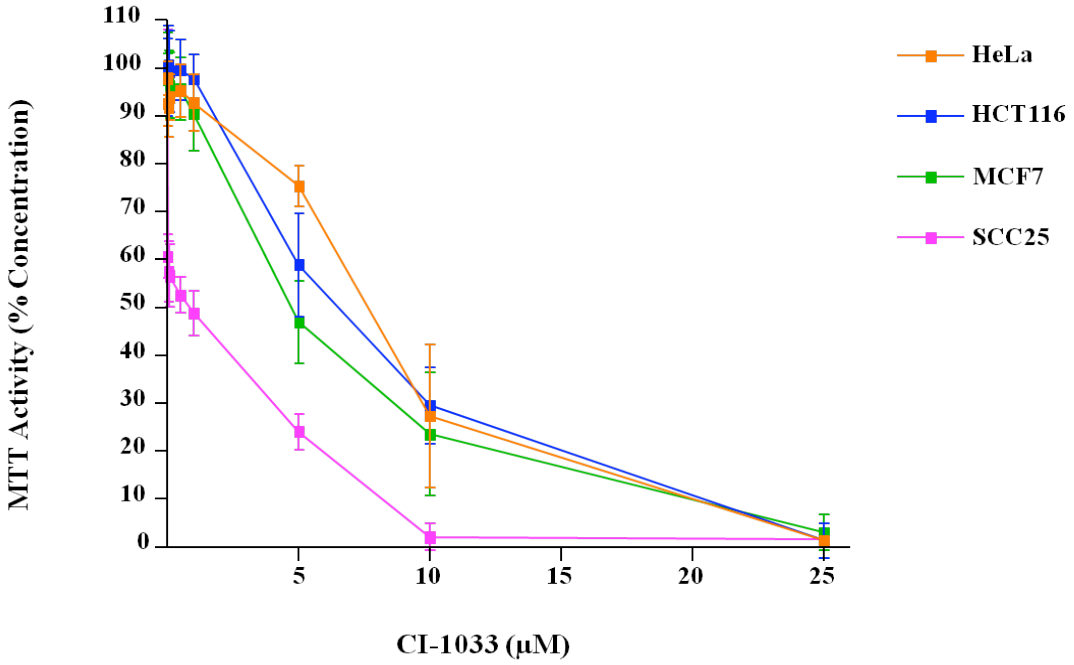

Figure 7. Cytotoxic effects of CI-1033 on a panel of cell lines, *in vitro*. MTT viability assay comparing the responses of HCT116, HeLa, MCF7 and SCC25 cells treated with 0 - 25µmol/L (six replicates per dose) CI-1033 for 48 hours. Each MTT was performed in triplicate. Graph represents pooled data.

3.2 Lovastatin potentiates the cytotoxic effects of CI-1033 in SCC25 cells. To determine whether lovastatin can enhance the cytotoxic effects of CI-1033, SCC25, MCF7, HeLa and HCT116 cells were treated with either 1 or 5μM lovastatin for 72 hours in combination with various concentrations of CI-1033 (0-25μM) for 48 hours and were evaluated by the MTT cytotoxicity assay. In all four cell lines, specific concentrations of lovastatin were able to potentiate the cytotoxic effects of CI-1033, and these values varied according to cell line. In the HeLa cell line, 1μM lovastatin in combination with 5 or 10μM CI-1033 resulted in a reduction of cell viability by approximately 30% and 80%, respectively as compared to untreated cells. This was a significant decrease in cell viability as compared to the individual treatments alone and to untreated cells ($p<0.05$ as determined by the standard t-test and one-way ANOVA). In the HCT116 cells, 1μM lovastatin in combination with 10μM CI-1033 resulted in a 70% reduction in cell viability and 5μM lovastatin in combination with 5 or 10μM CI-1033 resulted in a reduction of cell viability by approximately 55% and 80%, respectively. The reduction in cell viability was significant as compared to individual treatments and untreated cells ($p<0.05$ as determined by the standard t-test and one-way ANOVA). The MCF7 cells showed some response to the combination treatment with a decrease in cell viability of approximately 25% following treatment with 1μM CI-1033 in combination with both 1 and 5μM lovastatin. Decreases in cell viability at these combination concentrations were significant ($p<0.05$ as determined by the standard t-test and one-way ANOVA). SCC25 cells were highly responsive to the combination of lovastatin with CI-1033, showing decreased cell viability at concentrations as low as 0.5μM CI-1033 in combination with either 1 or 5μM lovastatin up to 5μM CI-1033 in combination with either 1 or 5μM lovastatin. Decreases in cell viability were 65% and 80% for 0.5μM CI-1033 in

combination with 1 or 5μM lovastatin, respectively, 70% and 80% for 1μM CI-1033 in combination with 1 or 5μM lovastatin, respectively and 85% and 90% for 5μM CI-1033 in combination with 1 or 5μM lovastatin, respectively. All of these combination treatments were statistically significant as compared to the individual treatments and non-treated cells ($p<0.05$ as determined by the standard t-test and one-way ANOVA) (Figure 8). Although all four cell lines were responsive to the combination treatment at some drug concentrations, only the SCC25 cells showed a consistent responsiveness to the combination at both 1 and 5μM lovastatin in combination with CI-1033 as compared to individual treatments and untreated cells.

The Trypan blue exclusion assay was performed on MCF7 and SCC25 cell lines in order to further validate the effects of lovastatin in combination with CI-1033 on cell viability. These cell lines were chosen to represent the most sensitive and least sensitive cells in the cell panel utilized to treatment with lovastatin. In the SCC25 cells, the combination of 5μM CI-1033 with 5μM lovastatin resulted in a 80% decrease in cell viability as compared to untreated cells and an approximate 15% and 30% reduction in cell viability as compared to the 5μM CI-1033 alone and 5μM lovastatin alone, respectively (Figure 9A). In MCF7 cells, the combination of 5μM CI-1033 with 5μM lovastatin resulted in an approximate 40% reduction in cell viability as compared to the control and to treatment with 5μM lovastatin alone, however, this decrease was not as dramatic when compared to treatment with 5μM CI-1033 alone, which resulted in a 30% decrease in cell viability on its own as compared to untreated cells (Figure 9B). Together, these results suggest the ability of lovastatin to potentiate the cytotoxic effects of CI-1033 in SCC25 cells.

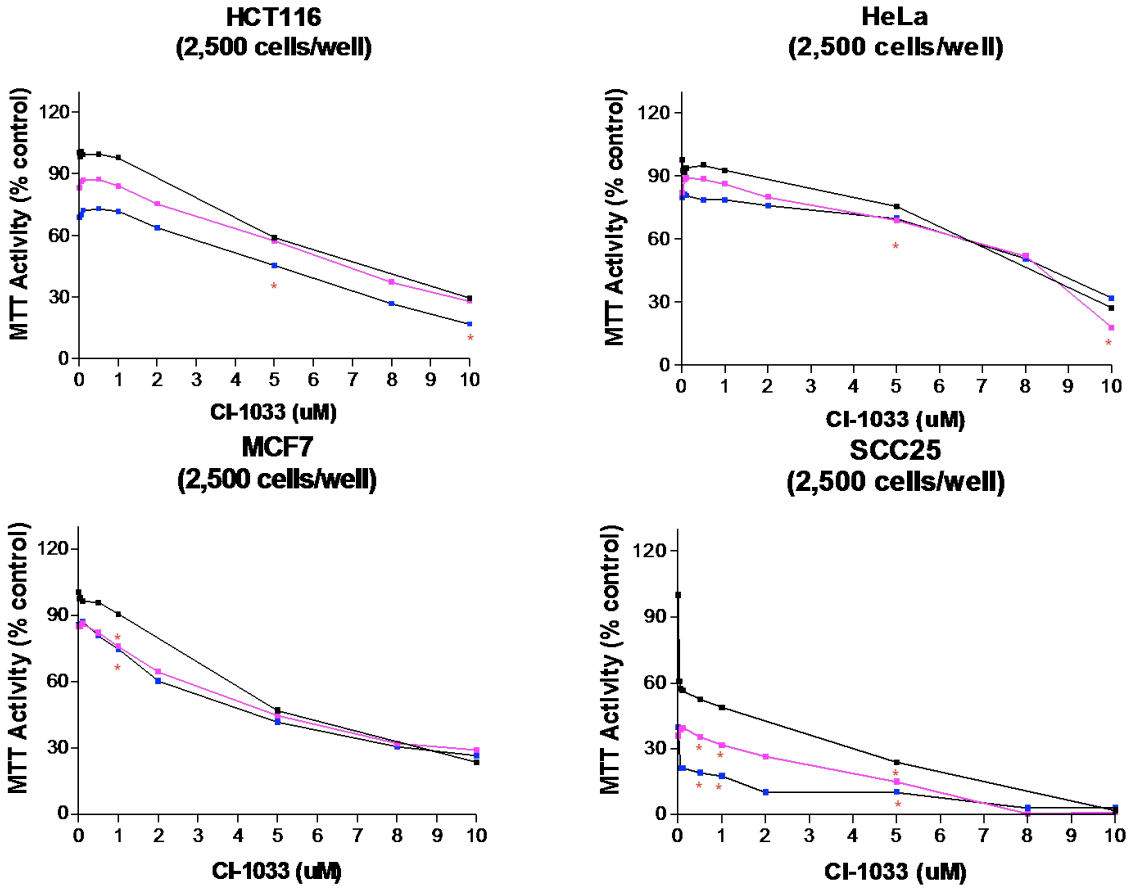

Figure 8. Lovastatin potentiates the cytotoxicity of CI-1033 in SCC25 cells, *in vitro*. MTT cell viability assays comparing the responses of HCT116, HeLa, MCF7 and SCC25 cells to treatment with 1 or 5µmol/L lovastatin (represented by the pink and blue lines, respectively) for 72 hours in combination with 0-25µmol/L CI-1033 for 48 hours (black line represents treatment with CI-1033 only and *denotes combinations of treatments that displayed significant differences in MTT activity compared to either agent alone and untreated cells; $p<0.05$, paired t-test and ANOVA). Each MTT was performed in triplicate.

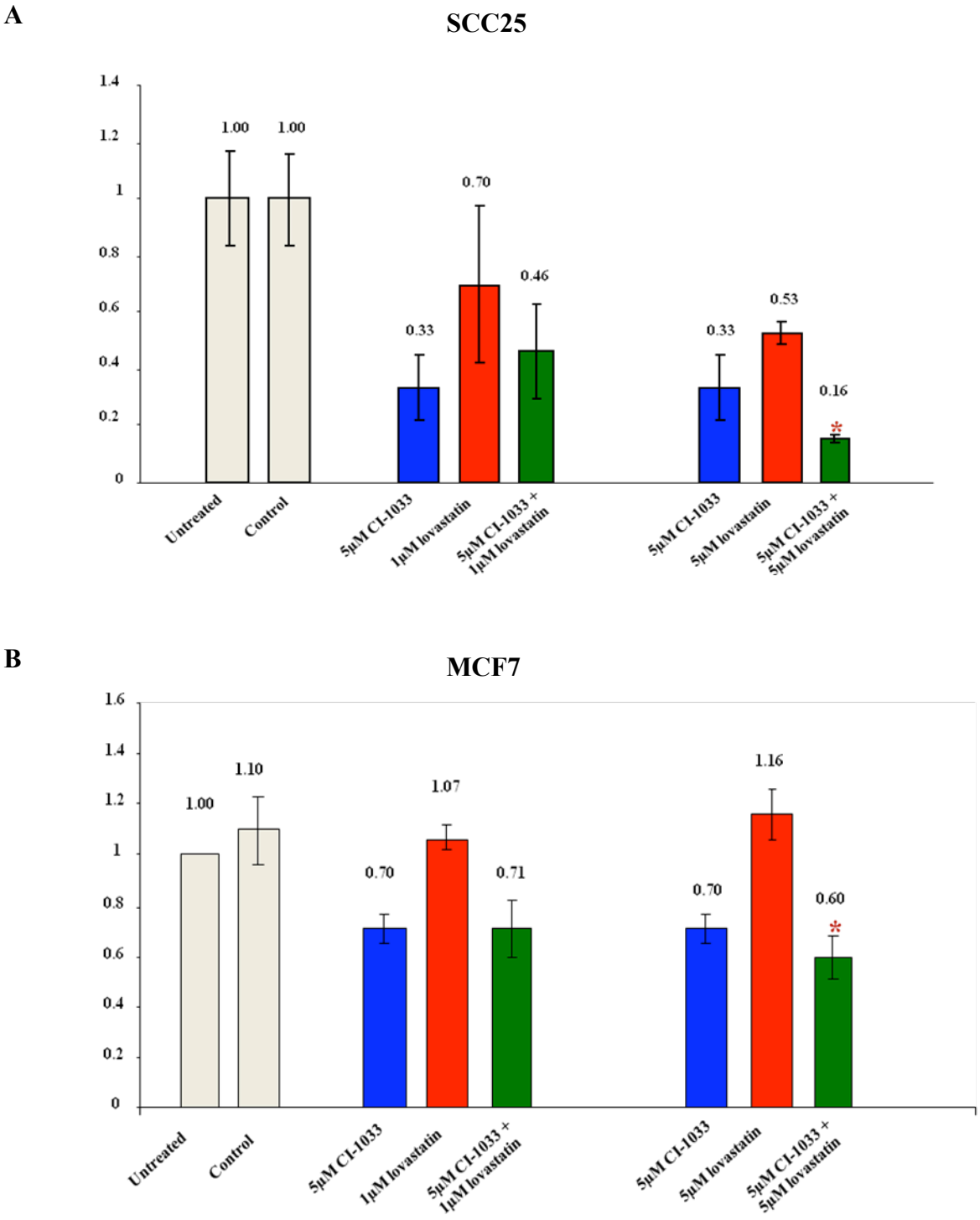

Figure 9. Lovastatin potentiates the cytotoxic effects of CI-1033 in SCC25 cells. Trypan Blue Exclusion Assay comparing the responses of MCF7 (A) and SCC25 (B), the most resistant and most sensitive cell lines in this study, respectively to 5μM CI-1033 in combination with 1 or 5μM 72 hour lovastatin treatment (* denotes significance with $p<0.05$).

3.3 Lovastatin in combination with CI-1033 induces apoptotic cell death in SCC25 cells.

Caspase activation is a hallmark of the induction of apoptosis (Duriez and Shah, 1997). Caspases regulate apoptosis through targeted degradation of cellular proteins like poly ADP ribose polymerase (PARP) (Duriez and Shah, 1997). Activation of the caspase cascade was evaluated in the HCT116, HeLa, SCC25 and MCF7 cell lines by western blot analysis. Following treatment of 1μM or 5μM CI-1033 alone, 5μM or 10μM lovastatin alone, 5μM CI-1033 in combination with 5μM or 10μM lovastatin or with solvent controls, cells were probed for cleavage of PARP. Death by apoptosis is induced by the upregulation of caspase-3 followed by the proteolytic cleavage of the 116kDa PARP protein to an 89kDa cleavage product (Duriez and Shah, 1997). Proteolytic cleavage of PARP was observed in SCC25 and HCT116 cells treated with either 1μM or 5μM CI-1033 alone and in the combination of 5μM CI-1033 with 5μM or 10μM lovastatin (Figure 10). The amount of cleaved PARP was higher in the combination treatments as compared to CI-1033 alone in SCC25, HCT116 and HeLa cells. Cleavage of PARP was not observed in MCF7 cells as these cells are caspase-3 deficient (Duriez and Shah, 1997). All of the treatments, including the controls resulted in cleaved PARP in the HeLa cells indicating potential over-confluence of these cells. These results demonstrate the ability of CI-1033 to induce apoptosis in HCT116 and SCC25 cells, although the results are more pronounced in the SCC25 cell line. These results also indicate the ability of lovastatin to enhance CI-1033 mediated cell death via apoptosis in SCC25 cells as previously shown by the MTT cell viability assay results.

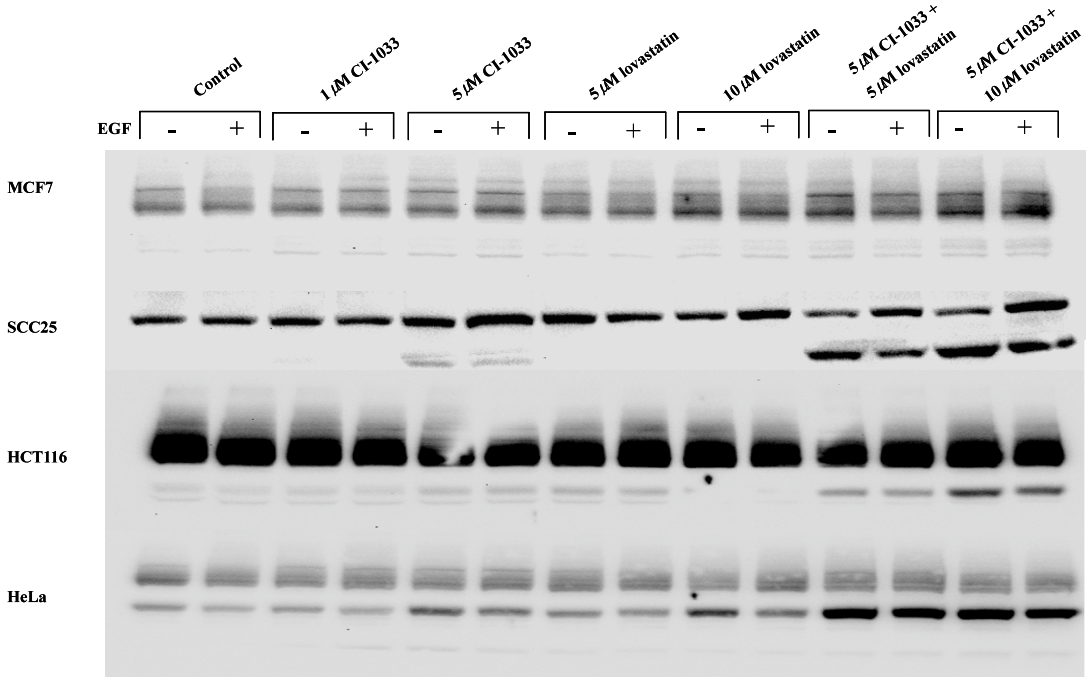

Figure 10. Lovastatin in combination with CI-1033 induces apoptotic cell death in SCC25 cells. MCF7, SCC25, HCT116 and HeLa cells were treated with lovastatin for 24 hours followed by treatment with CI-1033 for 2 hours. The EGFR was stimulated with EGF to induce receptor activation. Death by apoptosis was assessed by proteolytic cleavage of 116kDa PARP to an 89kDa cleavage product.

Cells were analyzed by phase contrast microscopy in order to observe the physical effects of the combination of lovastatin and CI-1033 on the cell lines. (Figure 11). These phase contrast images clearly depict the decreased viability of SCC25 cells following combination treatment with lovastatin and CI-1033 as compared to the individual treatments alone, treatment with solvent control (DMSO and 100% Ethanol) and non-treated cells. Cell death is observed through the rounding up of the SCC25 cells following the combination treatment. In the MCF7 cell line, cell-rounding indicative of cytotoxicity, was negligible. This data further demonstrates the ability of lovastatin to enhance the effects of CI-1033 in SCC25 cells.

3.4 Lovastatin in Combination with CI-1033 inhibits activation of the Epidermal Growth Factor Receptor. In order to validate the use of a pan-ErbB TKI on SCC25 cells, expression of all four ErbB family members was assessed via western blot analysis. As previously stated, SCC25 and MCF7 cells represent the most sensitive and most resistant cell lines in this panel to lovastatin, CI-1033 and their combination. Cell lines were treated with 1μM or 5μM CI-1033 alone, 5μM or 10μM lovastatin alone, 5μM CI-1033 in combination with 5μM or 10μM lovastatin or with drug solvents as controls. ErbB expression was assessed using anti-EGFR, anti-ErbB2, anti-ErbB3 and anti-ErbB4 antibodies (Figure 12). All four ErbB receptors were expressed in both the SCC25 and MCF7 cell lines at their respective molecular weights, which indicates that truncated receptors were not present in these cell lines. Exons 18 to 21 of the EGFR encompass the ATP binding site (Lynch et al., 2004; Paez et al., 2004) and these sites were sequenced to determine the mutational status of

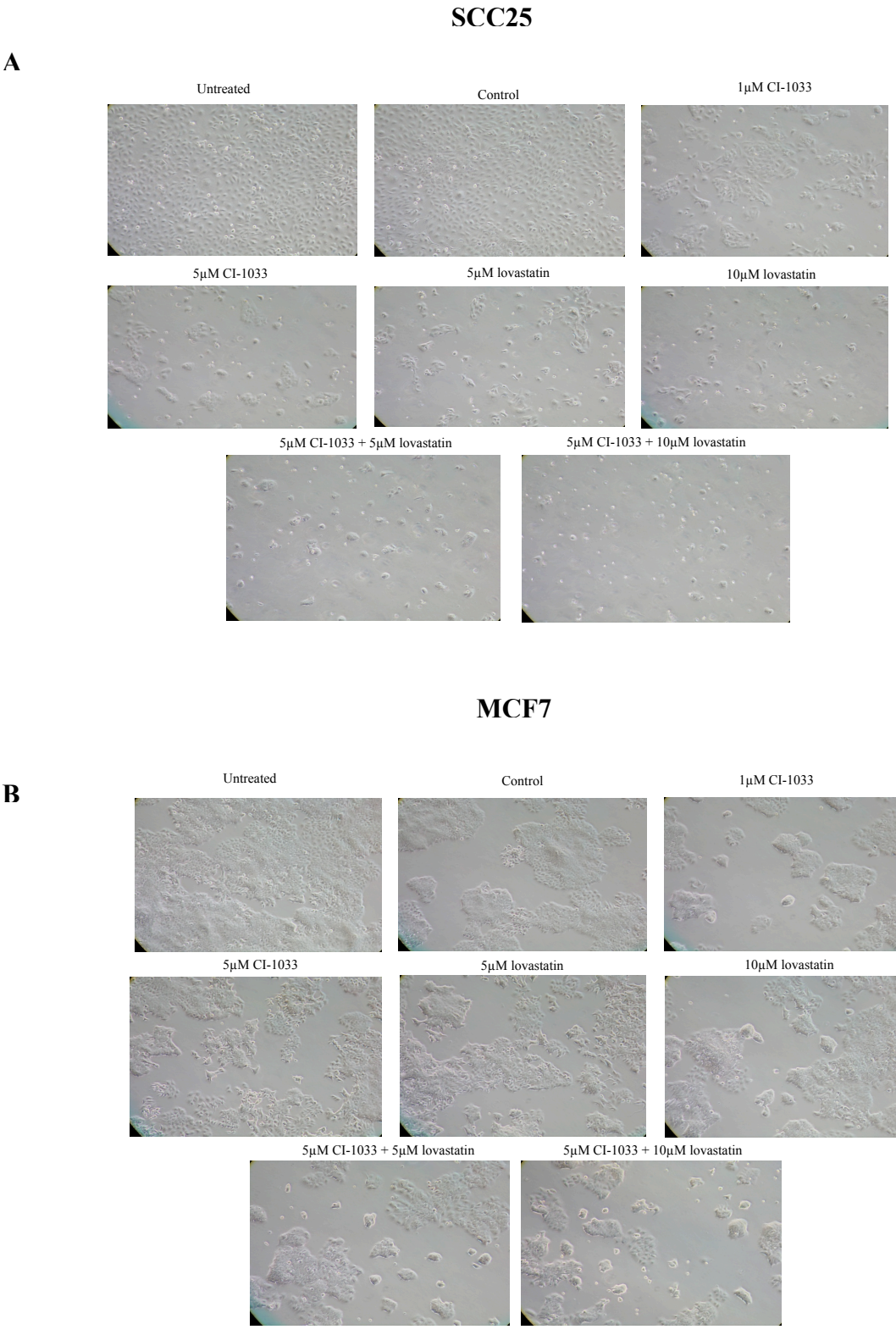

Figure 11. The physical effects of lovastatin in combination with CI-1033 on MCF7 and SCC25 cells. SCC25 (A) and MCF7 (B) cells were treated with CI-1033 alone for 48 hours, lovastatin alone for 72 hours and the two agents in combination. Phase contrast images were taken to observe the physical effects of these drugs.

this region in HCT116, SCC25, HeLa and MCF7 cells (Mantha et al., 2005). No point mutations or deletions were detected in the ATP binding sites of the EGFR's in each of the cell lines (Mantha et al., 2005). This is important because studies have shown that tumor cells harboring mutations in the ATP binding pocket of the EGFR intracellular Tyr kinase domain show more pronounced anticancer affects following treatment with the TKI, gefitinib (Lynch et al., 2004; Paez et al., 2004). Neutral polymorphisms were detected but did not result in alteration of the amino acid sequence of the EGFR (Mantha et al., 2005).

Western blot analysis was also used to determine the effects of the combination of lovastatin and CI-1033 on EGF-induced EGFR phosphorylation. The phospho-specific antibody employed detects a critical pTyr at Tyr residue 1,068 on the EGFR (Bishayee et al., 1999). MCF7 and SCC25 cells were serum starved and treated with 5 or 10μM lovastatin for 24 hours. As mentioned before, these are conditions that evoke significant inhibition of EGF-induced EGFR activation. After the 24 hour lovastatin treatment, cells were treated with CI-1033 for 2 hours and the EGFR was stimulated by EGF treatment for 15 minutes. The blots were then probed for pTyr at residue 1068. In the SCC25 cell line, CI-1033 alone and in combination with 5 or 10μM lovastatin resulted in inhibition of EGF-induced EGFR phosphorylation was observed (Figure 12). Although EGF-induced EGFR phosphorylation was also observed in MCF7 cells, the effects were more pronounced in the SCC25 cells.

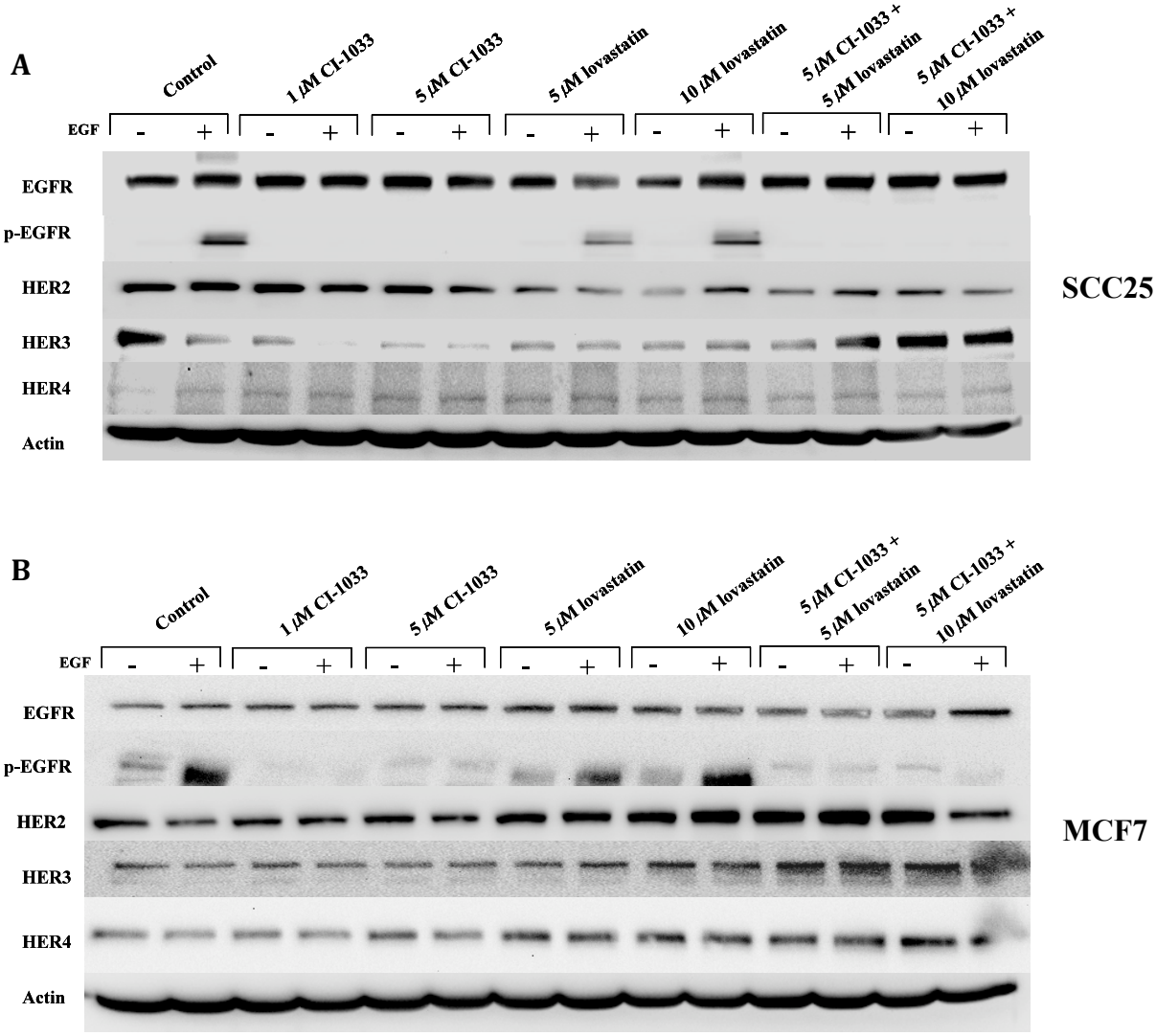

Figure 12. CI-1033 is a potent inhibitor of the EGFR in SCC25 cells. SCC25 (A) and MCF7 (B) were treated as described and were probed for expression of EGFR, ErbB2, ErbB3 and ErbB4 protein by western blot analysis. Activation of EGFR was evaluated by phosphorylation at Tyr 1068. Actin was used as a loading control.

3.5 CI-1033 Inhibition of Ras/MAPK and PI3K/AKT pathways is potentiated by lovastatin in SCC25 cells. Based on the ability of lovastatin to inhibit EGFR phosphorylation in SCC25 cells when in combination with CI-1033, signaling cascades downstream of the ErbB receptors were evaluated. Once again SCC25 and MCF7 cells were used to represent the most and least sensitive cell lines, respectively, to the combination of CI-1033 and lovastatin. The signaling pathways induced by these receptors include the PI3K/AKT and MAPK/Ras signaling pathways, which have previously shown to be affected by lovastatin treatment (Mantha et al., 2005). Serum starved SCC25 and MCF7 cells were treated with 5μM or 10μM lovastatin for 24 hours, conditions which cause significant inhibition of EGF-induced EGFR activation (Mantha et al., 2005). Following the lovastatin treatment, cells were treated with 1 or 5μM CI-1033 for 2 hours followed by ± EGF for 15 minutes to stimulate activation of the EGFR. Western blot analysis was employed to determine the functional activation of these pathways (Figure 13). Phosphospecific antibodies for active ERK and AKT as well as control antibodies for ERK and AKT were utilized. Lovastatin did not affect EGF-induced phosphorylation of ERK whereas both basal levels and EGF-induced levels of phosphorylated AKT were reduced in SCC25 and MCF7 cells, as previously published (Mantha et al., 2005). Following treatment with 1 or 5μM CI-1033, EGF-induced phosphorylated ERK (p-ERK) levels were reduced compared to basal protein levels in SCC25 cells. This reduction was also seen in the combination treated SCC25 cells as compared to basal protein levels. The MCF7 cells did not show reduced levels of EGF-induced p-ERK following treatment with lovastatin alone as compared to basal protein levels. ATF3 is induced by lovastatin in both the MCF7 and SCC25 cell lines as previously published (Niknejad et al., 2007). PI3K levels are steady as compared to basal

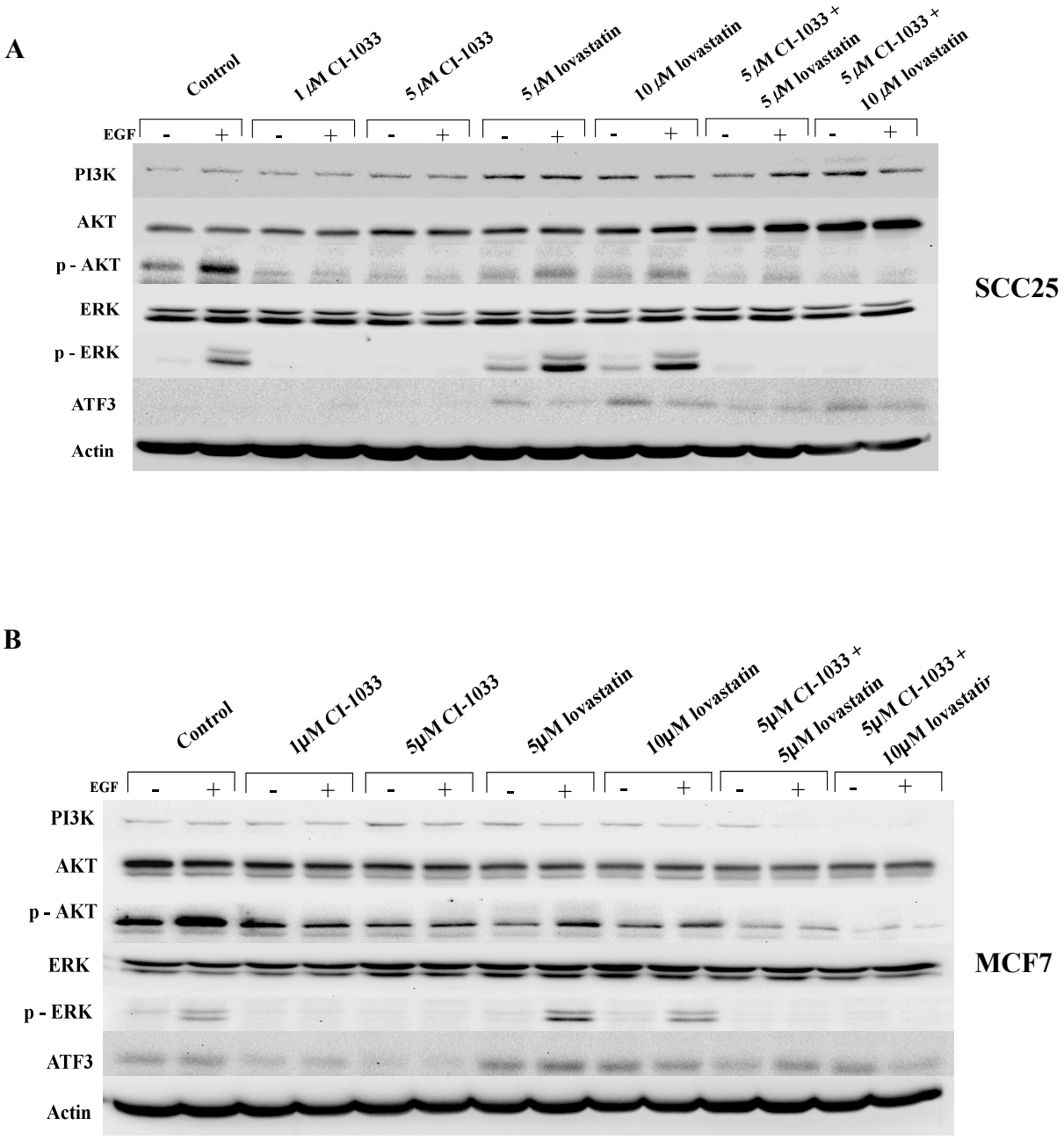

Figure 13. CI-1033 inhibition of Ras/MAPK and PI3K/AKT pathways is potentiated by lovastatin in SCC25 cells. Western blot analysis of 24-hour lovastatin treated-serum starved SCC25 (A) and MCF7 (B) cells. Cells were also treated with CI-1033 for 2 hours followed by the addition of 50ng/mL EGF. Cells were probed for PI3K (p85), AKT, p-AKT, ERK, p-ERK and ATF3. Actin was used as a loading control.

levels in both the MCF7 and SCC25 cell lines (cells probed for the p85 subunit). These results suggest that CI-1033 interferes with the transduction of EGFR signals to the Ras/MAPK pathway and the PI3K/AKT pathway in SCC25 cells.

4. DISCUSSION

Cancer is a leading cause of death worldwide and is predicted to cause an estimated 12 million deaths in 2030 as projected by the WHO. Despite advances in cancer therapy including improved surgical techniques, radiation oncology and the design of anti-cancer drugs, many patients with cancer fail to be cured (Dutta and Maity, 2007). SCC patients, specifically, have limited treatment options when presenting with metastatic disease (Greenlee et al., 2000). A number of anti-cancer therapies have been designed to target members of the ErbB family of RTK's, most specifically the EGFR (Dutta and Maity, 2007). The ErbB family of receptors are often mutated and/or overexpressed in cancerous tumors leading to deregulated cell proliferation, apoptosis, and angiogenesis, as well as increased tumor invasiveness and metastases (Baselga, 2002; Grandis and Sok, 2004). As such, therapies designed to target members of this family of receptors are relevant. Clinical trials using these agents in a variety of clinical settings have shown limited therapeutic efficacy, particularly when administered as individual therapies (Dutta and Maity, 2007). Combining these therapies with other treatment modalities, therefore, may lead to increased activity.

The purpose of this study was to investigate the potential of the HMG-CoA reductase inhibitor lovastatin to enhance the cytotoxic activity of the TKI CI-1033 through dual targeting and inhibition of the EGFR. In the literature, both CI-1033 and lovastatin have demonstrated the ability to inhibit EGFR activation as single agents through competitive inhibition with ATP for intracellular binding sites and inhibition of mevalonate metabolites, which has pleiotropic effects on the EGFR, respectively. The limited clinical responsiveness

following CI-1033 treatment is due to its specificity for overexpression of certain ErbB receptors in specific tumor types. Most phase II clinical trials conducted using CI-1033, have suggested the potential for this drug to work best as part of a combined therapeutic treatment modality as previously discussed. Limited clinical activity has also been demonstrated following individual treatment with lovastatin and it too may serve better as part of a combined modality approach (Mantha et al., 2005). Because CI-1033 is designed to compete with ATP for the intracellular domain ATP binding pocket on the ErbB RTK's, CI-1033 is expected to have various off target effects. These can be circumvented through combining this drug with other inhibitors of the EGFR and potentially lowering the concentration of CI-1033. Lovastatin, through inhibiting metabolites of the mevalonate pathway, has pleiotropic effects on not only various cellular pathways but the function of the EGFR as well.

The first objective of this study was to demonstrate the effects of these agents on a variety of cancer cell lines including MCF7, SCC25, HeLa and HCT116. These cell lines were chosen because of their previous responses to lovastatin alone and in combination with the TKI gefitinib (Dimitroulakos et al., 2006). Western Blot analysis demonstrated that all four of these carcinoma cell lines express the EGFR, ErbB2, ErbB3, and ErbB4 receptors and as such, their use was relevant to this study. These four cell lines responded differently to the individual treatments of both CI-1033 and lovastatin. The SCC25 cell line showed the highest sensitivity to both CI-1033 and lovastatin treatments alone. The MCF7 cell line was least sensitive to the effects of lovastatin treatment alone and the HeLa cell line was least responsive to treatment with CI-1033 alone. The HCT116 cell line was moderately responsive to both of these agents following individual treatment. As such, the SCC25 cell line was chosen to represent a cell line sensitive to both lovastatin and CI-1033 individual

treatments. The MCF7 cell line was chosen to represent a cell line that was non-responsive to treatment with lovastatin and CI-1033. This allowed for us to determine whether the combination of lovastatin with CI-1033 could enhance cytotoxicity when administered together on cell lines with varying responses to these agents individually.

Following combination treatment with CI-1033 and lovastatin, MCF7 cells were least sensitive to the combination of these two agents and the SCC25 cell line was the most sensitive to the combination of these agents. Although some significant differences were observed in the MCF7 cell line following combination treatment as compared to untreated cells, the reduction in cell viability was not as dramatic and did not cover the range of treatments as observed in the SCC25 cell line. The SCC25 cell line demonstrated consistent significant decreases in cell viability starting at 0.05μM CI-1033 to 5μM CI-1033 in combination with both 1 and 5μM lovastatin compared to individual treatments. Sensitivity to lovastatin alone and in combination with other TKI's such as gefitinib and erlontinib has previously been demonstrated in SCC cell lines (Mantha et al., 2005) suggesting that this type of treatment regimen may be best suited in this type of cancer.

Phase contrast images taken of MCF7 and SCC25 cells following treatment with lovastatin and CI-1033 alone as well as the combination of these two agents has allowed for visual determination of the effects of these two agents. Visually, the cells were rounded up and were detached from the plate following dual treatment with lovastatin and CI-1033 in comparison to either agent alone in the SCC25 cells. This sparked further interest, at a molecular level, as to how these agents were working together. Since it seemed this combination of agents induced morphological characteristics consistent with induction of

apoptosis in the SCC25 cells, cleavage of PARP was assessed to determine whether this combination treatment modality was causing cell death.

This novel combination of agents exhibited the ability to induce apoptotic cell death in SCC25 cells as determined by the cleavage of PARP. Interestingly, this combination increased apoptosis in the SCC25 cells as compared to lovastatin alone, and is enhanced as compared to treatment with CI-1033 as a single agent. This suggests that lovastatin is potentiating the ability of CI-1033 to induce apoptosis in these cells. The combination of these agents does not enhance PARP cleavage in the HeLa or HCT116 cell lines. Lovastatin has previously been shown to induce expression of activating transcription factor 3 (ATF3) through activation of the integrated stress response (Niknejad et al., 2007). ATF3 is thought to act as a tumor suppressor through inhibition of inhibitor of differentiation 1 (Id1) following binding of ATF3 to the promoter of Id1, while in complex with Smad3 (Kang et al., 2003). ATF3 is also able to induce the cyclin dependent kinase inhibitor p27, which is able to arrest the cell cycle at the G1 phase (Thompson et al., 2009). As such, we have observed induction of ATF3 in both the MCF7 and SCC25 cell lines following treatment with both lovastatin alone and lovastatin in combination with CI-1033. Induction of ATF3 demonstrates that lovastatin is active in each of these cell lines.

MCF7 is caspase-3 deficient (Onuki et al., 2003) and has thus provided a control for caspase induced apoptosis seen in the SCC25 cells. This deficiency causes failed inactivation of the caspase cascade and subsequent PARP cleavage in this cell line demonstrating that the caspase cascade is responsible for the apoptotic cell death seen in the SCC25 cell line. This will be important in determining tumor types appropriate for this treatment regiment, namely those with an intact caspase cascade will be best suited.

The combination of CI-1033 and lovastatin has exhibited the ability to affect EGF-induced activation of the EGFR. This has been demonstrated by the lack of phosphorylation at critical Tyr residue Tyr1068 located in the intracellular catalytic domain of the EGFR. This phenomenon was observed in both the MCF7 and SCC25 cell lines, however the effects were much more pronounced in the SCC25 cell line. As phosphorylation of EGFR is critical for downstream activation of signaling cascades, the effects of this dual treatment on components of the PI3K/AKT and Ras/MAPK pathways were observed. The combination of lovastatin and CI-1033 resulted in inhibition of activation of ERK as well as AKT as compared to EGF-induced basal levels in the SCC25 cell line. These results were not as dramatic in the MCF7 cells, however. It also appears that treatment of SCC25 cells alone with 1 or 5μM CI-1033 shows potent effects on these cells. As such, it was difficult to discern differences between the CI-1033 treatment alone and in combination with lovastatin. It would seem that these results indicate that CI-1033 is not being potentiated by the addition of lovastatin at these concentration levels of CI-1033. To address this issue, lower concentrations of CI-1033 would need to be tested in combination with lovastatin in future studies, to show a more moderate affect. Taken together, these results suggest successful inhibition of the EGFR as phosphorylation at critical tyrosines is not observed and as ERK and AKT, components of cascades downstream of the EGFR, are not activated.

Taking into consideration that CI-1033 alone is able to inhibit phosphorylated EGFR whereas treatment with lovastatin alone still results in phosphorylated EGFR and that lovastatin alone is able to induce apoptosis whereas CI-1033 treatment alone is not, this novel combinational approach suggests the co-operation of these drugs in targeting the EGFR at different levels. At one level, we observe CI-1033 causing irreversible inhibition of

the EGFR through blocking ATP binding and at another level we observe lovastatin-mediated induction of ATF3 and of the caspase cascade with the subsequent result of apoptosis in these tumor cell lines, *in vitro*.

As many cancer cell lines that overexpress ErbB2, ErbB3 and/or ErbB4 have responded well to CI-1033 and/or lovastatin, continuation of this study should include testing the effects of the combination of these two agents on PI3K/AKT and Ras/MAPK pathway components following stimulation with growth factors that correspond to these receptors.

These preliminary results suggest that this combination of agents seems to work best in squamous cell carcinomas of the head and neck. HNSCC represents the sixth most commonly diagnosed cancer in the world (Hoffman et al., 1998) and the tongue is the most common part of the oral cavity associated with SCC (Rusthoven et al., 2008). Tongue cancer has increased in incidence and has a poor survival rate compared to other parts of the oral cavity (Zelefsky et al., 1990). It has been previously described that members of the ErbB family of receptors play a role in the pathogenesis and progression of HNSCC (O-charoenrat et al., 2002). The EGFR is overexpressed in approximately 90% of HNSCC whereas the reports on ErbB2 contribution to HNSCC pathogenesis are conflicting (Slamon et al., 1989, Cavalot et al., 2007; Craven et al., 1992; Field et al., 1992; Xia et al., 1997). The overexpression of ErbB3 seems to play a role in the pathogenesis and progression of HNSCC, especially when coexpressed with other ErbB receptors (Shinthani et al., 1995; O-charoenrat et al., 2002; Xia et al., 1999; Ibrahim et al., 1997) and plays an important role in patient prognosis in patients with SCC of the tongue (Del Sordo et al., 2009). The prognostic role of ErbB4 has not been fully characterized in tumors expressing this receptor such as breast, colon, prostate, lung, ovary, pancreas, endometrium, bronchus, cervix, stomach,

thyroid and esophagus (Del Sordo et al., 2009; Srinivasan et al., 1999; Haugen et al., 1996; Merimsky et al., 2001; Graber et al., 1999; Gilmour et al., 2001; Srinivans et al., 2000; Xu et al., 2008). It has, however, shown the ability to enhance the invasive properties of HNSCC cells following heterodimer formation with other members of the ErbB family (O-charoenrat et al., 1999; O-charoenrat et al., 2000). Considering that squamous cell carcinomas of the head and neck seem to be most sensitive to these particular anti-cancer agents, future direction of this project should also focus on expanding these results on other HNSCC cell lines to further validate the use of this combination of agents on these cancer types. As previously mentioned, lower concentrations of CI-1033 should also be used in combination with lovastatin to circumvent the effects of CI-1033 treatment alone on these cells.

Following the potential expansion of this study to include other HNSCC cell lines and ligand stimulation of the ErbB2, ErbB3 and ErbB4 receptors, this *in vitro* study may potentially move towards *in vivo* work in the future. Animal models of HNSCC can be utilized to test the effects of the combination of lovastatin and CI-1033 on *in vivo* tumor progression. These results will be valuable in the potential progression to a phase I clinical trial of this novel combinational therapeutic approach. Although these results look somewhat promising, more work will need to be done prior to progression to clinical trials.

In this study, the ability of this dual agent therapeutic modality to inhibit the function of the EGFR has been demonstrated. Furthermore, the combination of these agents results in apoptotic cell death and significantly reduced cytotoxicity as compared to either agent alone. These results seem to be manifested in squamous cell carcinomas of the tongue as represented by the SCC25 cell line in this study and present the possibility of this treatment on cancers of this nature. The results also demonstrate the ability of this combination to

inhibit activation of the EGFR and subsequently affect the PI3K/AKT and Ras/MAPK signaling pathways. These pathways play important roles in mitogenic and cell survival responses mediated by the ErbB receptors (Boulougouris and Elder, 2001; Mendelsohn and Baselga, 2000). Taken together, these findings suggest that combining mevalonate pathway and tyrosine kinase inhibitors may represent a novel combinational therapeutic approach in variety of human cancers. Through irreversible targeting of all members of the ErbB family of receptors, CI-1033 has demonstrated the ability to inhibit activation of the EGFR in response to EGF stimulation suggesting its potential ability to inhibit activation of other members of the ErbB receptor family.

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