Mechanism and Therapeutic Potential of Statin-Mediated Inhibition of Tyrosine Kinase Receptors

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Abstract

Receptor tyrosine kinases (RTK) are key regulators of growth, differentiation and survival of epithelial cells and play a significant role in the development and progression of cancers derived from these tissues. In malignant cells, these receptors and their downstream signalling pathways are often deregulated, leading to cell hyper-proliferation, enhanced cell survival and increased metastatic potential. Furthermore, endothelial expressed RTKs regulate tumor angiogenesis allowing for tumor growth and maintenance by promoting their vascularization. Epithelial malignancies such as squamous cell carcinomas (SCC), non-small cell lung (NSCLC) and malignant mesotheliomas have very limited treatment options when presenting as metastatic disease. RTKs, particularly the epidermal growth factor (EGFR) and the vascular endothelial growth factor (VEGFR) receptors, have been shown to play significant roles in the pathogenesis of these tumor types. Statins are potent inhibitors of HMG-CoA reductase, the rate limiting enzyme of the mevalonate pathway, that are widely used as hypercholesterolemia treatments. The mevalonate pathway produces a variety of end products that are critical for many different cellular pathways, thus, targeting this pathway can affect multiple signalling pathways. Our laboratory has previously shown that lovastatin can induce tumor specific apoptosis especially in SCC and that 23% of recurrent SCC patients treated with lovastatin as a single agent showed disease stabilization in our Phase I clinical trial. Subsequently, our lab was able to demonstrate that lovastatin in combination with gefitinib, a potent inhibitor of the EGFR showed co-operative cytotoxicity when combined (Chapter 2). Furthermore, the pro-apoptotic and cytotoxic effects of these agents were found to be synergistic and to be manifested in several types of tumor cell lines including SCC, NSCLC and glioblastoma. I was able to expand upon these important

findings and demonstrated that lovastatin, through its ability to disrupt the actin cytoskeleton, inhibited EGFR dimerization and activation (Chapter 3). This novel mechanism targeting this receptor has clinical implications as lovastatin treatment combined with gefitinib showed co-operative inhibitory effects on EGFR activation and downstream signalling. The RTK family of proteins share similar features with respect to activation, internalization and downstream signalling effectors. I further demonstrated that lovastatin can inhibit the VEGFR-2 in endothelial cells and mesotheliomas, where VEGF and its receptor are co-expressed driving their proliferation, and induces synergistic cytotoxicity in mesothelioma cells in combination with VEGFR-2 tyrosine kinase inhibitors (Chapter 4). These findings suggest that statins may augment the effects of a variety of RTK inhibitors in a similar fashion representing a novel combinational therapeutic approach in a wide repertoire of human cancers. More importantly, based on this work, we initiated a Phase I/II study evaluating high dose rosuvastatin and the EGFR inhibitor tarceva in SCC and NSCLC patients at our institute. This clinical evaluation will provide invaluable data that will play a role in developing this novel therapeutic strategy. Together, the work embodied in this thesis provides a model for the regulation of EGFR/VEGFR-2 activation and signalling by targeting the rho family of proteins that demonstrates a novel mechanism that can be exploited to refine current therapeutic paradigms.

Acknowledgments

Many people have made it possible for me to complete this chapter of my professional life. They include friends, family, and colleagues. Thanks go to my committee members, Drs. Bruce McKay and Christina Addison, for support, advice, and reagents. Of course, all of this would not have been possible without the absolute support and never waning optimism of my supervisor, Dr. Dimitroulakos.

Dedications

To my dearest parents, who have supported me through the good and the bad and made all these possible…

To Bunny (my beloved son) and Caramel who have filled my heart with love…

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List of Abbreviations

4EBP1	4E binding protein 1
ABD	Actin binding domain
AML	acute myelocytic leukemia
bFGF	Basic fibroblast growth factor
CC	Cervical cancer
CI	Combination-Index
CFP	Cyan fluorescent protein
c-MET	Hepatocyte growth factor receptor
Dok-R	Dok related docking protein
DSS	Disease specific survival
ECM	Extracellular matrix proteins
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF4E	eukaryotic translation initiation factor 4E
Eps15	Epidermal growth factor receptor substrate 15
ERK	Extracellular regulated kinase
FRET	Fluorescence resonance energy transfer
FPP	Farnesyl pyrophosphate
FPTase	Farnesyl transferase
FT	Farnesyl transferase
GGPP	Geranylgeranyl pyrophosphate
GGPTase	Geranylgeranyl transferase
GGTaseI	Geranylgeranyl transferase I
GPCR	G protein-coupled receptor
GRB-2	Growth factor receptor-bound protein 2
HB-EGF	Heparin-binding EGF like growth factor
HDL	High-density lipoprotein
HUVEC	Human umbilical vein endothelial cells
Ig	Immunoglobulin-like
IGF-1R	Insulin-like growth factor receptor-1
JAK	Janus kinase
LD50	50% lethality
LDLs	Low-density serum lipoproteins
MAPK	Mitogen activated protein kinase
MEK	Mitogen-activated extracellular-regulated kinase
MM	malignant mesothelioma
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NSCLC	Non small cell lung cancer
OS	Overall survival
PDGFR	Platelet-derived growth factor receptor
PI3-K	Phosphoinositide 3-kinases
PKC	Protein kinase C

PLC	Phospholipase C
PLGF	Placenta growth factor
PTB	Phosphotyrosine binding
PTEN	Phosphatase and tensin homolog
PTP1B	Protein tyrosine phosphates 1B
ROCK	Rho-associated coiled-coil forming protein serine/threonine kinase
HR	Hazard ratio
RTK	Receptor tyrosine kinases
S6K1	S6 kinase 1
SCC	Squamous cell carcinomas
SCCHN	Head and neck SCC
SH2	Src homology-2
SHC	Src homology 2 domain containing protein
Sos	Son of sevenless
STAT	Signal transducer and activator of transcription
TGF-α	Transforming growth factor α
TK	Tyrosine kinase
TKIs	Tyrosine kinase inhibitors
TSAd	T-cell-specific adapter molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular Permeability Factor
VRAP	VEGF-receptor-associated protein
YFP	Yellow fluorescent protein

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CHAPTER 1

General introduction

Receptor tyrosine kinases (RTK) are key regulators of growth, differentiation and survival of epithelial and endothelial cells and play a significant role in the development and progression of cancers derived from these tissues [1-9]. In malignant cells, these receptors and their downstream signalling pathways are often deregulated, leading to cell hyper-proliferation, enhanced cell survival and increased metastatic potential [1-9]. Epithelial malignancies such as squamous cell carcinomas (SCC) and malignant mesotheliomas have very limited treatment options when presenting as metastatic disease [10-15]. Furthermore, endothelial expressed RTKs regulate tumor angiogenesis allowing for tumor growth and maintenance by promoting their vascularization [16, 17]. RTKs, particularly the epidermal growth factor (EGFR) and the vascular endothelial growth factor (VEGFR) receptors, have been shown to play significant roles in the pathogenesis of these tumor types, however, targeting of these receptors in these settings has limited therapeutic efficacy [4-6, 18, 19].

This chapter gives a detailed introduction to the EGFR/VEGFR and their roles in cancer. The clinical relevance of the inhibition of these receptors and their downstream signalling pathways are also discussed.

1. Receptor tyrosine kinases (EGFR VEGFR)

1.1 ErbB receptors and their binding ligands

The ErbB family of receptor tyrosine kinases includes the Epidermal Growth Factor Receptor ErbB1 (EGFR/HER1), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4) [20-22]. The EGFR is a 170 kDa transmembrane glycoprotein of a single polypeptide chain [20, 22]. It has a heavily glycosylated extracellular domain that is responsible for ligand binding and a single α-helical transmembrane domain of 23 amino acids [20, 22]. The intracellular domain contains an uninterrupted kinase site and multiple autophosphorylation

sites clustered at the C-terminus [20, 22]. EGFR has several ligands with similar structures, including epidermal growth factor (EGF), transforming growth factor α (TGF-α), heparin-binding EGF like growth factor (HB-EGF), amphiregulin, betacellulin, and epiregulin [23, 24]. EGF was discovered by Stanley Cohen of Vanderbilt University along with Rita Levi-Montalcini for which both received the Nobel prize in Physiology or Medicine in 1986. High EGFR expression has been associated with advanced tumor stage, resistance to standard therapies (chemotherapy and radiation) and, in some tumors, with poor patient prognosis [4].

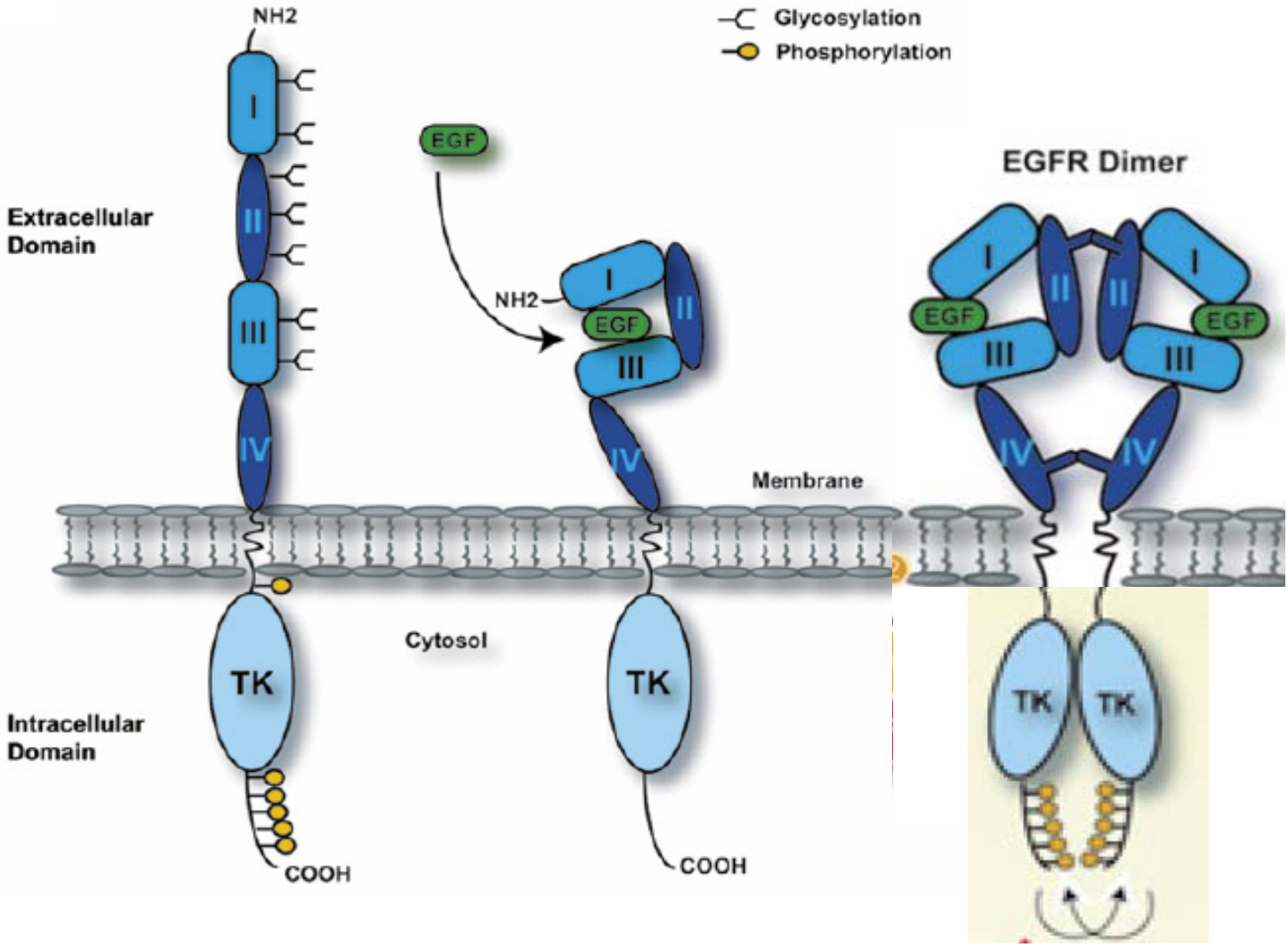
1.2 EGFR structure, dimerization and activation

The extracellular portion of the EGFR has been subdivided into four domains (Chapter 1 Figure 1A) [25]. Domains I and III have 37% sequence identity, whereas domains II and IV are rich in cysteines [25]. Domain I and III have been shown to bind directly with EGF, and then two molecules of the monomeric receptor-ligand complex interact with each other to form a dimeric complex (Chapter 1 Figure 1A) [25].

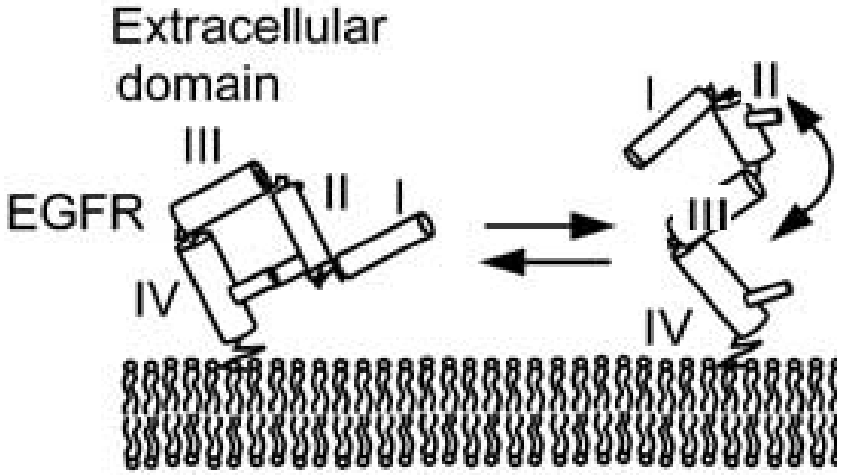
Ligand-induced dimerization is a common feature of most growth factor receptors for their activation and induction of downstream signal transduction cascades [26-28]. EGFR is proposed to interact with its ligand in either reversible or irreversible mechanisms [26-28]. According to the irreversible mechanism, binding with EGF induces irreversible conformational changes in EGFR that keep it in a dimeric and kinase-active form [26-28]. According to the reversible mechanism, EGFR is in fast equilibrium between monomer and dimer, and binding of EGF would shift this equilibrium toward dimer formation [26-28]. Another proposed model is that in the absence of ligands, the extracellular regions of EGFR exist in equilibrium between the closed (where the arm of domain II that is required for

Chapter 1 Figure 1. EGFR structure. A, EGFR is a highly glycosylated membrane spanning protein, which consists of three main domains: an extracellular domain, a transmembrane domain and an intracellular domain containing the tyrosine kinase (TK). The extracellular domain is composed of four subdomains designated I, II, III and IV. The domains I and III bind directly with EGF. Binding of ligand to EGFR leads to receptor dimerization. Adapted from Zandi 2007. B, In the absence of ligands, the extracellular regions of EGFR exist in equilibrium between the closed (where the arm of domain II that is required for dimerization is buried in domain IV) and open conformations (where domain II is exposed and available for dimerization). Adapted from Teramura 2006.

A


NH2
Glycosylation
Phosphorylation
EGF
EGFR Dimer
Extracellular Domain
I
II
III
IV
NH2
EGF
Membrane
Cytosol
TK
Intracellular Domain
COOH

B


Extracellular domain
EGFR
III
II
I
IV

dimerization is buried in domain IV) and open conformations (where domain II is exposed and available for dimerization) (Chapter 1 Figure 1B) [28]. It has been estimated that 95 percent of EGFR exists in the closed conformation in the absence of ligand binding [27]. Ligand binding stabilizes the EGFR extracellular region in the open conformation, thereby facilitating receptor dimerization and receptor autophosphorylation [26-28]. Both binding mechanisms suggest that the EGFR dimer possesses much higher affinity for ligand and higher tyrosine kinase activity thus implying that it is the active form of the kinase.

Ligand binding can also induce dimerization of the receptor with other members of the ErbB family [20, 23, 29]. EGFR, ErbB2, and ErbB4 contain catalytically competent kinase domains and can form heterodimeric pairs with each other [23, 29]. ErbB3 contains an inactive kinase domain, but it can pair with and activate the other members of the family [23, 29]. ErbB2 has no ligand and the structure of its extracellular region differs significantly from that of EGFR [23, 24]. In the absence of a ligand, ErbB2 has a conformation that resembles the ligand-activated state, making this receptor constitutively active [23, 24]. Furthermore, this finding may rationalize the observation that ErbB2 has an enhanced capacity of heterodimerization and is the preferred dimerization partner for the other activated ErbB receptors [23, 24]. Among all possible ErbB2 containing heterodimeric receptor complexes, ErbB2/ErbB3 heterodimer is the most potent signalling module in terms of cell proliferation and in vitro transformation [30]. Generally speaking, homo- and heterodimers of the ErbB family members can initiate a wide variety of different signalling pathways to produce signals with different duration and intensity.

1.3 VEGFR and its ligand VEGF

In 1983, Senger and his collaborators described the partial purification of a protein of tumoral origin, able to provoke vascular leakage in pig skin. This protein, called Vascular Permeability Factor (VPF) then is now referred to as VEGF. VEGF is a key regulator of angiogenesis and can efficiently stimulate the proliferation and the differentiation of endothelial cells [31]. VEGF denotes a large family of dimeric glycoproteins that consist of five mammalian (VEGF-A, VEGF-B, VEGF-C, VEGF-D, Placenta growth factor (PLGF)) and one virus-encoded members [5, 6, 32]. VEGF-A was the first member to be discovered and has been shown to participate in a variety of processes, with both physiological and pathological functions [33]. In humans, six VEGF-A splice variants have been detected: VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A183, VEGF-A189 and VEGF-A206 [32]. Although VEGF-A121, VEGF-A183 and VEGF-A189 are expressed in various tissues, VEGF-A165 is the most abundant and biologically active form, whereas VEGF-A145 and VEGF-A206 are relatively rare [5, 6, 32]. VEGF-A165 is expressed as a 46 kDa homodimer composed of two 23 kDa monomers linked by two inter-subunit disulfide bridges [5, 6, 32].

VEGFs function through their cell surface receptor tyrosine kinases VEGFRs. There are three different types of VEGFRs: VEGFR-1, 2 and 3 [34-36]. VEGFR-1 (Flt-1) is expressed on haematopoietic stem cells, monocytes, macrophages and vascular endothelial cells [34-36]. VEGFR-2 (Flk-1/KDR) is expressed on both vascular and lymphatic endothelial cells, while VEGFR-3 (Flt-4) expression is restricted to lymphatic endothelial cells [34-36]. Of the two receptors expressed on vascular endothelial cells, only VEGFR-2 contributes to intracellular signalling, while VEGFR-1 functions in sequestering excess VEGF [34-36].

In the case of the VEGF/VEGFR system, the ligand (VEGF molecule) is bivalent and the receptor (VEGFR) is monovalent, i.e. one VEGF molecule can bind two VEGFRs [5, 6, 32].

VEGFR-2 has an extracellular domain consisting of seven immunoglobulin-like (Ig) repeats, a single transmembrane region, and a tyrosine kinase domain in the intracellular region that is interrupted by a kinase insert domain (Chapter 1 Figure 2) [5]. VEGF-A binds to the second and third extracellular Ig-like domains of VEGFR-2 [37]. The fourth Ig-like domain is believed to mediate receptor dimerization [38]. The fifth and sixth Ig-like domains are crucial for VEGF retention after binding, whereas the first Ig-like domain might regulate the ligand binding because its removal improves VEGF association [39]. The seventh Ig-like domain stabilizes the dimer when held in close proximity with another [5, 6, 32].

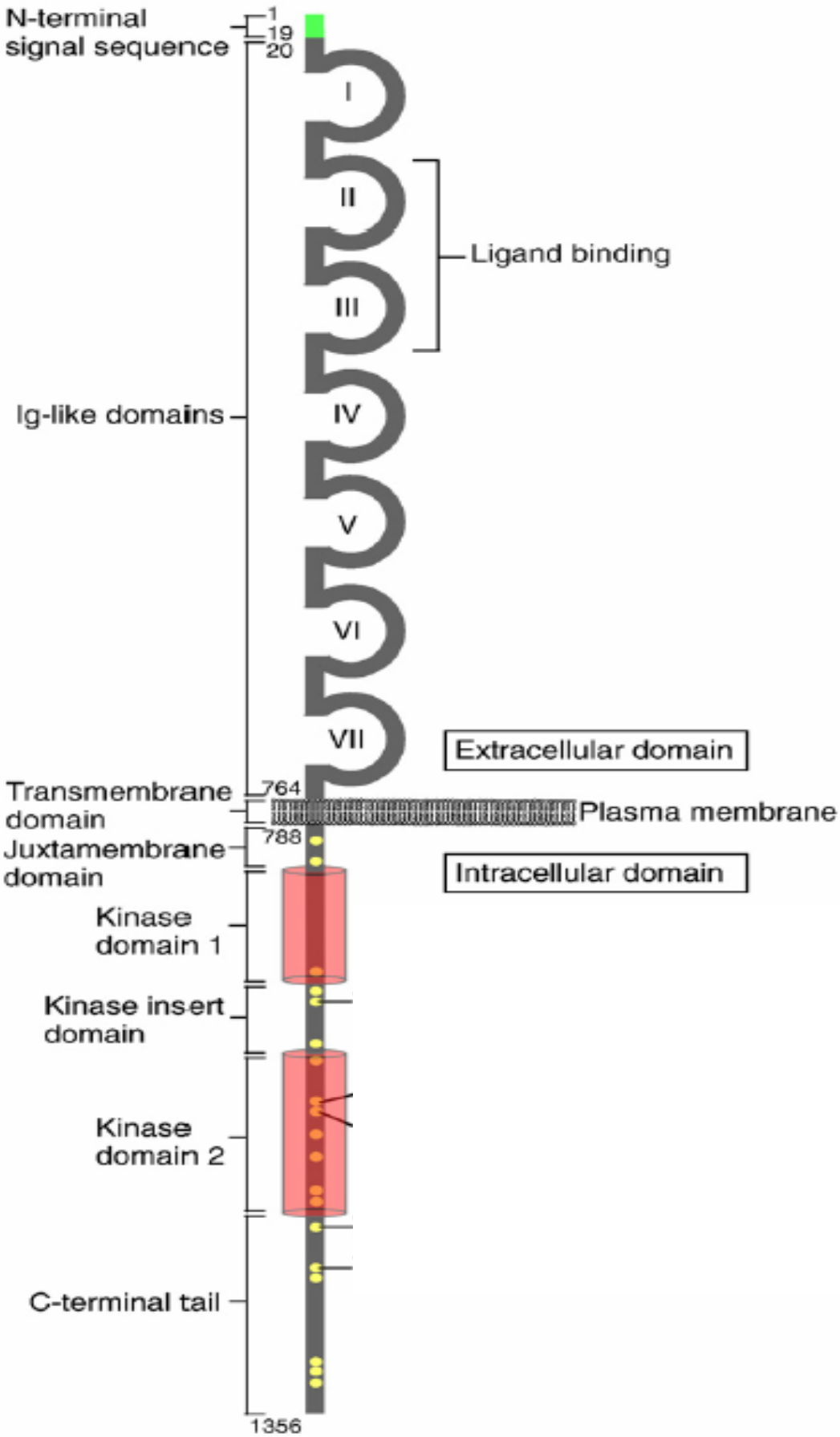
The VEGF receptors are receptor tyrosine kinases and, like other members of this family, dimerization is required for their activation. Signalling is initiated when one VEGF ligand binds two receptor monomers, resulting in the transactivation of the intracellular kinase domains of the receptors.

2. Signal transduction via EGFR and VEGFR

2.1 Physical association between EGFR and signalling proteins

Multiple residues of the EGFR cytoplasmic kinase domain are autophosphorylated upon EGFR dimerization and thereafter recruit a variety of downstream signalling proteins containing Src homology-2 (SH2) or phosphotyrosine binding (PTB) domains, leading to the activation of intracellular signalling pathways [20, 22]. Substrates for these autophosphorylated tyrosine include an adaptor growth factor receptor-bound protein 2 (GRB-2) that binds pY1068 and pY1086, the Shc adaptor that binds pY1148 and pY1173, the Dok related docking protein (Dok-R) adaptor that binds pY1086 and pY1148, phospholipase (PLC-γ) which is recruited by pY1173 and pY992, phosphatase (PTB-1B)

Chapter 1 Figure 2. Structure of human VEGFR-2 (KDR). VEGFR-2 is a transmembrane kinase receptor. The extracellular domain comprises seven Ig-like domains (I–VII), of which the second and third domains bind VEGF-A. The intracellular domain contains two kinase domains, which are split by a kinase-insert domain of 70 amino acids. Adapted from Holmes 2007.


N-terminal signal sequence
1
19
20
I
II
III
Ligand binding
Ig-like domains
IV
V
VI
VII
Extracellular domain
Transmembrane domain
764
Plasma membrane
788
Juxtamembrane domain
Intracellular domain
Kinase domain 1
Kinase insert domain
Kinase domain 2
C-terminal tail
1356

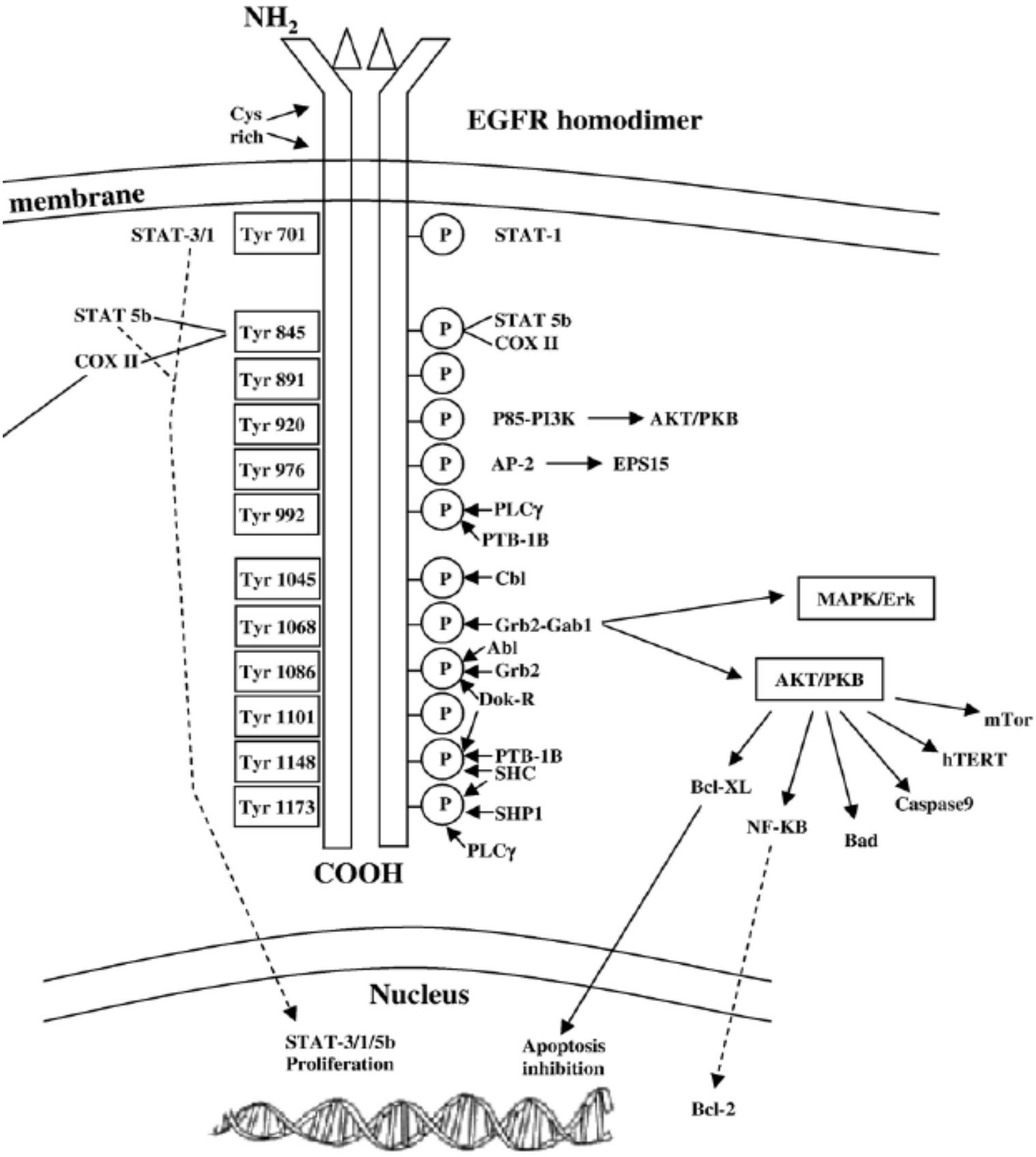
which can interact with pY992 and pY1148, c-Cbl which binds pY1045, the SHP-1 phosphatase which binds pY1173, and the Abl tyrosine kinase which binds pY1086 (Chapter 1 Figure 3) [20, 22].

2.2 Major signalling pathways are integrated at the EGFR c-terminus

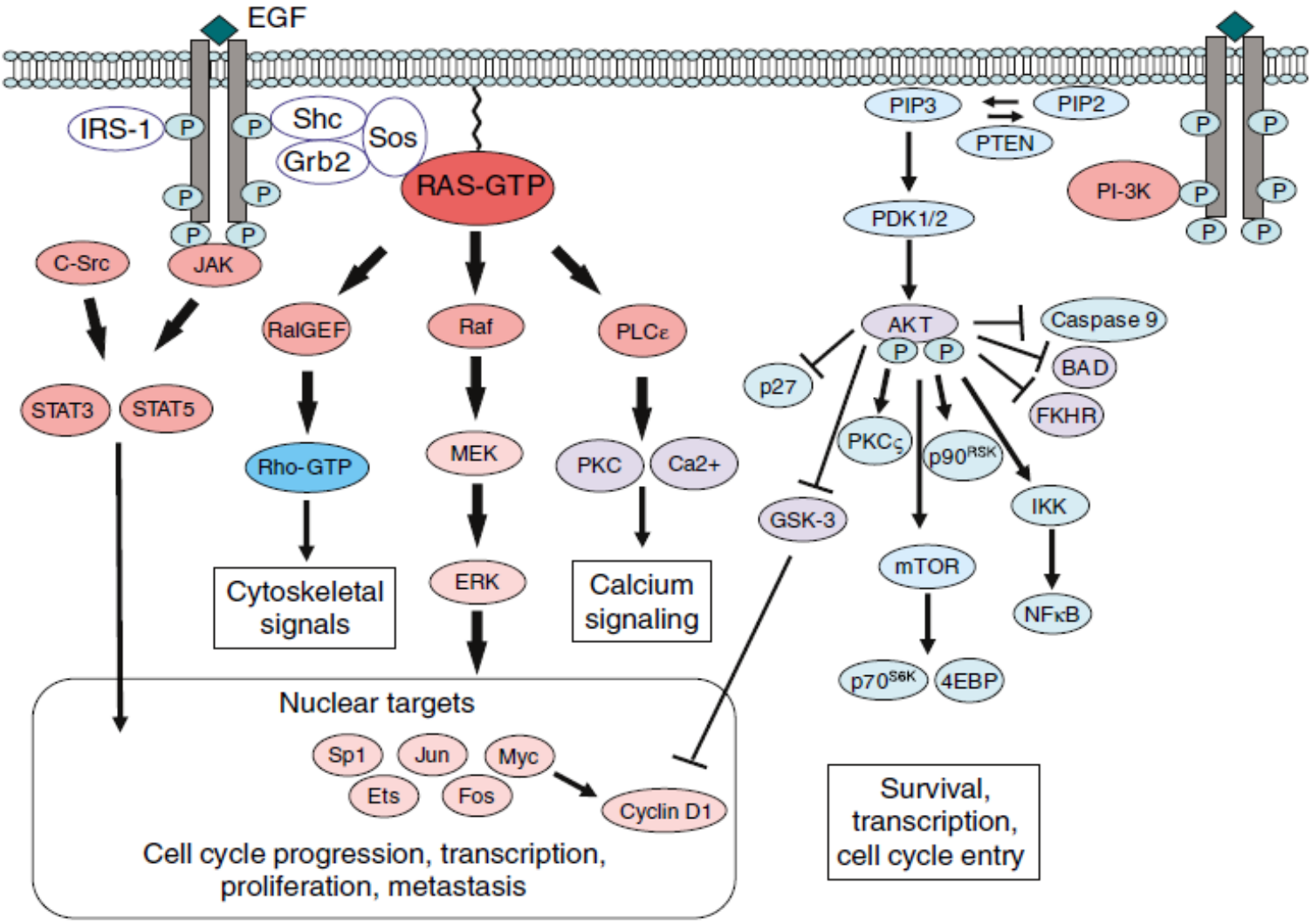
Ligand binding to the EGFR leads to the activation of a number of cell signalling pathways such as Raf/mitogen activated extracellular regulated kinase (MEK)/extracellular regulated kinase (ERK), phosphoinositide 3-kinases (PI3-K)/Akt, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and PLCγ/protein kinase C (PKC) mediated pathways (Chapter 1 Figure 4) [21, 40-43].

The Raf/MEK/ERK pathway can be activated by a variety of factors such as growth factors, stress and inflammatory stimulus and G protein-coupled receptor (GPCR) activation [20, 22, 42, 43]. One of the many signalling molecules involved in this pathway is Grb2 which is constitutively bound to the guanyl nucleotide exchange factor-Son of sevenless (Sos) [20, 21]. Grb2 and Sos are normally localized in the cytosol [20, 21]. When EGFR becomes activated, autophosphorylation at numerous sites on the C-terminus allows Grb2 to bind the Y1068 and Y1086 of EGFR via its SH2 domain [44, 45]. Subsequently, Grb2 and Sos are recruited to the plasma membrane and brought into proximity with the guanine nucleotide-binding protein Ras [46]. Sos accelerates the exchange of GDP for GTP and transforms Ras into its active conformation [46]. Ras is then able to recruit the serine/threonine kinase Raf to the plasma membrane where Raf can be phosphorylated and activated [46]. As a result, MEK and ERK also get phosphorylated and activated [46]. In turn, the active ERK can regulate the expression of many downstream genes such as transcription factors (Myc, Ets, and CREB) and apoptotic regulatory molecules(Bad, Bcl-2

Chapter 1 Figure 3. The EGFR kinase domains. Schematic representation of major tyrosine residues that can get phosphorylated at the EGFR kinase domain after EGFR stimulation by EGF related ligands or autophosphorylated during homodimerisation. Possible adaptors and signalling proteins can get activated from each phosphorylated tyrosine residues and major signalling pathways can be generated. Adapted from Sebastian 2006.


NH2
Cys rich
EGFR homodimer
membrane
STAT-3/1
Tyr 701
P
STAT-1
STAT 5b
COX II
Tyr 845
STAT 5b
COX II
Tyr 891
Tyr 920
P85-PI3K
AKT/PKB
Tyr 976
AP-2
EPS15
Tyr 992
PLCγ
PTB-1B
Tyr 1045
Cbl
Tyr 1068
Grb2-Gab1
MAPK/Erk
Abl
Tyr 1086
Grb2
AKT/PKB
Dok-R
Tyr 1101
mTor
Tyr 1148
PTB-1B
SHC
hTERT
Bcl-XL
Tyr 1173
SHP1
Caspase9
NF-KB
Bad
PLCγ
COOH
Nucleus
STAT-3/1/5b
Proliferation
Apoptosis
inhibition
Bcl-2

Chapter 1 Figure 4. EGFR activation of some major signal transduction pathways. EGFR activates many kinase cascades which result in activation/inactivation of transcription factors which regulate gene expression to either stimulate or repress proliferation. Activation of several signalling cascades is triggered predominately by the RAS-to-MAPK and the PI-3K/Akt pathways, resulting in enhanced tumor growth, survival, invasion and metastasis. Adapted from Reuter 2007.


EGF
IRS-1
P
Shc
Grb2
Sos
RAS-GTP
JAK
C-Src
STAT3
STAT5
RalGEF
Rho-GTP
Raf
MEK
ERK
PLCε
PKC
Ca2+
Cytoskeletal signals
Calcium signaling
PIP3
PIP2
PTEN
PI-3K
PDK1/2
AKT
Caspase 9
BAD
FKHR
p27
PKCς
p90RSK
GSK-3
IKK
mTOR
NFκB
p70S6K
4EBP
Nuclear targets
Sp1
Jun
Myc
Ets
Fos
Cyclin D1
Cell cycle progression, transcription, proliferation, metastasis
Survival, transcription, cell cycle entry

and caspase 9) [47, 48]. Activation of the Ras/Raf/MEK/ERK by the EGFR has been suggested to play a role in the transformation of cells from a variety of tissues [20, 22, 42, 43]. Inhibition of this pathway may not only inhibit cell proliferation, but may also act to sensitize cells to cancer treatment.

The PI3-K/Akt pathway is involved in cellular processes such as proliferation, survival, adhesion and migration [49, 50]. PI3-K is composed of a p85 adapter subunit and a p110 kinase subunit [49, 50]. Activation of the EGFR tyrosine kinase creates docking sites on the C-terminus of EGFR for the SH2 domain of the p85 subunit [49, 50]. The binding of the p85 subunit to EGFR recruits the p110 subunit, localizing it to the inner surface of the plasma membrane [49, 50]. This brings the PI3-K catalytic subunit into close proximity with its phosphotidylinositol substrate and subsequent phosphorylation and activation of the Serine/Theorine kinase Akt [49, 50]. Activation of Akt results in survival, proliferation, and prevention of apoptosis by phosphorylating and inactivating several proteins such as Bad, caspase-9, Forkhead transcription factor, and Raf-1 [50, 51]. Deletion of phosphatase and tensin homolog (PTEN), a negative regulator of the PI3-K signalling pathway, is common in many cancers and is thought to cause increased PI3-K activity [49, 50].

The STAT family is a group of transcription factors. In humans, there are seven STAT genes: STAT 1, 2, 3, 4, 5a, 5b and 6 [49]. STATs appear to be constitutively associated with EGFR [52]. The SH2-binding domain of the inactive STAT transcription factors binds to phosphotyrosine residues on the EGFR [52]. Inactive STATs are associated with the intracellular portion of EGFR in its resting state [52]. When the receptor is activated, the STATs become activated, form homo- and heterodimers, and translocate to the nucleus [52].

STATs 1, 3 and 5 appear to be involved in EGFR signalling [52, 53]. Activation of STATs leads to proliferation and differentiation.

EGFR mediated signalling pathways have also been shown to regulate both angiogenesis and metastasis [54]. A PLC-γ dependent pathway is believed to directly participate in the regulation of metastasis through phosphorylation of Tyr992 in the EGFR cytoplasmic kinase domain [55]. EGFR Tyr992 is the direct docking site of PLC-γ and EGF receptor mediated activation of PLC-γ is believed to be critical for the formation of actin stress fibers and focal adhesion, which may result in tumor cell invasion and migration [55].

2.3 Biological responses mediated by EGFR activation

ErbB receptors signalling activate various transcription factors such as c-fos, c-Jun, c-myc, STAT, NF-kB, zinc finger transcription factor and Ets family members [20, 22, 56, 57]. The activated gene expression ultimately induces cellular responses, such as cell proliferation, differentiation, invasion, migration, adhesion, survival and cellular repair [20, 22, 56, 57]. Signals are terminated when EGFR is endocytosed and degraded [20, 22, 56, 57].

2.4 EGFR endocytosis

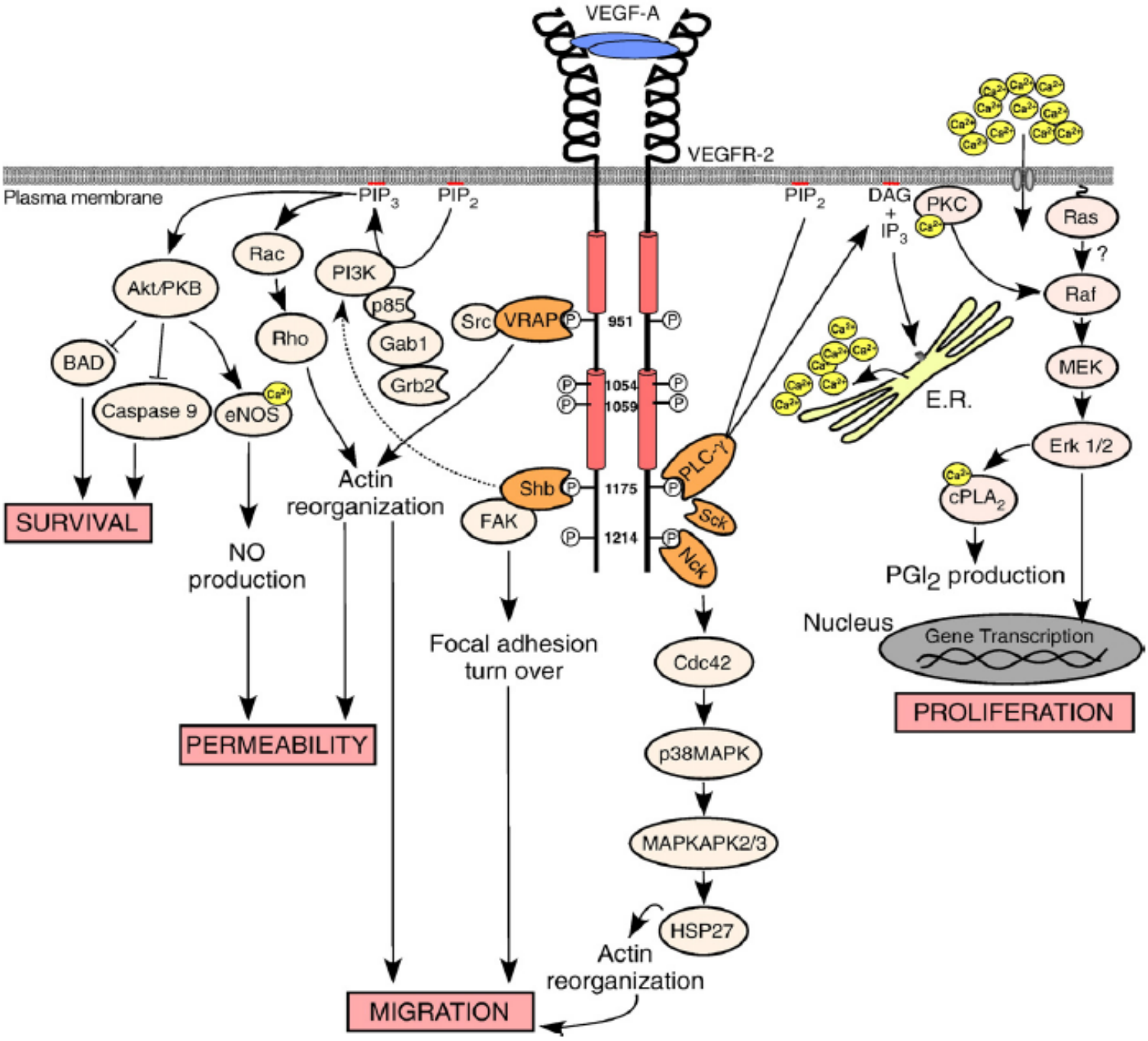
During signal termination, activated EGFR complexes migrate from the caveolae/raft component of the cell membrane to the bulk membrane component and cluster into clathrin-coated pits that are subsequently internalized in a kinase dependent manner [22, 23, 58-60]. Two distinct processes determine the fate of the internalized receptors. One is regulated by the ubiquitin ligase known as Cbl [22, 23, 58]. After activation of the EGFR by ligands, Cbl is recruited rapidly to the EGFR and mediates its ubiquitination [58]. Cbl proteins can bind to the EGFR via two types of interactions: they can interact directly via their PTB domain with a phosphorylated tyrosine (Y1045) in the EGFR cytoplasmic tail or they can interact

indirectly via binding to Grb2 [22, 23, 58]. Recruitment of Cbl to ligand-receptor complexes in early endosomes targets receptors for lysosomal degradation by promoting receptor ubiquitination [58]. In the absence of Cbl, receptors are instead recycled to the plasma membrane [22, 23, 58]. The fate of the internalized receptors can also be determined by the relative stability of the different activated ligand–receptor complexes in the mildly acidic endosomal environment, a second process [22, 23, 58]. Activated EGFR homodimers that are relatively stable and remain bound to Cbl will result in their endocytic sorting to lysosomes and degradation [22, 23, 58]. In contrast, EGFR–HER2 heterodimers are less stable and more easily uncoupled in early endosomes, causing Cbl to dissociate from the receptor complex [22, 23, 58]. These receptors then recycle through the default pathway that returns them to the cell surface. Besides the ligand-mediated EGFR endocytosis mechanisms described above, EGFR undergoes spontaneous metabolic turnover with a half-life of approximately 10–14 hours in both fibroblasts and epithelial cells and of 20–48 hours in transformed cells [22, 23, 58]. Thus, on average, a receptor will cycle through the endocytic pathway dozens of times during its life span, with a low probability of being degraded.

2.5 Signal transduction mediated through VEGFR

VEGF and VEGFR play key roles in angiogenesis and tumor growth. More recent work has revealed that the major phosphorylation sites are Y951 in the kinase-insert domain, Y1054 and Y1059 within the kinase domain, and Y1175 and Y1214 in the C-terminal tail of the VEGF receptor (Chapter 1 Figure 5) [5, 6, 32]. Phosphorylation of specific tyrosine residues in the receptor creates a consensus sequence for the recruitment of specific intracellular proteins, via their SH2 domains [5, 6, 32]. For example, phosphorylation of Y951 creates a binding site for VEGF-receptor-associated protein (VRAP) also called T-cell-

Chapter 1 Figure 5. Schematic illustration of VEGFR-2 intracellular signalling. Binding of VEGF to the receptor induces dimerisation and autophosphorylation of specific intracellular tyrosine residues. Activation of intracellular signalling cascades results in proliferation, migration, survival and increased permeability. Adapted from Holmes 2007.


VEGF-A
VEGFR-2
Plasma membrane
PIP3
PIP2
Rac
PI3K
p85
Gab1
Grb2
Akt/PKB
BAD
Caspase 9
eNOS
Rho
Src
VRAP
951
1054
1059
1175
1214
Shb
FAK
PLC-γ
Sck
Nck
SURVIVAL
NO production
Actin reorganization
PERMEABILITY
Focal adhesion turn over
MIGRATION
Cdc42
p38MAPK
MAPKAPK2/3
HSP27
Actin reorganization
DAG + IP3
PKC
E.R.
Ras
Raf
MEK
Erk 1/2
cPLA2
PGI2 production
Nucleus
Gene Transcription
PROLIFERATION

specific adapter molecule (TSAd) [5, 6, 32]. Phosphorylation of Y1175 creates a binding site for a number of signalling proteins such as PLC-γ, the adaptor protein Shb and the adaptor protein Sck [5, 6, 32]. Phosphorylation of Y1214 creates a binding site for the adaptor protein Nck [5, 6, 32].

A number of studies have shown that VEGFR-2 is the principal mediator of several physiological and pathological effects of VEGF-A on endothelial cells [7, 9, 35, 36, 61-63]. Similar to EGFR, ligand binding to the VEGFR leads to the activation of a number of cell signalling pathways. These include the Raf/MEK/ERK pathway (proliferation), PI3-K/Akt pathway (survival and permeability), PLCγ/PKC pathway (proliferation) and cdc42/P38 mitogen activated protein kinase (MAPK) pathway (migration) [7, 9, 35, 36, 61-63].

A recent study indicated that VEGFR-2 undergoes ligand-dependent downregulation and degradation in a novel mechanism involving non-classical PKCs [62, 64]. The serines 1188 and 1191 in the carboxyl terminus of VEGFR-2 are phosphorylated either directly by PKC or by serine kinases whose activity is regulated by PKCs [62, 64]. Upon phosphorylation these sites may recruit an E3 ligase, leading to ubiquitinylation and degradation of VEGFR-2 [62, 64]. The exact E3 ligase responsible for such ubiquitinylation of VEGFR-2 is still unknown. Unlike in EGFR endocytosis, c-Cbl is not the E3 ligase involved in ubiquitinylation and downregulation of VEGFR-2 [62, 64].

3. Receptor tyrosine kinases (EGFR, VEGFR) in cancer

3.1 Dysregulation of EGFR expression in human carcinomas

EGFR signalling is tightly controlled in normal cells. However, EGFR is overexpressed in many human tumors occurring in head and neck, breast, lung, glioblastoma, bladder, colorectal, prostate and ovarian carcinoma etc [3, 4, 29, 43, 65, 66]. High EGFR expression

has been associated with advanced tumor stage, poor prognosis, and resistance to standard therapies (hormonal therapy, chemotherapy, and radiation) [4]. Blocking EGFR reduces tumor cell proliferation, promotes tumor cell apoptosis, inhibits production of angiogenic factors and improves survival in animal models bearing tumor cell xenografts [67]. Dysregulated EGFR expression and signalling can occur by a number of different mechanisms.

One mechanism is by overexpression of the receptor on the cell surface. This occurs as a result of amplification of the gene encoding EGFR and is found in over half of glioblastoma [68]. In glioblastoma, there is also alteration of EGFR structure [69, 70]. For example, truncation of the negative regulatory extracellular domain of EGFRvIII (a mutant form of EGFR) in glioblastoma gives rise to a well known truncated EGFR variant in which amino acids 6–273 are replaced by a single glycine residue, resulting in a 145-kDa glycoprotein with constitutive, ligand-independent activation of the receptor tyrosine kinase [71-73].

Another mechanism that may result in dysregulation and increased EGFR signalling is the loss of phosphatase activity. Phosphatases, such as PTEN, are important for dephosphorylation of active kinases [74]. When phosphatases fail to function properly because of either decreased expression or mutation, induced signalling pathways remain active for a relative longer time [74]. One recent report indicated that functional PTEN is required for the action of trastuzumab, an antibody against ErbB2 [75]. Loss of PTEN results in trastuzumab resistance [75]. This is a highly significant finding as PTEN deficiency has been reported in up to 50% of breast cancers [75].

Due to the high expression frequency of an individual ErbB receptor type in human carcinoma, co-expression of different receptors often occurs in most tumors [76-79]. Indeed,

tumors that co-express different ErbB receptors are often associated with a more aggressive phenotype and a worse prognosis [77, 80, 81]. In addition, co-expression of different EGF-like growth factors is a common phenomenon in human carcinogenesis [21]. To date, others have demonstrated that simultaneous blockade of different growth factors and/or receptors of the ErbB family produces a more significant growth inhibition as compared with inhibition of a single component of this network [82, 83]. Indeed, signalling complexity due to interactions among the four receptors and their binding with many ligands makes it difficult to measure signalling output for each receptor and within different human tumors. How these components relate to tumor progression and ErbB receptor targeted therapies requires further investigation.

3.2 VEGF and its receptor in tumor angiogenesis

Angiogenesis, the process of developing new blood vessels from existing ones, is critical for tumor cell growth, survival, invasion and metastasis [54, 84, 85]. Tumors that are 1-2 millimeters in size can generally obtain sufficient nutrients and oxygen by simple diffusion; however, they require new blood vessels if they grow beyond that size [86]. Angiogenesis is tightly controlled by pro-angiogenic and anti-angiogenic factors released by the tumor cells as well as adjacent host cells [86]. Ultimately, tumor growth will induce the angiogenic response. As a result, endothelial cells grow from nearby blood vessels, cross the basement membrane, and migrate into the tumor mass where they proliferate and form capillaries [87]. However, these new tumor vessels differ morphologically from normal blood vessels in that they (i) are heterogeneous and leaky, (ii) contain disorganised and irregularly shaped endothelial cells, (iii) have abnormal basement membranes [88]. Blood flow into the tumor

is restricted by these features. It is also restricted by the increased interstitial pressure caused by the leaky vessels [88].

Numerous growth factors work in the tumor microenvironment to promote angiogenesis [88]. Of these, VEGF may be one of the most important factors in angiogenesis and acts at several steps in the angiogenic process [88]. For example, VEGF mobilises bone marrow-derived endothelial cell precursors, stimulates endothelial cell proliferation, inhibits their apoptosis and modulates their migration to sites of angiogenesis [88]. In addition, VEGF stimulates production of enzymes that degrade the extracellular matrix [54]. VEGF also potently increases vascular permeability [54]. However, whereas VEGF is one important factor involved in angiogenesis early in tumor growth, other proangiogenic factors may become involved as the tumor continues to grow [88].

Oncogenic activation, loss of tumor suppressor factors and tumor hypoxia all lead to upregulation of VEGF production [89-92]. Both VEGF and its receptors have emerged as anti-cancer targets on the basis of their central and specific role in angiogenesis. In principle, VEGF-targeted therapy may inhibit tumor growth by blocking new vessel growth. Recent data show that VEGF induces not only tumor angiogenesis, but also promotes the maintenance and survival of new vessels in tumors [89-92]. Therefore, the VEGF pathway plays a key role in regulation of tumor angiogenesis, and tumor development. Thus, it would be highly feasible to inhibit tumor growth by blocking the VEGF pathway. It has been demonstrated that both tumor angiogenesis and tumor growth are repressed when VEGF mediated signal transduction is inhibited [5, 93, 94]. The inhibition of VEGF function also prevents metastasis, because of a reduced contact between tumor cells and blood vessels [5, 93, 94]. Not all angiogenic tumors produce metastases, but the inhibition of angiogenesis can

prevent the growth of tumor cells at both primary and secondary sites [5, 93, 94]. Inhibition of tumor angiogenesis may therefore prove to be an effective therapeutic intervention, and several potential inhibitors of angiogenesis have been reported [95].

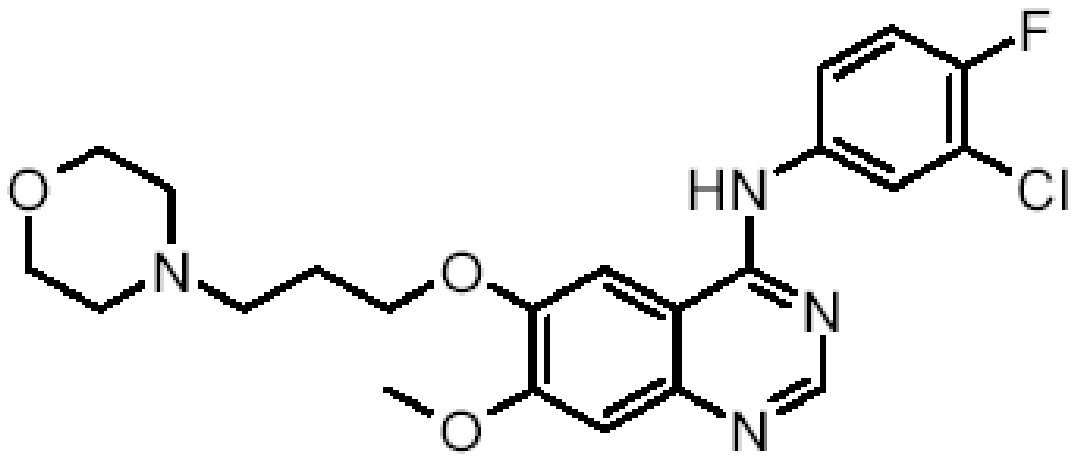
4. Targeting receptor tyrosine kinases

4.1 EGFR Tyrosine Kinase as a target for anticancer therapy

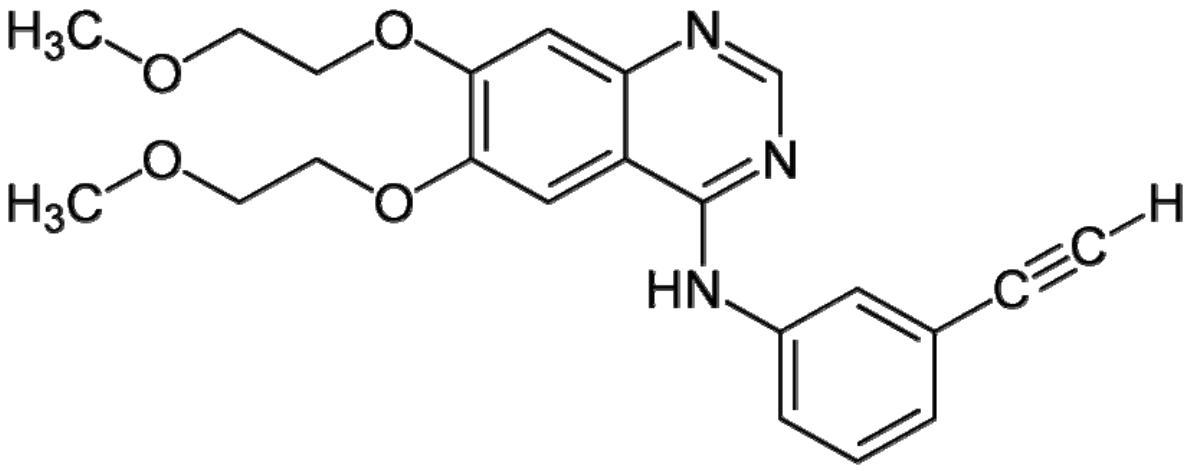
EGFR is an excellent target in cancer therapy given that it is frequently overexpressed and/or abnormally activated in tumors including head and neck squamous cell carcinomas (SCCHN), colorectal cancer, glioblastoma, or non-small-cell lung cancer [96]. Moreover, early studies with anti-EGFR monoclonal antibodies against the EGFR demonstrated clinical benefit [97-99]. EGFR can be targeted by monoclonal antibodies that compete for the extracellular ligand domain of receptor tyrosine kinases through receptor binding (e.g. cetuximab, panitumumab) [97-99]. These antibodies block the binding of actual ligands and inhibit the activation of subsequent signal cascades [97-99]. In contrast to monoclonal antibodies, small molecule tyrosine kinase inhibitors (TKIs) inhibit the phosphorylation of intracellular tyrosine residues located on transmembrane receptor TKs (e.g., erlotinib) through blocking their ATP-binding sites [100, 101].

Two TKIs of the EGFR are now in clinical development and they have been evaluated in SCCHN: Gefitinib (ZD1839, Iressa; AstraZeneca, London, UK) and OSI-774 (OSI-774, formerly known as CP-358, 774, Tarceva; Genentech, San Francisco, CA) (Chapter 1 Figure 6) [102]. Gefitinib is a novel, synthetic, low molecular weight anilinoquinazoline -4- (3-chloro-4- fluoroanilino)-7-methoxy-6- (3-morpholinopropoxy) –quinazoline [103]. It was discovered in studies designed to characterize the catalytic mechanism of EGFR-TK

Chapter 1 Figure 6. Chemical structure of Iressa and Tarceva.



Iressa
AstraZeneca



Tarceva (OSI774)
Roche/Genentech/OSI

inhibition [104]. Of several candidate compounds synthesized and tested, gefitinib has been proven to be a clinically effective drug [103]. Gefitinib can inhibit EGFR tyrosine kinase by binding to the ATP-binding site of the enzyme, suppress EGFR phosphorylation, inhibit MAPK activation, reduce keratinocyte proliferation, increase expression of the cyclin-dependent kinase inhibitor p27/Kip1 and induce apoptosis [102]. Studies show that gefitinib has high enzyme selectivity and minimal activity against other TKs, such as the structurally related HER-family EGFR-TK ERBB2, or VEGFR-1 and VEGFR-2 [105].

Preclinical studies with gefitinib have confirmed its anti-tumor activity in a variety of cultured tumor cell lines and in human tumor xenografts, either as a single agent or in combination with other chemotherapy agents [106, 107]. Clinical Phase I studies also have demonstrated that daily administration of gefitinib is safe, although with a high degree of interpatient variability [108, 109]. Clinical studies showed that peak plasma concentrations following administration of single and repeated oral doses of gefitinib were reached after 3–7 hours, with a half-life of 27–49 hours [110]. Several Phase II studies of gefitinib as monotherapy or in combination with other therapies have been conducted in a variety of tumor types, including non-small cell lung cancer (NSCLC), breast cancer, esophageal cancer, prostate cancer, head and neck cancer, colorectal cancer, ovarian cancer, and renal cell cancer [111, 112]. More, interestingly, gefitinib demonstrated a much higher response rate and survival benefit in Asian patients than in American patients [113]. The most frequent adverse events associated with gefitinib administration include acne-like rash, nausea, and diarrhea, but these are usually mild [40, 114, 115]. Less than 1% of patients require interruption of treatment due to adverse effects [40, 114, 115]. Although drug interactions can occasionally occur when multi-drugs are administered, pre-clinical and clinical data

strongly suggests that gefitinib is one of most potent and promising drug candidates that specifically targets aberrant EGFR signalling in several carcinomas [106-115].

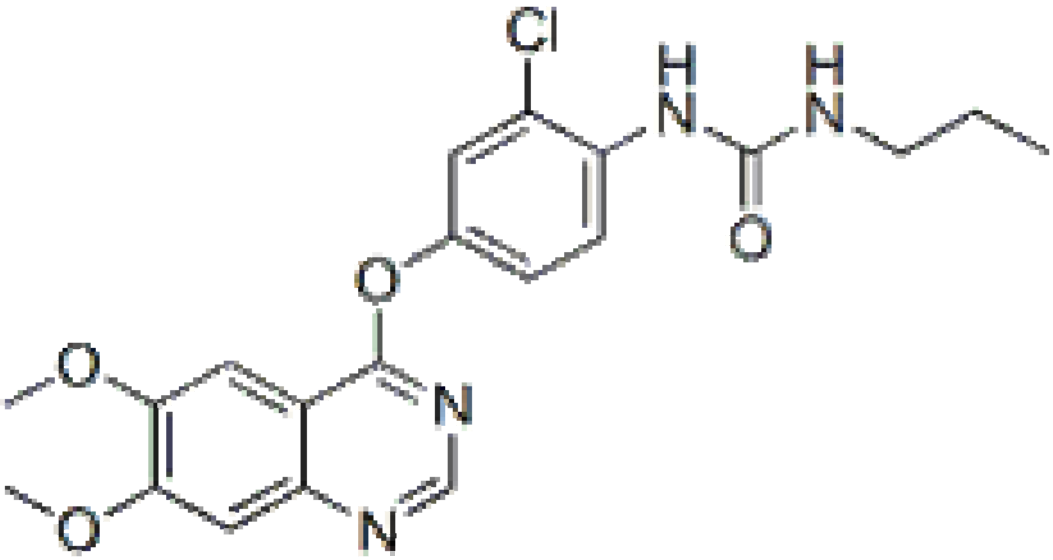
4.2 VEGFR as a target for anticancer therapy

VEGFR-2 has a key role in mediating VEGF-induced responses. Many selective agents for targeting VEGFR-2 have been brought into the clinic [95]. KRN633 (late-stage preclinical development), is a quinazoline derivative with high activity against the VEGFR (IC50=11.7nM) and c-Kit kinases (IC50=6.5nM) [116]. ZM323881, a novel RTK inhibitor, is a potent and selective inhibitor of human VEGFR2 tyrosine kinase in vitro and it can inhibit VEGFA-induced proliferation of human endothelial cells (Chapter 1 Figure 7) [117]. Both of these two VEGF/VEGFR pathway inhibitors have a class-specific toxicity profile. Their toxicity generally does not overlap with chemotherapy [118].

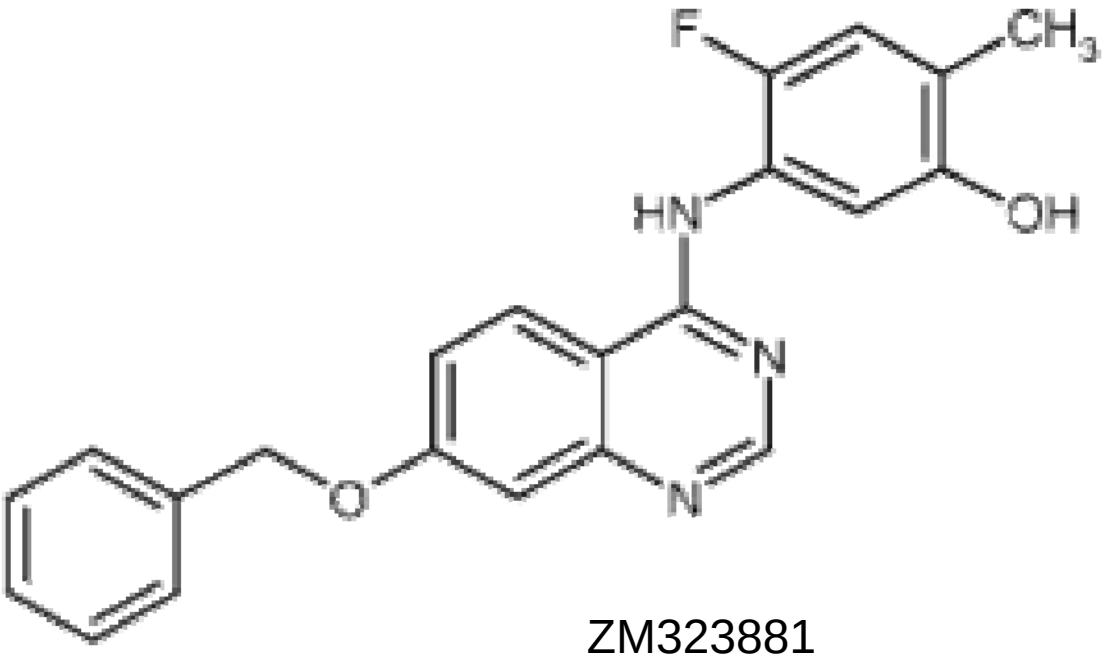
4.3 Tumor resistance to EGFR inhibitors

Despite the possible outcomes of TKI, some tumors can develop resistance to certain EGFR inhibitors. Several mechanisms may account for such resistance. One is to activate tumor-induced angiogenesis. In cancer cells, the EGFR autocrine pathway partially controls the production of several pro-angiogenic growth factors, including VEGF and basic fibroblast growth factor (bFGF) [119, 120]. Activation of EGFR by its specific ligands, such as TGF-β and EGF, can also up-regulate VEGF expression in colorectal cancer, prostate cancer, human glioma and head and neck squamous cell cancer cells in vitro [14, 119, 121]. The link between EGFR and VEGF signalling is also attested by the fact that inhibition of EGFR activity by selective anti-EGFR agents often results in downregulation of tumor-induced, VEGF-mediated angiogenesis [122-124]. These data support the idea that in cancer cells altered control of angiogenesis is a mechanism responsible for resistance to EGFR

Chapter 1 Figure 7. Chemical structure of KRN633 and ZM323881.



KRN633
Kirin Brewery Co., Ltd



ZM323881
Tocris Bioscience

inhibitors in vivo [122-124]. It is possible that tumor cells overcome the harmful effects of EGFR inhibitors by up-regulation of tumor angiogenesis-promoting growth factors such as VEGF [110, 125-128]. Therefore, it is reasonable to hypothesize that acquired resistance to EGFR antagonists might arise from enhanced VEGF expression rather than dysregulated EGFR signalling in many tumor cells. Cancer cells treated chronically with EGFR inhibitors may escape from growth inhibition through alternative survival pathways such as VEGF [110, 125-128]. As a result, the development of resistant tumors with altered angiogenic pathways highlights the need for novel approaches to cancer therapy [110, 125-128]. Combined molecular therapies targeting several pathways such as antiangiogenic therapy with EGFR inhibitor therapy, may minimize this resistance effect and should be taken into consideration in cancer treatment.

Another mechanism of resistance is to activate alternative tyrosine kinase receptors that bypass the EGFR pathway. EGFR is able to control and enhance tumor cell survival, proliferation and invasion through multiple downstream signalling pathways. However, it is now known that some other TK receptors such as insulin-like growth factor receptor-1 (IGF-1R), VEGFRs, platelet-derived growth factor receptor (PDGFR), or hepatocyte growth factor receptor (c-MET) can also drive similar signalling pathways [125, 129-131]. In fact, in human cancer cells, multiple growth factor receptors from different receptor families can be simultaneously activated, leading to the initiation of multiple overlapping signal transduction pathways [129-131]. Moreover, the genetic instability of cancer cells under drug treatment gives them the ability to turn to alternative survival mechanisms when crucial pathways for cell survival are inhibited or blocked (i.e. Multiple similar growth-controlling pathways can be activated in cancer cells treated with certain selective signal transduction inhibitors such

as anti-EGFR agents) [132, 133]. An alternative survival system in cancer cells to overcome the block of EGFR inhibitors is represented by the activation of other TK receptor systems which are not EGFR related.

The third mechanism of resistance results from constitutive activation of the signalling pathway independent of EGFR-ligand interactions. Constitutive activation of intracellular signalling elements such as PI3K represents one of the most common mechanisms of resistance to EGFR inhibitors. EGFR-independent increased activity of PI3K could result from direct gene amplification, activating mutations of the p85 subunit, overexpression of downstream effectors such as Akt, as well as inactivating mutations or loss of function of PTEN, a phosphatase that acts as a negative regulator of PI3K [49, 74]. These alterations are common events during cancer formation and progression and could possibly result in constitutive activation of oncogenic signals through Akt, MAPK or both [49, 74]. Constitutive activation of PI3K/Akt pathway has been demonstrated to play a crucial role in the development and maintenance of tumor resistance to EGFR inhibitors [134, 135]. Therefore, treatment with EGFR inhibitors alone may be far from adequate in a subset of cancer patients with constitutively activate Akt.

The last but not least mechanism may be due to EGFR gene mutations. Loss of EGFR expression or altered function due to EGFR gene mutations can be one reason why tumor cells become resistant to EGFR antagonists. Glioblastoma cell lines expressing the mutated variant EGFRvIII have been shown to be relatively resistant to gefitinib since higher doses and longer exposure to gefitinib are necessary to significantly decrease EGFRvIII phosphorylation [136]. Cell cycle analysis shows G0/G1 accumulation of EGFR-expressing cells following geftinib treatment, whereas no detectable changes are observed in EGFRvIII-

expressing cells [136]. The protective effect of EGFRvIII may be partially due to phosphorylation of Akt, which is inhibited in EGFR expressing cells after treatment with gefitinib, but is unaffected in EGFRvIII expressing cells [136].

5. Combination therapies targeting multiple receptor tyrosine kinases

Combination therapies can overcome some of the tumor resistance to TKI and are discussed below.

5.1 Combined inhibition of signalling from EGFR and VEGFR

Combined inhibition may be important for optimal suppression of solid tumor growth. Many parallel pathways exist between the VEGFR and EGFR signalling cascades, and these pathways are clearly linked in tumors [137-139]. Thus, EGFR and VEGFR share common downstream signalling pathways and may have direct and indirect effects on tumor cell and endothelial cell growth. Although some patients initially respond to EGFR TKIs in monotherapies, nearly all eventually become refractory to treatment [140, 141]. Therefore, combined inhibition has the potential to overcome resistance to monotherapies.

Individual inhibition of either EGFR or VEGFR pathway has shown limited clinical efficacy, possibly because of incomplete suppression of angiogenic and/or proliferative pathways resulting from intrinsic resistance and mutation by the tumor [141-143]. In addition, the lack of overlapping toxicities between inhibition of the VEGFR (e.g., hypertension, proteinuria) and the EGFR (e.g., rash, diarrhea) pathways suggests that simultaneous inhibition of both of these pathways has the potential to be tolerated by cancer patients [144, 145]. Thus, it is an appealing notion to combine the antiproliferative effects of EGFR inhibitors with the antiangiogenic effects of VEGFR inhibitors to achieve greater suppression and possibly delay appearance of resistant tumors. In fact, preclinical and early clinical data

provide a strong rationale for combined inhibition of both the VEGFR and EGFR pathways [146, 147].

5.2 Combined therapy with EGFR and mTOR inhibitors

The mammalian target of rapamycin (mTOR) is a PI3K related serine/threonine kinase and plays a central role in regulating cell growth, proliferation, and survival [148-150]. It also regulates initiation step of mRNA translation through interactions with other proteins such as Raptor (forming mTOR complex 1 (mTORC1)) and Rictor (forming mTOR complex 2 (mTORC2)) [150]. PI3K/Akt signalling represents a major cell survival pathway and its activation has long been associated with malignant transformation and apoptotic resistance [134, 151, 152]. It is generally thought that mTOR (i.e., mTORC1) functions downstream of PI3K/Akt and is phosphorylated (or activated) in response to stimuli that activate the PI3K/Akt pathway. However, a recent discovery demonstrated that mTORC2 is also an Akt Ser473 kinase and could function upstream of Akt [150, 153, 154]. mTORC2 is thought to be insensitive to rapamycin. Interestingly, others have shown that mTOR inhibitors activate Akt while suppressing mTORC1 signalling in different types of cancer cell lines and clinical human tumor samples [153-156]. To date, it is still unclear how mTOR inhibitors activate Akt survival signalling, although it may involve mTORC2. In addition to the intrinsic resistance of cancer cells to mTOR inhibition by rapamycin, cancer cells can acquire resistance to rapamycin [155]. Therefore, understanding the mechanisms by which cells become resistant to mTOR inhibitors such as rapamycin has been a long-standing goal in the field and may eventually guide the development of successful mTOR-targeted cancer therapies by overcoming cell resistance to mTOR inhibition.

5.3 Combined inhibition of signalling from EGFR and Ras

The critical role of Ras in the signal transduction from cell surface to downstream molecular effectors and its relationship with development of resistance against EGFR antagonists demonstrate the importance of the Ras pathway as a target of novel anticancer combination therapy [56, 157]. Ras mutations can induce its constitutive activation that persistently stimulate tumor cell proliferation and inhibit apoptotic cell death. These constitutive activation mutations are not rare and have been identified in several human malignancies [158]. Since activation of the Ras/Erk pathway through mutations in the Ras gene contributes to resistance of NSCLC cells to EGFR inhibitors, it has been proposed that inhibition of Ras/Raf/ERK signalling with farnesyl transferase (FT) inhibitors (inhibits Ras activation) may enhance the antitumor activity of EGFR inhibitors [159, 160]. Indeed, treatment of gefitinib in combination with the FT inhibitor SCH66336, results in inhibition of farnesylation and Ras protein activation, significantly reduces the growth of A549 (Human lung adenocarcinoma epithelial cell line) cells compared to treatment with single agents [161].

In conclusion, cancer is a disease resulting from multiple mutations and it is thus unlikely that only one signalling pathway drives the oncogenic processes of tumor cells. Therefore, an individual selective agent targeting a single signal transduction pathway has limited anti-tumor effect in most solid cancers. Furthermore, the crosstalk of the multiple intracellular signalling pathways justifies the need to interfere at different stages to overcome escape mechanisms in cancer cells. A better therapeutic effect may be achieved in a combination treatment with two or multiple inhibitors for separate pathways known to be critical to the survival of the tumor. The combination of agents that target multiple different

functional pathways might have additive or synergistic activity (i.e. combination of HMG-CoA reductase inhibitor with TKI). Chapter 2, 3 and 4 will address this in detail.

6. HMG-CoA reductase inhibitors

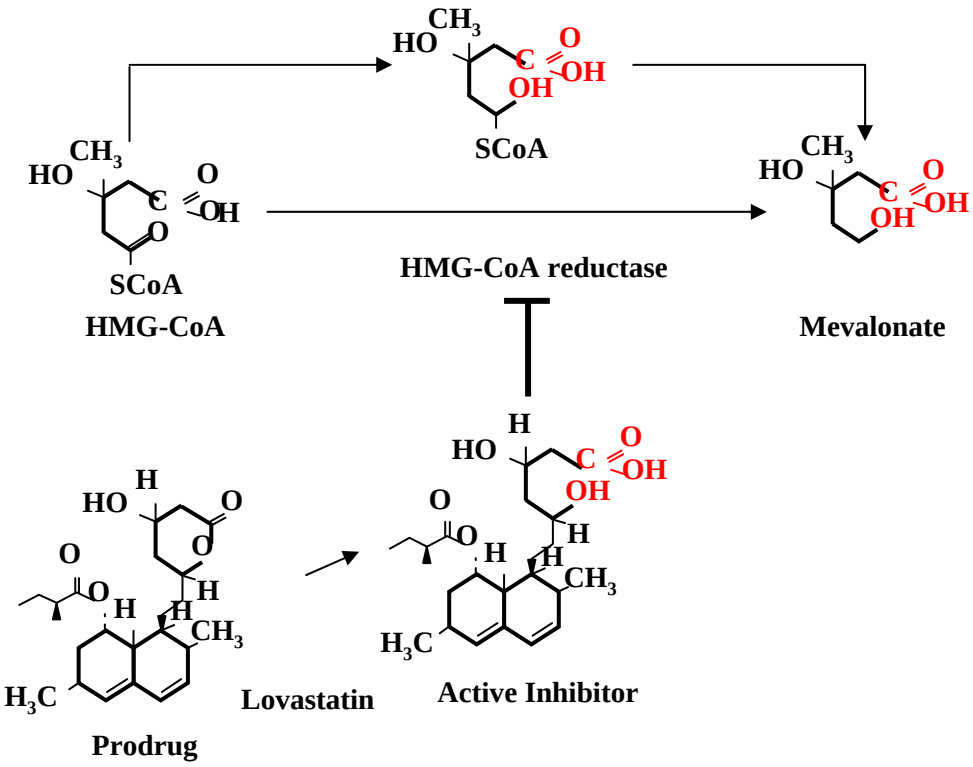
6.1 The statin family

Deregulated or elevated activity of HMGCoA reductase has been observed in a range of different tumors including hepatocellular carcinoma, leukemia, lymphoma, colorectal and lung adenocarcinoma [162-167]. Studies have shown that statins (HMG-CoA reductase inhibitors) can reduce cancer incidence by 28–33% [168, 169]. Indeed, recent analyses demonstrate that inhibitors of HMG-CoA reductase can directly block tumor cell growth both *in vitro* and *in vivo* [170-174].

The statin family is composed of eight unique compounds that are either natural or chemically synthesized [175-179]. Statins derived from fungal fermentation include pravastatin, simvastatin, and lovastatin, whereas fluvastatin, atorvastatin, cerivastatin, rosuvastatin and pitavastatin are synthetic compounds [180-187]. The common structural characteristic of all statins is a side chain that exists either in a closed ring (inactive, lactone pro-drug) or an open ring (active, acid) form (Chapter 1 Figure 8) [188, 189]. The former undergoes activation *in vivo* by carboxyesterases in plasma and liver [190, 191]. The open ring form blocks catalytically active HMG-CoA reductase by mimicking a reaction intermediate formed within the active site of this enzyme (Chapter 1 Figure 8) [188]. Statins are highly competitive inhibitors as they can bind HMGCoA reductase 1000-fold more strongly than the natural substrate [176, 188].

The statin family of drugs was initially found to block hepatic synthesis of cholesterol, predominantly lowering the low-density serum lipoproteins (LDLs) and hence improving

Chapter 1 Figure 8. Lovastatin. The open ring activated form of lovastatin is a molecular mimic of the intermediate formed during the conversion of HMG-CoA to mevalonate. Lovastatin binds to the active site of HMG-CoA reductase with a greater than 1000 fold efficiency compared to the natural substrate. The stability and binding efficiency of lovastatin make it a potent inhibitor of this enzyme. The members of the statin family of inhibitors share this open ring structure.


HO
CH3
O
C
OH
SCoA
HMG-CoA
HMG-CoA reductase
Mevalonate
Lovastatin
Prodrug
Active Inhibitor
H3C

clinical cholesterol profiles [192, 193]. Briefly, statin action for hypercholesterolemia leads to a decreased hepatic cholesterol production, which in turn leads to increased LDL receptor turnover, enhanced hepatic LDL-cholesterol uptake, and ultimately decreased plasma LDL-cholesterol levels [190, 191]. Overall, plasma LDL-cholesterol levels can be substantially decreased by 20–60%, along with mild elevation in high-density lipoprotein (HDL)-cholesterol and a reduction in triglyceride levels [190, 191]. Independent of their effect on cholesterol homeostasis, there is growing experimental evidence indicating that statins have anticancer effects ranging from antiproliferative, pro-apoptotic, differentiating, anti-invasive and radiosensitising properties, depending on the particular cell type and circumstances under which they are studied [192-195]. It has been well established that exposure of certain transformed cells to statins in vitro can lead to growth arrest, by activating a well defined G1/S cell cycle checkpoint through an as yet unknown mechanism [192, 196]. In recent years it has become clear that inhibitors of HMGCoA reductase can trigger a subset of tumor-derived cells to undergo apoptosis. Most importantly, statins can trigger apoptosis in a tumor specific manner (i.e. leukemia, melanoma, hepatoma, pancreatic, lung and neuroblastoma) [196-204].

The difference in metabolism among the various statins leads to different distributions of the drugs in the liver or peripheral tissues at similar doses [205]. For example, the concentration of pravastatin was found lower in the liver but higher in the peripheral tissues, including kidney, spleen, testis, adrenal gland, and non-glandular stomach as compared with lovastatin or simvastatin [205]. It was thought that the lipophilic properties of the lovastatin or simvastatin pro-drugs confer their selectivity to liver [206]. Similarly, lovastatin and

simvastatin were shown to cross the blood-brain and placental barriers but pravastatin and fluvastatin do not [206].

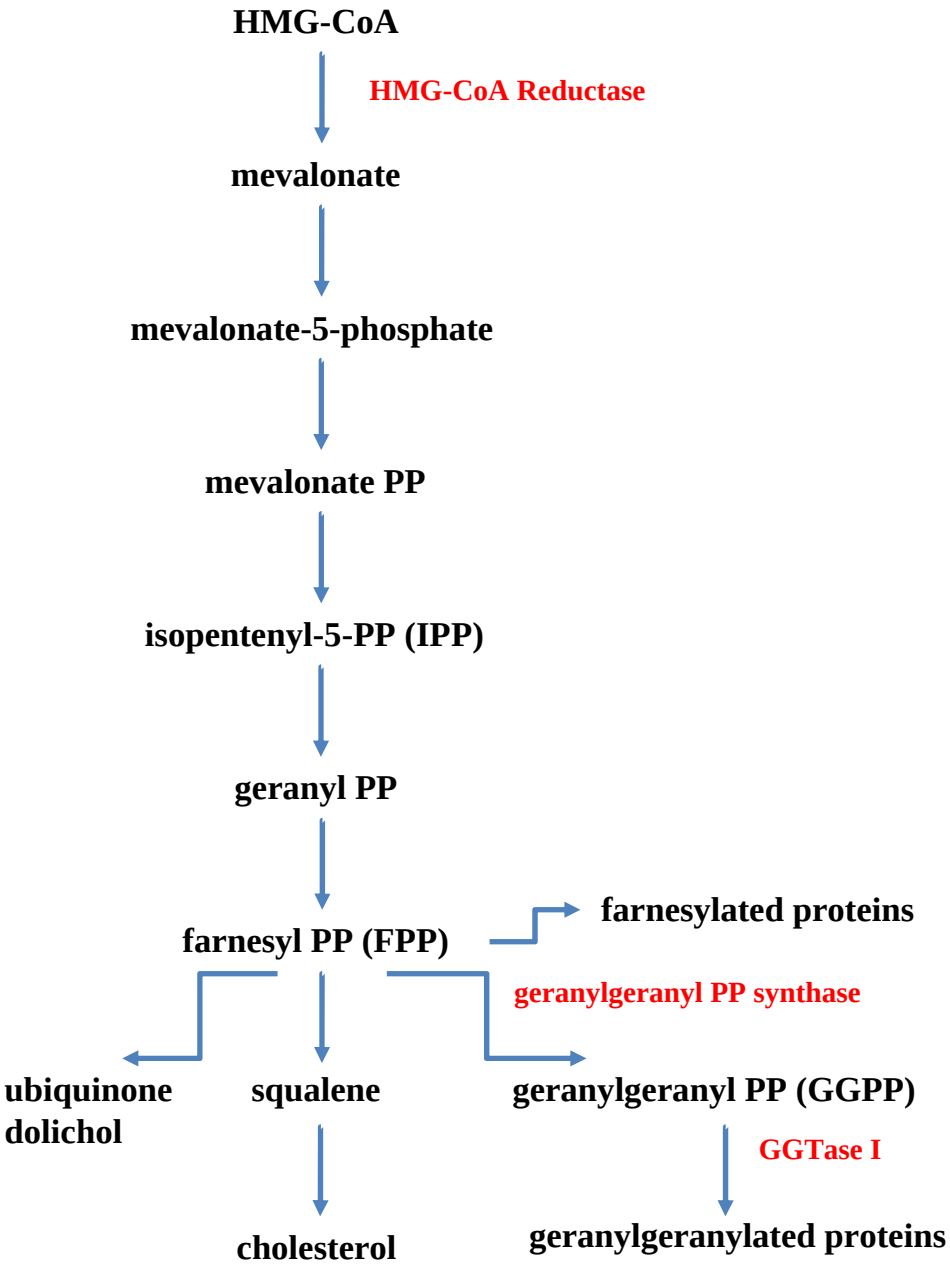
The safety of this family as therapeutic drugs has been documented extensively and they are remarkably well tolerated [175, 179, 207, 208]. Most minor adverse side effects include constipation, flatulence, dyspepsia, nausea, gastrointestinal pain, and elevated serum transaminase levels [175, 191]. In addition, interactions with other drugs must be thoroughly considered to ensure systemic concentrations are strictly controlled.

6.2 Lovastatin

Lovastatin, one of the first-generation statins, is a specific and irreversible competitive inhibitor of HMG-CoA reductase (Chapter 1 Figure 8) [176]. Its active open ring form can effectively block this critical metabolic pathway and has been used extensively for the treatment of hypercholesterolemia [176]. Lovastatin appears to be a promising pro-apoptotic agent and exhibits a cytotoxic effect in a number of transformed/tumor cell lines from head and neck SCC and cervical cancer (CC) [197, 209]. However, other dividing cells such as normal bone marrow progenitors are not affected by lovastatin [196]. In the subset of tumors that are sensitve to lovastatin, lovastatin-induced apoptosis appears to be cell type dependent and may result from the depletion of critical mevalonate metabolites (Chapter 1 Figure 9) required for cellular survival [210]. However it is also possible that lovastatin may inhibit cell growth by a mechanism independent of this rate-limiting enzyme (HMG-CoA reductase) of the mevalonate pathway. Clearly, understanding the sensitivity and specificity of tumor responsiveness to lovastatin as well as the molecular mechanism of lovastatin-induced apoptosis requires further investigation. These questions are addressed in Chapter 2, 3 and 4.

6.3 The Mevalonate Pathway

Chapter 1 Figure 9. The Mevalonate Pathway. The end products of the mevalonate pathway include sterols, especially cholesterol, involved in membrane structure; ubiquinone, involved in electron transport; dolichol, required for glycoprotein synthesis; farnesyl and geranylgeranyl isoprenoids that are covalently bound to various proteins to facilitate membrane localization.


HMG-CoA
HMG-CoA Reductase
mevalonate
mevalonate-5-phosphate
mevalonate PP
isopentenyl-5-PP (IPP)
geranyl PP
farnesyl PP (FPP)
farnesylated proteins
geranylgeranyl PP synthase
ubiquinone
dolichol
squalene
geranylgeranyl PP (GGPP)
GGTase I
cholesterol
geranylgeranylated proteins

Mevalonate pathway metabolites are critical for the function and/or expression/localization of RTKs and the effectors of their downstream signalling cascades [211-213]. The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate, which is catalyzed by HMG-CoA reductase [211]. The mevalonate pathway (Chapter 1 Figure 9) produces various end products that are critical for many different cellular functions. These products include cholesterol, dolichol, ubiquinone, geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP) [211]. Cholesterol is essential in maintaining cellular membrane structure and integrity. It also serves as a precursor for the synthesis of steroid hormones and bile acid [211]. Dolichol works as a carrier molecule of oligosaccharides in N-linked protein glycosylation for the production of glycoproteins [211]. Ubiquinone is involved in mitochondrial respiration while isopentenyladenine is an essential substrate for the modification of certain tRNAs [211].

Mevalonate metabolites also mediate prenylation of signalling proteins that play critical roles in transducing EGFR signals. Prenylated proteins are post-translationally modified at or near the carboxyl terminus by formation of cysteine thioethers with the isoprenoid lipid substrates, FPP or GGPP [212, 213]. Three distinct enzymes, farnesyl transferase (FPTase), geranylgeranyl transferase I (GGPTase I) and geranylgeranyl transferase II (GGPTase II) that transfer these isoprenoid substrates to recipient proteins have been identified [214]. These transferases depend on FPP and GGPP substrates to modify many target proteins, including small GTP binding proteins, such as Ras, Rho, Rab, Rac, and Rap etc [215]. These small GTP binding proteins participate in a variety of important cellular processes, such as the regulation of proliferation, signal transduction, and programmed cell death [215]. To be activated, some of these proteins must first undergo prenylation (addition of a farnesyl

moiety (*e.g.*, Ras) or a geranylgeranyl moiety (*e.g.*, Rho) to the carboxy-terminus of the proteins) to associate with the plasma membrane [212, 213]. Activated proteins can then regulate RTK activity on cell proliferation, cell survival, intracellular trafficking and cell motility [212, 213]. Lovastatin has been shown to induce apoptotic cell death through inhibiting the prenylation of some small GTP binding proteins in different cell types [195, 216-218].

7. Rationale and Hypothesis

Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products, and thus has significant influences on many critical cellular functions. Malignant cells appear highly dependent on the sustained availability of the end-products of the mevalonate pathway [172]. Deregulated or elevated activity of HMG-CoA reductase has been observed in a range of different tumors [172]. In addition, given its pivotal role in the well-defined mevalonate pathway, HMG-CoA reductase has been a potential molecular target for cancer therapy. Numerous clinical trials were carried out to assess the benefits of HMG-CoA reductase inhibitors [219-221]. Our clinical trial group has previously shown that lovastatin can induce tumor specific apoptosis especially in SCC and that 23% of recurrent SCC patients treated with lovastatin as a single agent showed disease stabilization in our Phase I clinical trial [222]. Subsequently, our lab was able to demonstrate that lovastatin in combination with gefitinib, a potent inhibitor of the EGFR showed co-operative cytotoxicity when combined [223]. Furthermore, the pro-apoptotic and cytotoxic effects of these agents were found to be synergistic and to be manifested in several types of tumor cell lines including SCC and NSCLC [223].

7.1 Hypothesis

Targeting the mevalonate pathway will inhibit the activity of RTKs and act co-operatively with known inhibitors of these receptors. The mechanism by which statins affect RTK function will likely be regulated by inhibiting the function of prenylated rho family proteins.

The work embodied in this thesis provides a novel model for the regulation of EGFR/VEGFR activation and signalling by targeting rho family of proteins which provides a novel, exploitable mechanism and more refined therapeutic approaches.

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CHAPTER 2

Lovastatin enhances gefitinib activity in glioblastoma cells irrespective of EGFRvIII and PTEN status

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Running Head: Lovastatin enhances gefitinib activity

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Abstract

The epidermal growth factor receptor (EGFR) is commonly amplified and mutated in glioblastoma, making it a compelling therapeutic target. Recent reports have demonstrated clinical activity of the EGFR inhibitors gefitinib and erlotinib in a subset of glioblastoma patients. Co-expression of EGFRvIII, a constitutively active mutant receptor expressed in 50% of tumors, and PTEN, an inhibitor of PI3K activity, by glioblastoma cells is associated with clinical response to these EGFR kinase inhibitors. PTEN loss and resulting increased PI3K pathway activity appears to act as a resistance factor. A critical therapeutic challenge is to identify agents that enhance the anti-cancer effects of these agents and promote responsiveness to EGFR kinase inhibitors in a broader spectrum of glioblastoma patients. For example, combining gefitinib with inhibitors of the PI3K/AKT pathway show enhanced cytotoxicity in glioblastoma derived cell lines. Here, we show that targeting HMG-CoA reductase with lovastatin, that can affect the activity of multiple cell signalling pathways, significantly enhanced the sensitivity of glioblastoma cells to the EGFR kinase inhibitor gefitinib in the five cell lines tested. In an isogenic model system, U87MG glioblastoma cells expressing EGFRvIII and PTEN in relevant combinations, we show that combined gefitinib and lovastatin treatments induce potent synergistic cytotoxicity irrespective of EGFRvIII and PTEN status. These studies demonstrate the potential of lovastatin to augment the cytotoxic effects of gefitinib and provide a rationale for combined statin/EGFR targeted therapies in glioblastoma patients.

Key Words: epidermal growth factor receptor, lovastatin, mevalonate pathway, PTEN

Introduction

Communication between individual cells is essential for the regulation and coordination of complex cellular processes such as growth, differentiation, migration and apoptosis [1]. Signal transduction networks mediating these biological processes are regulated in part by polypeptide growth factors that activate cell surface receptors either in paracrine or autocrine manner [1]. The primary mediators of these cell responses are receptor tyrosine kinases that couple ligand binding to downstream signalling cascades and gene transcription [2]. The ErbB family of receptor tyrosine kinases play key roles in the growth, differentiation, migration and cell survival of epithelial tissues [3, 4]. The ErbB receptors consist of four distinct family members that are highly homologous: ErbB1/EGFR, ErbB2, ErbB3, and ErbB4 [5]. Ligand binding to the EGFR promotes either homodimerization (EGFR/EGFR) or heterodimerization between the bound receptor and other members of the ErbB family (predominately EGFR/ErbB2), activating the receptor tyrosine kinase [6]. This sequence of events results in the autophosphorylation of specific tyrosine residues within their tyrosine kinase domains [3] triggering a series of downstream signalling cascades. These cascades regulate the effects of these receptors on cell proliferation and cell survival that includes the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways, respectively [3, 7]. In malignant cells, this receptor and its downstream signalling pathways often are deregulated, leading to cell hyper proliferation, enhanced cell survival and increased metastatic potential [3, 8]. The dependence of these tumor cells on activated EGFR may render tumors susceptible to inhibitors of these kinases [9, 10]. In fact, a small subgroup of patients with lung cancer harboring specific mutations in the EGFR kinase domain, have been associated with responsiveness [11, 12].

Among patients with glioblastoma, the most common primary malignant brain tumor of adults [13], a small subgroup also seems to benefit from the EGFR kinase inhibitors erlotinib and gefitinib [14]. However, the infrequency of mutations in the EGFR kinase domain in glioblastomas suggests that such *EGFR* mutations cannot account for responsiveness to EGFR kinase inhibitors [15]. The *EGFR* gene is commonly amplified in glioblastoma, but this abnormality also does not correlate with responsiveness to EGFR kinase inhibitors [14]. Glioblastomas often express EGFRvIII, a constitutively active genomic deletion variant of EGFR [16]. This variant lacks the ligand binding domain and is constitutively active that strongly and persistently activates the PI3K/AKT signalling pathway, which provides critical information for cell survival, proliferation, and motility [16]. By promoting chronic dependence on PI3K/AKT signalling, EGFRvIII may sensitize glioblastoma cells to EGFR kinase inhibitors [17]. The PTEN (phosphatase and tensin homologue deleted in chromosome 10) tumor suppressor protein, an inhibitor of the PI3K signalling pathway, is commonly lost in glioblastoma [18]. This loss may promote cellular resistance to EGFR kinase–inhibitor therapy by dissociating EGFR inhibition from downstream PI3K/AKT pathway inhibition [19]. In fact, a strong association exists between the co-expression of EGFRvIII and PTEN in glioblastoma cells responsive to EGFR kinase inhibitors [20].

Recently, inhibitors of the mammalian target of rapamycin (mTOR) kinase, a downstream target of AKT, have been shown to enhance the responses of both erlotinib and gefitinib in PTEN-deficient glioblastoma cells [21]. In our recent studies, we have demonstrated that the inhibition of HMG-CoA reductase with lovastatin, which results in mevalonate depletion, can potentiate the cytotoxic effects of gefitinib in a variety of tumor

derived cell lines [22]. Furthermore, the pro-apoptotic and cytotoxic effects of these agents were found to be synergistic rather than additive, to potentially involve the PI3K/AKT signalling pathway, and to be independent from the occurrence of EGFR mutations known to alter its ATP binding site and its interaction with gefitinib [22]. In this study, we evaluated the effects of this combination in a variety of glioblastoma cell lines and in a U87MG isogenic model system expressing EGFRvIII and PTEN in relevant combinations.

Material and methods

Tissue culture

The U87MG (referred to as U87 for ease of presentation), A172, DBTRG, MO59J and U118 glioblastoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cell lines and the U87 derived variants U87- pLPCX, U87-PTEN, U87-EGFRvIII-pLPCX and the U87-EGFRvIII-PTEN were maintained in Dulbecco's-MEM (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal, QC). Derivation of the U87-EGFRvIII cells has been described [23]. The PTEN variants express a PTEN clone obtained from ATCC, sequenced for verification and sub cloned into the pLPCX retroviral vector. We used MLV-based vectors and packaging components [24] to generate retroviruses for stable transduction of the PTEN cDNA. Amphotropic retrovirus was packaged by 293T cells and the viral-containing supernatant used to infect cells in the presence of 8μg/ml Polybrene (Sigma, St. Louis, MI). Post-selection transduction efficiency was verified by Western blotting using a rabbit monoclonal antibody to PTEN (Cell Signalling Technology, Beverly, MA). Cells were exposed to solvent control or to 0-50μM lovastatin (generously provided by Apotex, Mississauga, Canada, diluted from a 10mM stock in ethanol prepared as previously described [25]). Gefitinib (generously provided by AstraZeneca, Macclesfield, England) was diluted from a 50mM stock in DMSO, human recombinant EGF (Sigma) was diluted from a 50μg/ml stock in 10mM acetic acid/0.1% bovine serum albumin (Sigma) and mevalonate (Sigma) was diluted from a 100mM stock in ddH_2O.

Western blot analysis

Total cellular protein was extracted using a buffer that consisted of 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 0.2mM sodium orthovanadate (Sigma) and 0.2mM phenyl methyl sulphonyl fluoride (Sigma) in 2xPBS. Approximately 200µl of extraction buffer was used to treat 10^6 cells. Total protein was quantified with the Biorad Protein Assay using bovine serum albumin (Sigma) as standard. Protein extracts representing 50µg total protein were separated on a 10% SDS-PAGE gel and electrophoretically transferred onto PVDF membranes (Amersham, Toronto, ON). Membranes were blocked in 5% skim milk powder in PBS overnight at 4^0C. Primary antibody, diluted in 5% skim milk powder in PBS, was incubated with the membrane for 1hr at room temperature. The antibodies used for protein detection were specific for phosphotyrosine (clone PY20) (Santa Cruz Biotechnology, Santa Cruz, CA); EGFR, PTEN, AKT and pAKT (Cell Signalling Technology, Danvers, MA); and actin (Sigma). The secondary antibodies (Amersham, Mississauga, Canada) were applied at a 1:5000 dilution in 3% BSA, 10% FBS in PBS and incubated for 1hr at room temperature (washes following antibody incubations are 3x5min in PBS/0.05% Tween 80 (Sigma) then processed for chemiluminescent detection (Amersham). The images were acquired using the Gene Gnome Imaging System (Syngene Bio-imaging, Frederick, MD).

MTT Assay

In a 96 well flat bottom plate (Nunc, Naperville, Il.) approximately 5,000 cells/150µl of cell suspension was used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following treatment, 50µl of a 5mg/ml solution in phosphate buffered saline of the MTT tetrazolium substrate (Sigma) was added and incubated for 6 hrs at 37^{O}C. The resulting violet formazan precipitate was solubilized by the

addition of 100µl of a 0.01M HCl/10% SDS (Sigma) solution shaking overnight at 37^{o}C. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies at 570nm to determine the optical density of the samples.

Flow Cytometry

Cell cycle parameters were determined by flow cytometry using propidium iodide labeling of single cells as described previously [26]. Single cell suspensions were labeled with 50µg/ml propidium iodide (Sigma) and approximately 10^{6} cells in 100µl analyzed by flow cytometry. Ten thousand cells were evaluated and the percentage of cells in subG1 phase determined using the Modfit LT program (Verity Software House, Topsham, Maine).

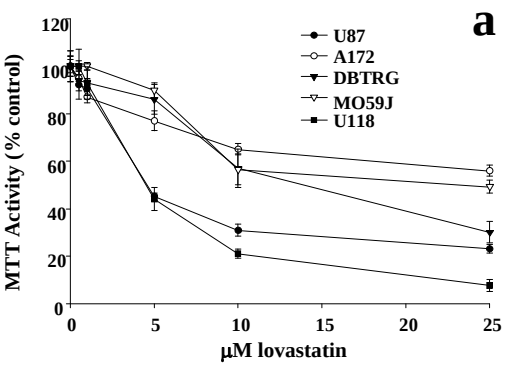
Results

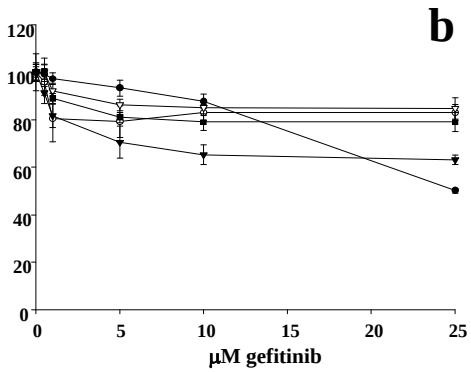
Co-operative cytotoxicity with lovastatin and gefitinib in combination

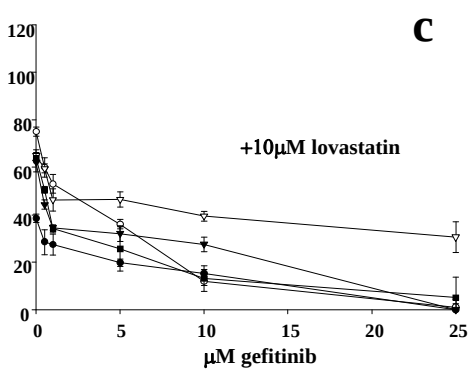
Previous studies have demonstrated that lovastatin can induce cytotoxicity in a variety of glioblastoma derived cell lines [27]. In this study, we evaluated the effects of 72hrs lovastatin treatment, 48hrs gefitinib treatment and the combination of 10μM lovastatin with 0-25μM gefitinib for 48hrs. For all treatment regimens, cells were pretreated for 24hrs with lovastatin followed by the combination for 48hrs as our previous study demonstrated synergy with this combination using this schedule [22]. Employing the MTT cell viability assay, we demonstrated that the U87 and the U118 cell lines showed significant cytotoxicity following 0.1-25μM lovastatin treatment for 72hrs. The dose of lovastatin that corresponded to 50% lethality (LD50) under these conditions was less than 5μM for these two glioblastoma cell lines. The A172, DBTRG and MO59J cell lines were less sensitive with LD50 values greater than 10μM observed (Figure 1a). Similar to recent studies, gefitinib did not significantly affect cell viability in these glioblastoma derived cell lines (Figure 1b) [21]. However, 10μM lovastatin in combination with gefitinib (24hr pretreatment with lovastatin followed by the combination for 48hrs) showed significant co-operative effects in all of the five cell lines evaluated (Figure 1c and d). Evaluation of the LD50 values in the A172, DBTRG and MO59J cell lines with either agent alone compared to the combination of 10μM lovastatin with gefitinib demonstrated synergistic cytotoxicity of these agents in combination (Figure 1e).

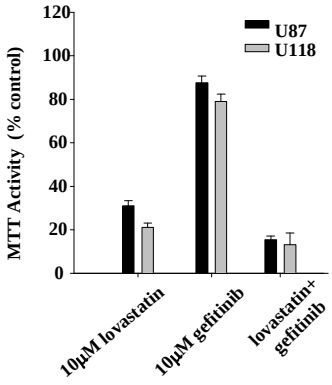
Using flow cytometric analysis of propidium iodide stained U87 and A172 cell lines, we evaluated the role of the induction of apoptosis as the mechanism by which this

Chapter 2 Figure 1. MTT assay showing co-operative cytotoxicity with lovastatin and gefitinib combination treatment. MTT cell viability assay comparing the response of U87, A172, DBTRG, MO59J and U118 glioblastoma derived cell lines to lovastatin, gefitinib and combination treatments. The cell lines were treated with 0-25μM lovastatin for 72hrs (a), 0-25μM gefitinib for 48hrs (b) and 24hr pre-treatment with 10μM lovastatin followed by the combination of 10μM lovastatin with 0-25μM gefitinib for 48hrs (c). Cell viability was assessed with the activity of untreated cells taken to be 100%. Co-operative cytotoxicity in the lovastatin sensitive U87 and U118 cell lines (d) as well as the synergistic cytotoxicity demonstrated by LD50 evaluations of this combination in A172, DBTRG and MO59J (e). In all experiments, error bars indicate standard deviation.









d

e

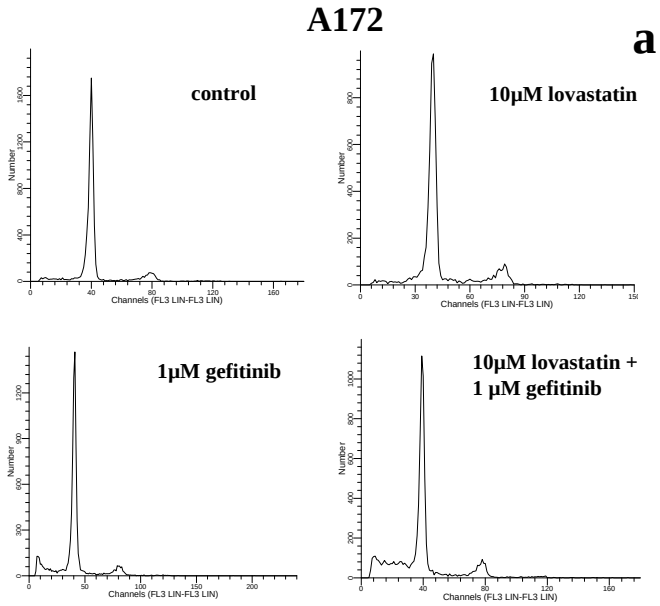
	µM lovastatin	µM gefitinib	µM gefitinib +10µM lovastatin
LD50			
A172	**>25**	**>25**	**2.0**
DBTRG	**12**	**>25**	**0.3**
MO59J	**25**	**>25**	**0.7**

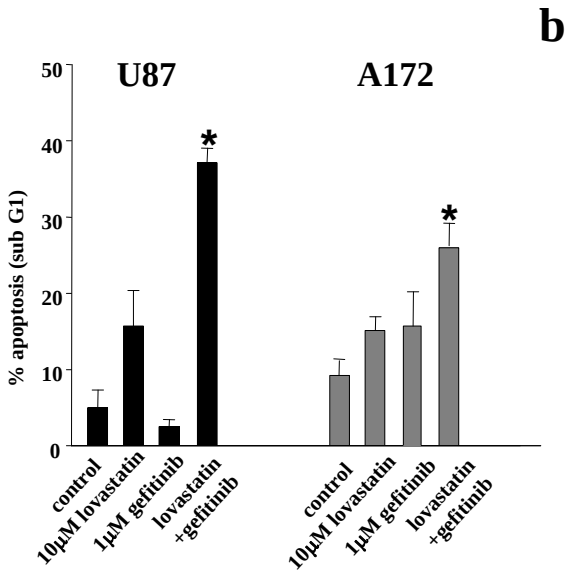
combination of agents act co-operatively to enhance their cytotoxicity. Apoptotic bodies that contain nuclear fragments are visualized as a pre-G1 peak in the DNA histogram that is characteristic of this programmed cell death modality [28]. Treating U87 and A172 cell lines with 10μM lovastatin and 1μM gefitinib, therapeutically relevant concentrations of these anti-cancer agents [9, 29], showed a significant enhanced induction of apoptosis with this combination compared to either agent alone (Figures 2a and b). To confirm that the cytotoxic and the combined synergistic effects with gefitinib of lovastatin were due to its targeting of HMG-CoA reductase, A172 treated cells were supplemented with mevalonate. The co-administration of mevalonate reversed lovastatin but not gefitinib's effect on A172 cell viability (Figure 3). Mevalonate addition also reversed lovastatin's combined cytotoxic effects with gefitinib in A172 cells. Therefore, the combination of lovastatin and gefitinib displayed co-operative cytotoxicity in glioblastoma cells by inducing a potent apoptotic response through targeting of HMG-CoA reductase.

Lovastatin enhances gefitinib AKT inhibition in EGFRvIII expressing cells

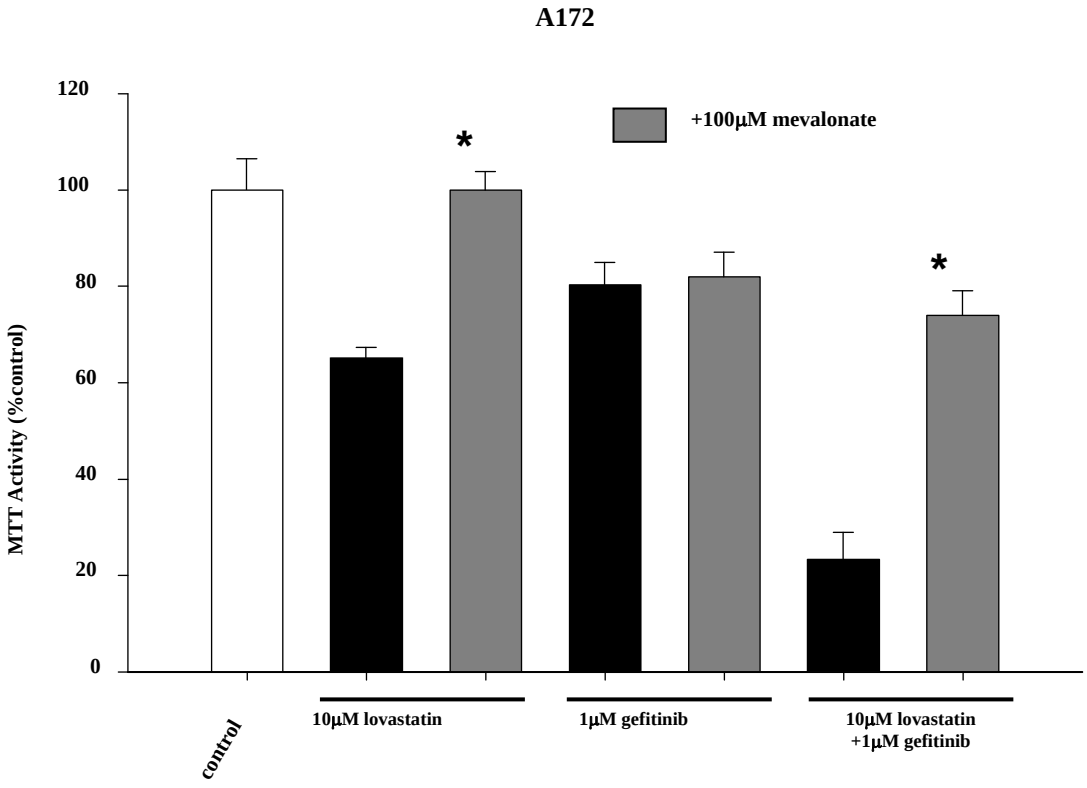
The activity of EGFR-TKIs like gefitinib in glioblastomas has been associated with the presence of EGFRvIII protein and PTEN expression that regulates the PI3K/AKT pathway [20]. This pathway plays a significant role in the proliferative and anti-apoptotic effects induced by the EGFR. Parental U87 cells lack EGFRvIII and PTEN expression potentially providing a viable isogenic cell line model to evaluate their effects on lovastatin treatments alone and in combination with gefitinib. Using retroviral transduction, we expressed EGFRvIII and PTEN in relevant combinations that included the following stable cell lines: U87-pLPCX (empty vector control), U87-PTEN, U87-EGFRvIII-pLPCX and U87-EGFRvIII-PTEN. Up to 1μM gefitinib treatments for 2hrs with or without pretreatment

Chapter 2 Figure 2. Flow cytometric analysis showing co-operative cytotoxicity with lovastatin and gefitinib combination treatment. (a and b) Flow cytometric analysis of subG1 apoptotic fraction as determined by propidium iodide staining of cellular DNA content comparing the response of U87 and A172 cell lines to lovastatin, gefitinib and combination treatments. Both cell lines were treated with 10μM lovastatin for 72hrs, 1μM gefitinib for 48hrs and 24hr pre-treatment with 10μM lovastatin followed by the combination of 10μM lovastatin with 1μM gefitinib for 48hrs. *Combination of treatments that displays significant differences in %apoptosis compared to either agent alone ($P<0.05$, Paired T-test). In all experiments, error bars indicate standard deviation.


A172
a
control
10µM lovastatin
1µM gefitinib
10µM lovastatin +
1 µM gefitinib
Number
Channels (FL3 LIN-FL3 LIN)


b
U87
A172
% apoptosis (sub G1)
50
40
30
20
10
0
*
*
control
10µM lovastatin
1µM gefitinib
lovastatin
+gefitinib

Chapter 2 Figure 3. Mevalonate addition reversed lovastatin's combined cytotoxic effects with gefitinib. MTT cell viability assay comparing the response of the A172 cell line to lovastatin, gefitinib and combination treatments with or without co-administration of mevalonate. A172 cells were treated with 10μM lovastatin for 72hrs, 1μM gefitinib for 48hrs and 24hr pre-treatment with 10μM lovastatin followed by the combination of 10μM lovastatin with 1μM gefitinib for 48hrs alone or with 100μM mevalonate. Cell viability was assessed with the activity of untreated cells taken to be 100%. *Mevalonate co-administrations that display significant differences in MTT determined cell viability ($P<0.01$, Paired T-test). In all experiments, error bars indicate standard deviation.

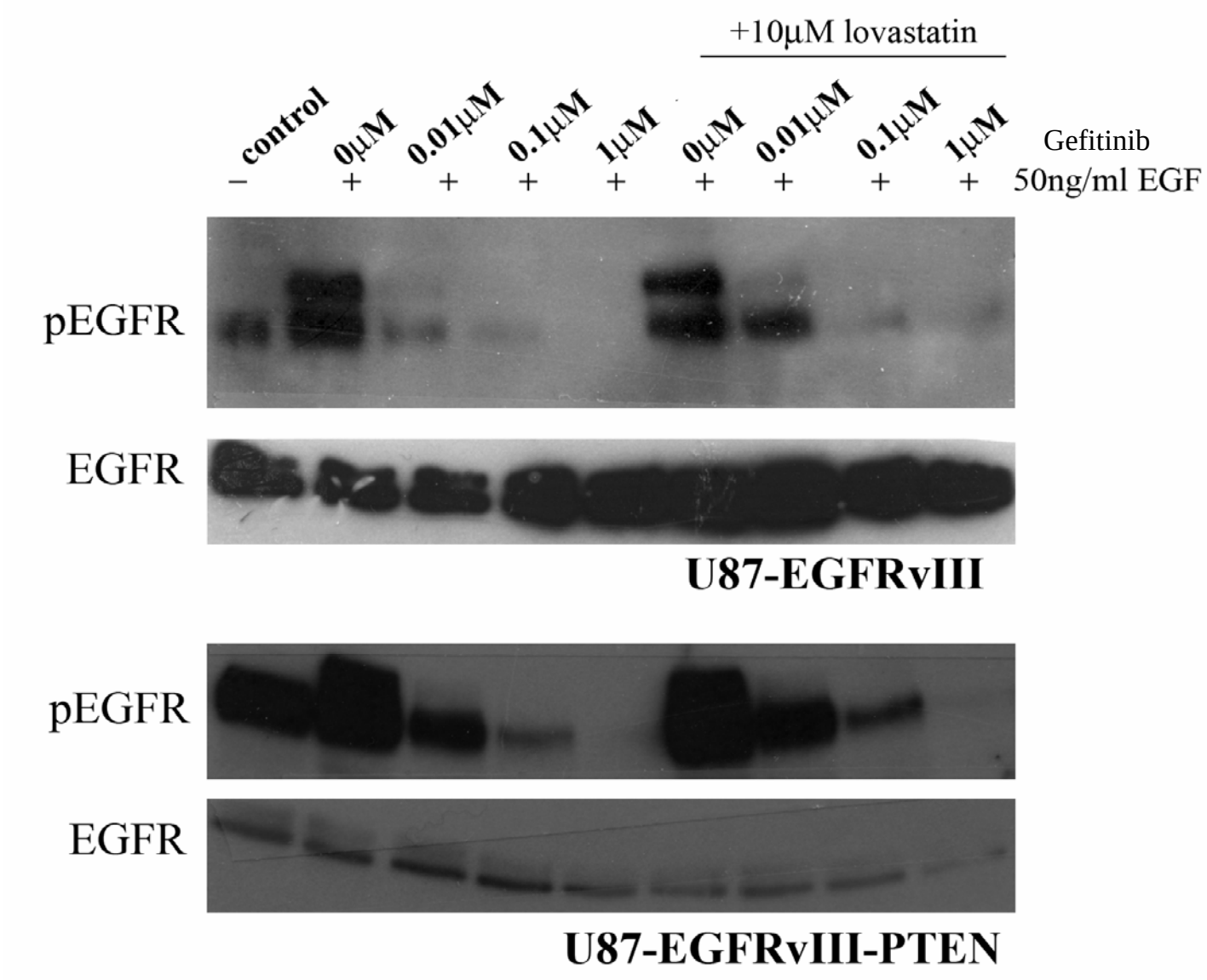

A172
120
100
80
60
40
20
0
MTT Activity (%control)
+100μM mevalonate
*
*
control
10μM lovastatin
1μM gefitinib
10μM lovastatin
+1μM gefitinib

with 10μM lovastatin for 24hrs and subsequently stimulated for 15min with 50ng/ml EGF were analyzed.

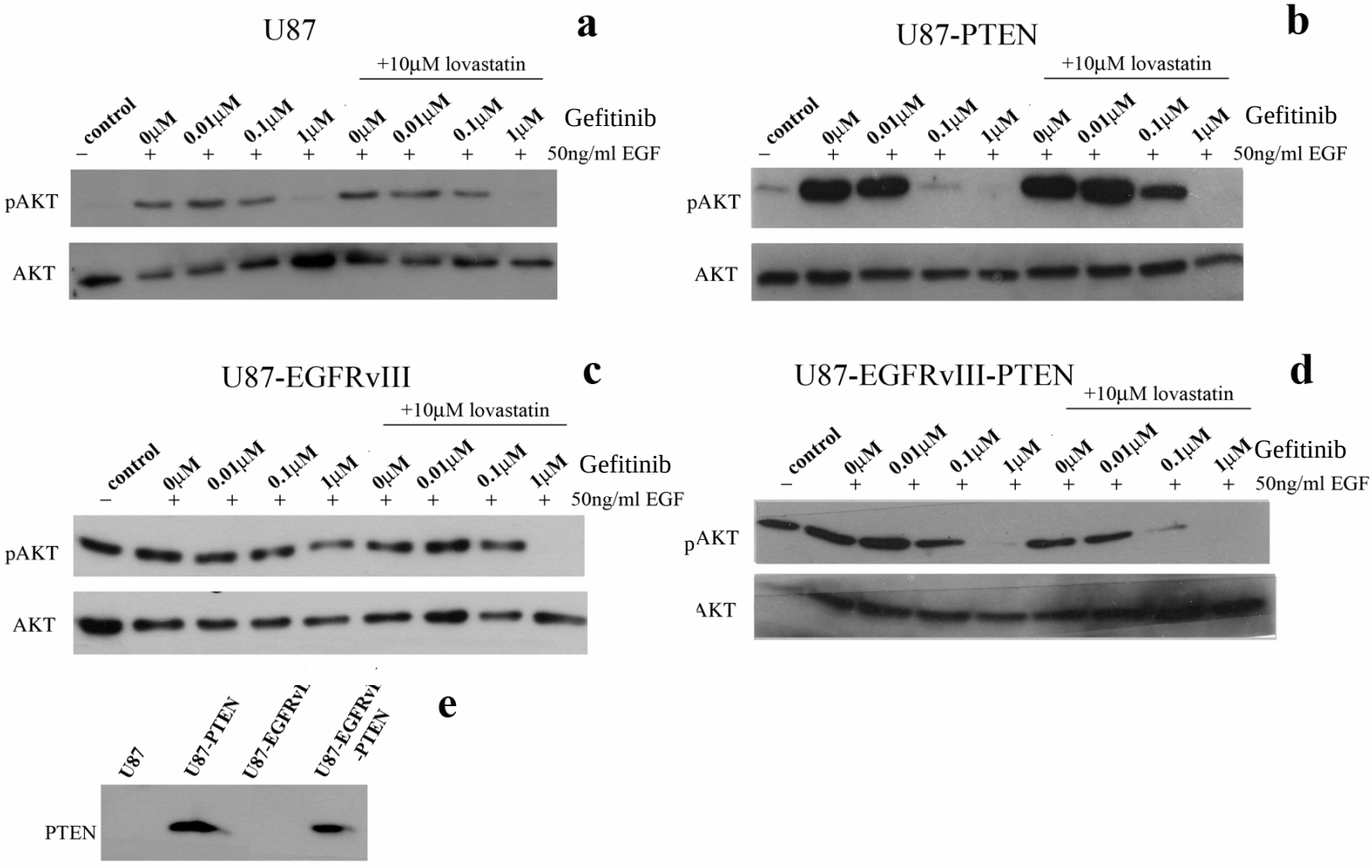
As previously reported, we were unable to detect endogenous expression of EGFR in U87 cells that lacked exogenous EGFRvIII expression [21]. In U87-EGFRvIII and U87EGFRvIII-PTEN cells both the endogenous EGFR and EGFRvIII were expressed and visualized as doublets with the lower band corresponding to EGFRvIII that lacks the ligand binding domain. Constitutive activation of EGFRvIII was demonstrated in both cell lines as untreated cells showed significant levels of phosphorylated EGFRvIII (Figure 4). Addition of 50ng/ml of EGF for 15min induced the activation of EGFR in both cell lines. Gefitinib treatment induced potent inhibition of ligand induced EGFR activation with 0.01μM 2hr treatment in both U87-EGFRvIII and U87-EGFRvIII-PTEN cells. Constitutive activation of EGFRvIII was also inhibited by gefitinib but required a dose of 1μM for 2hrs treatment. Both cell lines showed similar results and there was no observable difference with 10μM 24hrs lovastatin pretreatments (Figure 4).

We further evaluated the role of reconstituted PTEN expression in U87 and U87-EGFRvIII cells on AKT activation. Compared to U87 cells, U87-PTEN cells demonstrated a more potent inhibition of ligand induced AKT phosphorylation with significant inhibition observed with 0.1μM gefitinib treatment for 2hrs. Pretreatment with 10μM lovastatin for 24hrs did not have an effect on ligand induced AKT activation in these cells (Figure 5a and b). Similarly, in EGFRvIII expressing PTEN reconstituted U87 cells, enhanced gefitinib inhibition of AKT activation was shown compared to EGFRvIII-PTEN deficient U87 cells (Figure 5c and d). Only the EGFRvIII and the EGFRvIII-PTEN expressing cells did lovastatin pretreatment (10μM, 24hrs) enhance the effects of gefitinib on AKT activation

Chapter 2 Figure 4. Both cell lines showed similar results with or without lovastatin treatment. Western blot analysis of 0-1μM of gefitinib treatment for 2hrs with or without a 24hr 10μM lovastatin pre-treatment of serum starved U87-EGFRvIII-pLPCX and U87-EGFRvIII-PTEN cell lines with EGFR and phospho-EGFR (PY20) antibodies. Following addition of 50ng/ml of EGF for 15min, the activation EGFR and EGFRvIII were evaluated.


+10μM lovastatin
control
0μM
0.01μM
0.1μM
1μM
0μM
0.01μM
0.1μM
1μM
Gefitinib
− + + + + + + + + 50ng/ml EGF
pEGFR
EGFR
U87-EGFRvIII
pEGFR
EGFR
U87-EGFRvIII-PTEN

Chapter 2 Figure 5. lovastatin enhances gefitinib AKT inhibition in EGFRvIII expressing cells. Western blot analysis of 0-1μM of gefitinib treatment for 2hrs with or without a 24hr 10μM lovastatin pre-treatment of serum starved U87 (a), U87-PTEN (b), U87-EGFRvIII (c) and U87-EGFRvIII-PTEN (d) cell lines with AKT and phospho-AKT (serine 473) antibodies. Following addition of 50ng/ml of EGF for 15min, the activation of AKT was evaluated. PTEN expression was confirmed in the U87-PTEN and U87-EGFRvIII-PTEN lines (e).

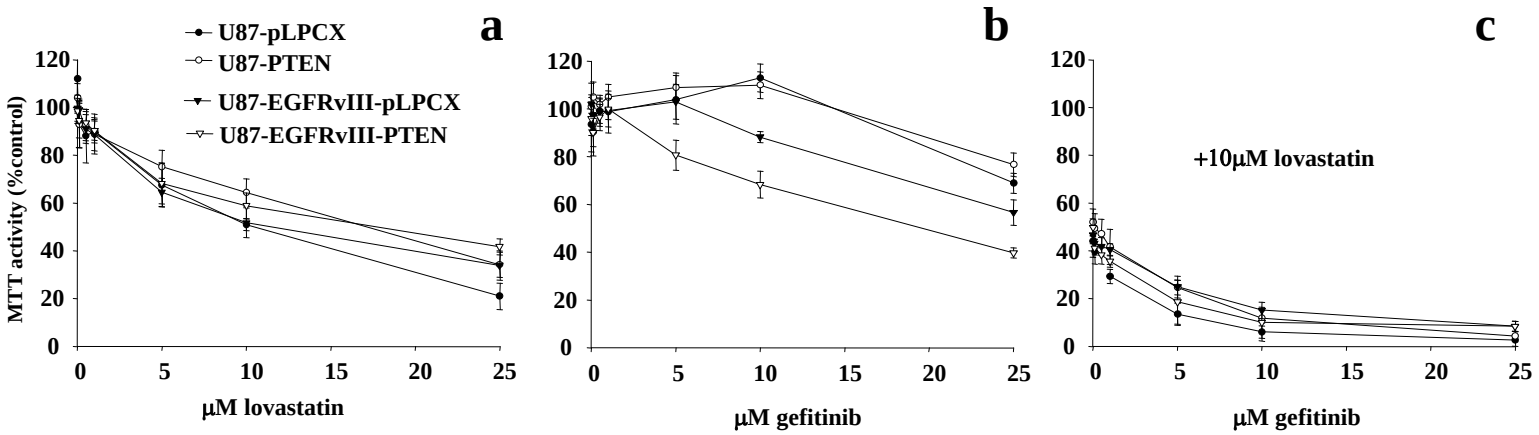

a
U87
+10μM lovastatin
control
0μM
0.01μM
0.1μM
1μM
0μM
0.01μM
0.1μM
1μM
Gefitinib
50ng/ml EGF
pAKT
AKT
b
U87-PTEN
+10μM lovastatin
Gefitinib
50ng/ml EGF
pAKT
AKT
c
U87-EGFRvIII
+10μM lovastatin
Gefitinib
50ng/ml EGF
pAKT
AKT
d
U87-EGFRvIII-PTEN
+10μM lovastatin
Gefitinib
50ng/ml EGF
pAKT
AKT
e
U87
U87-PTEN
PTEN

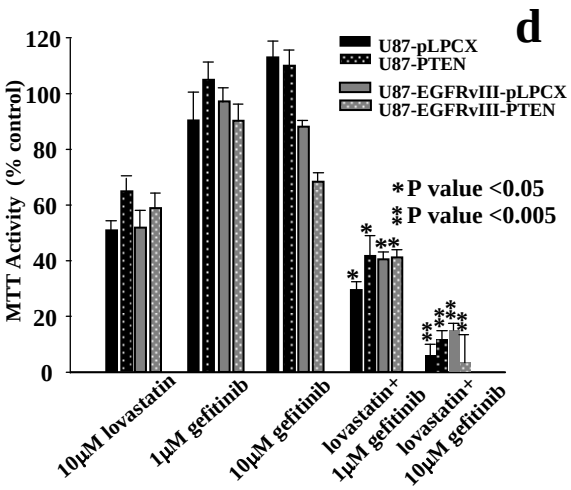
(Figure 5). Therefore, gefitinib inhibits AKT activation more significantly in PTEN expressing U87 cells while lovastatin enhanced gefitinib's inhibitory effects on AKT activation only in EGFRvIII expressing U87 cells.

Enhanced lovastatin and gefitinib cytotoxicity irrespective of EGFRvIII and PTEN status

Employing the MTT cell viability assay, we demonstrated that all four of these U87 derived cell lines showed significant cytotoxicity following 0.1-25μM lovastatin treatment for 72hrs (Figure 6a). The response to lovastatin-induced cytotoxicity was similar irrespective of EGFRvIII or PTEN expression status. Similar to recent studies, gefitinib (0.1-25μM) sensitivity was more pronounced in the U87-EGFRvIII-PTEN cells (Figure 6b) and was in line with the clinical data that has shown enhanced activity of gefitinib in patients whose glioblastomas express both EGFRvIII and PTEN [20, 21]. We then evaluated the potential of lovastatin to enhance the cytotoxic effects of gefitinib in these U87 cell line variants. As previously described, a 24hr pretreatment with 10μM lovastatin was followed by the combination of 10μM lovastatin and 0.1-25μM gefitinib for 48hrs. In all four U87 derived cell lines, lovastatin and gefitinib showed significant co-operative cytotoxic effects (Figure 6c). This enhanced cytotoxicity was irrespective of EGFRvIII and PTEN status, was dose dependent and significant differences in cytotoxicity were observed when comparing the combinations to either agent alone (Figure 6d). Therefore, lovastatin in combination with gefitinib induced a significant and potent cytotoxic response in U87 cells regardless of their EGFRvIII and PTEN status.

Chapter 2 Figure 6. Enhanced lovastatin and gefitinib cytotoxicity irrespective of EGFRvIII and PTEN status. MTT cell viability assay comparing the response of U87-pLPCX, U87-PTEN, U87-EGFRvIII-pLPCX and U87-EGFRvIII-PTEN expressing cells to lovastatin, gefitinib and combination treatments. Both cell lines were treated with 0-25μM lovastatin for 72hrs (a), 0-25μM gefitinib for 48hrs (b) and 24hr pre-treatment with 10μM lovastatin followed by the combination of 10μM lovastatin with 0-25μM gefitinib for 48hrs (c). Cell viability was assessed with the activity of untreated cells taken to be 100%. Statistically significant differences were determined using Paired T-test (d). In all experiments, error bars indicate standard deviation.


a
U87-pLPCX
U87-PTEN
U87-EGFRvIII-pLPCX
U87-EGFRvIII-PTEN
MTT activity (%control)
μM lovastatin
b
μM gefitinib
c
+10μM lovastatin
μM gefitinib


d
U87-pLPCX
U87-PTEN
U87-EGFRvIII-pLPCX
U87-EGFRvIII-PTEN
MTT Activity (% control)
*P value <0.05
**P value <0.005
10μM lovastatin
1μM gefitinib
10μM gefitinib
lovastatin+ 1μM gefitinib
lovastatin+ 10μM gefitinib

Discussion

Glioblastoma is the most aggressive human brain tumor with a dismal prognosis [13]. Novel therapeutic approaches are necessary as chemotherapy and radiation have not significantly affected outcome [13]. One particularly attractive approach revolves around targeting the EGFR as 40% of glioblastomas show amplification of the EGFR gene locus [30]. Half of these tumors express EGFRvIII, a mutant receptor that is constitutively active due to deletion of the extra cellular ligand-binding domain [16]. Based on recent clinical trials, 10% to 20% of unselected glioblastoma patients respond to EGFR tyrosine kinase inhibitors [14]. The majority of responding tumors expressed EGFRvIII, however, EGFRvIII expression was also found in drug resistant tumors [14]. Loss of the tumor suppressor PTEN was highly correlated with treatment failure and co-expression of EGFRvIII and PTEN strikingly predicted treatment responses [20]. Thus, EGFRvIII, much like EGFR kinase domain mutations in lung cancer, sensitize tumor cells to EGFR kinase inhibitor therapy, but apparently requires functional PTEN. PTEN serves as negative regulator of the PI3K/AKT pathway by removing the third phosphate from the inositol ring of the second messenger PIP3 [19]. PTEN inactivation, present in 50% of glioblastomas, results in accumulation of PIP3 levels and persistent signalling through AKT [18]. PTEN loss could thus promote resistance to EGFR tyrosine kinase inhibitors by deregulation of the AKT signalling pathway [19]. Therefore, in certain tumor cell lines, intervention at multiple points in a signalling cascade may be necessary to most effectively inhibit cell growth and survival. For example, inhibition of mTOR, a downstream target of AKT that regulates protein translation cell growth and survival [31], with rapamycin in combination with EGFR tyrosine kinase inhibitors induced synergistic growth inhibition in PTEN deficient glioblastoma cells [21].

In this study, we evaluated the potential of lovastatin to augment the effects of gefitinib in a variety of glioblastoma cell lines as well as the U87 cell line expressing relevant combinations of EGFRvIII and PTEN. The combination of lovastatin and gefitinib treatments displayed significant co-operative cytotoxicity through the induction of a potent apoptotic response in the five glioblastoma cell lines evaluated. In the U87 isogenic cell line model, lovastatin induced cytotoxicity was pronounced and equivalent in the U87 cells that did not express EGFRvIII or PTEN, expressed EGFRvIII alone, expressed PTEN alone or expressed both. While lovastatin pre-treatment enhanced the inhibitory activity of gefitinib on AKT, this effect was limited to the EGFRvIII expressing cells. However, the synergistic cytotoxic effects observed with lovastatin and gefitinib in U87 cells was irrespective of EGFRvIII and PTEN status. This suggests that lovastatin's ability to target other cell signalling pathways may mediate its combinational effects with gefitinib. Nonetheless, the potent synergistic cytotoxic response observed with these two agents that have an extensive clinical history with good safety profiles [9, 10, 29] can be easily evaluated as a novel therapeutic approach in glioblastoma patients. Since this synergistic cytotoxicity was readily observed irrespective of EGFRvIII and PTEN status, addition of statins to an EGFR tyrosine kinase regimen in glioblastoma may enhance the efficacy of this approach.

Statins are potent competitive reversible inhibitors of HMG-CoA reductase, the rate limiting enzyme of the mevalonate pathway, that are widely used as hypercholesterolemia treatments [32]. Malignant cells are dependent on the sustained availability of the end products of the mevalonate pathway [33]. Deregulated or elevated activity of HMG-CoA reductase has been shown in a range of different tumors [33]. The mevalonate pathway produces various end products that are critical for many different cellular functions [34].

These products include cholesterol, dolichol, ubiquinone, isopentenyladenine, geranylgeranyl pyrophosphate, and farnesyl pyrophosphate. Cholesterol is essential in maintaining cellular membrane structure and integrity and plays an important role in EGFR activation and downstream signalling. It also serves as a precursor for the synthesis of steroid hormones and bile acid. Dolichol works as a carrier molecule of oligosaccharides in N-linked protein glycosylation for the production of glycoproteins. Ubiquinone is involved in mitochondrial respiration while isopentenyladenine is an essential substrate for the modification of certain tRNAs [34]. Geranylgeranyl transferase and farnesyl transferase use geranylgeranyl pyrophosphate and farnesyl pyrophosphate, respectively, for post-translational modifications of a wide variety of cellular proteins [35, 36]. These include Ras, nuclear lamins, and many small GTP-binding proteins such as members of the Rab, Rac, and Rho families [35, 36]. These proteins regulate cell proliferation, intracellular trafficking and cell motility and this post-translational modification functions as a membrane anchor critical for their activity [35, 36]. Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products and, thus, may have significant influences on many critical cellular signalling pathways.

Due to the ability of statins to target a number of cell signalling pathways their potential as anti-cancer agents has also been evaluated. Statin treatment can directly block tumor cell growth, invasion and metastases both *in vitro* and *in vivo* [21, 37]. However, in initial clinical analyses of statins as anti-cancer therapeutics, no significant anti-tumor responses were observed [29]. New optimism regarding the use of statins, however, has emerged from recent studies. We and others have demonstrated that a number of human tumor types including glioblastoma cells, are particularly susceptible to lovastatin-induced

apoptosis [26, 27, 38, 39]. The identification of specific tumor types that are sensitive to lovastatin-induced apoptosis, spurred further clinical evaluation by our group and a Phase I trial in recurrent squamous cell carcinoma patients was undertaken. Although no tumor regressions were observed, 23% of patients exhibited stable disease suggesting further clinical evaluation is warranted [40]. The therapeutic potential of statins would likely as part of a combinational therapeutic approach. To this end, we demonstrated the synergistic cytotoxic effects of statins in combination with gefitinib in squamous cell and colon carcinoma as well as in non-small cell lung cancer cells [22]. In this study, we expanded on this work and demonstrated that statins also potentiate the effects of gefitinib in glioblastoma cells suggesting potential clinical utility of this approach.

Acknowledgments

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CHAPTER 3

Lovastatin inhibits EGFR dimerization and AKT activation in squamous cell carcinoma cells: Potential regulation through targeting rho proteins

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Running Title: EGFR function and the Mevalonate Pathway

Abbreviations: RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; HMG-CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; SCC, squamous cell carcinoma; NSCLC, non-small cell lung carcinoma; ROCK, rho-associated kinase; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; OS, overall survival; DSS, disease specific survival

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Author contributions : TTZ and JD wrote the manuscript. TTZ designed experiments. TTZ, BGL (part of Figure 1 and 5) performed experiments. GG, KD, PAB contributed to the clinical data.

Abstract

We recently demonstrated the ability of lovastatin to inhibit the function of the epidermal growth factor receptor (EGFR) and its downstream signalling of the PI3K/AKT pathway. Combining lovastatin with gefitinib, a potent EGFR inhibitor, induced synergistic cytotoxicity in various tumor derived cell lines. In this study, lovastatin treatment inhibits ligand-induced EGFR dimerization in squamous cell carcinoma (SCC) cells and its activation of AKT and its downstream targets 4EBP1 and S6K1. This inhibition was associated with global protein translational inhibition demonstrated by a decrease in RNA associated polysome fractions. The effects of lovastatin on EGFR function were reversed by the addition of geranylgeranyl pyrophosphate that acts as a protein membrane anchor. Lovastatin treatment induced actin cytoskeletal disorganization and the expression of geranylgeranylated rho family proteins that regulate the actin cytoskeleton, including rhoA. Lovastatin-induced rhoA was inactive as EGF stimulation failed to activate rhoA and inhibition of the rho-associated kinase, a target and mediator of rhoA function, with Y-27632 also showed inhibitory effects on EGFR dimerization. The ability of lovastatin to inhibit EGFR dimerization is a novel exploitable mechanism regulating this therapeutically relevant target. To explore the potential clinical significance of this combination, we evaluated the effect of statin on the overall survival (OS) and disease specific survival (DSS) of patients with advanced non-small cell lung cancer enrolled to NCIC Clinical Trials Group phase III clinical trials BR21 (EGFR tyrosine kinase inhibitor erlotinib versus placebo) and BR18 (carboplatin and paclitaxel with or without the metalloproteinase inhibitor BMS275291). In BR18, use of statin did not affect OS or DSS. In BR21, patients showed a trend for

improvement in OS (HR: 0.69, p = 0.098) and DSS (HR: 0.62, p = 0.048), and there was no statin × treatment interaction effect (p=0.34 and p=0.51for OS and DSS; respectively).

Key Words: epidermal growth factor receptor; mevalonate pathway; AKT; lovastatin; rho GTPases

Introduction

Epithelial malignancies such as squamous cell carcinomas (SCC) have very limited treatment options when presenting as metastatic disease [1, 2]. Receptor tyrosine kinases (RTK), particularly the epidermal growth factor receptor (EGFR), have been shown to play significant roles in the pathogenesis of SCC [3]. As such, targeting EGFR function has been an intensive focus of anti-cancer therapeutic approaches. Tyrosine kinase inhibitors (TKI) prevent the autophosphorylation of the intracellular tyrosine kinase domain of RTK [4, 5]. These molecules are generally reversible competitors with ATP for binding to the intracellular catalytic domain of the tyrosine kinase [4, 5]. These selective inhibitors of RTKs represent attractive therapeutic approaches [6-8]. As such, TKI have been developed targeting the EGFR that have shown promising but limited clinical activity including gefitinib and erlotinib [6, 7].

Activation of the EGFR is stimulated by ligand binding that results in receptor dimerization and its subsequent autophosphorylation required to activate its downstream signalling targets that mediate its effects on growth, differentiation, migration and cell survival [5, 9]. Levels of these receptors at the cell surface, affinity for ligand binding, its dimerization partners and activity of attenuation mechanisms that include receptor internalization and phosphatase activity regulate the extent of EGFR autophosphorylation and activation [5]. Dimerization of EGFR following ligand binding is critical for its autophosphorylation and activation of its downstream signalling cascades. EGFR can either form homo-dimers or hetero-dimers where Erb2 is the most common binding partner of EGFR in this case [10]. Ligand activated EGFR is rapidly internalized into early endosome vesicles as a primary mechanism to attenuate its signal [5]. Receptor dimerization and

internalization are linked due to the necessity of dimerization to induce receptor autophosphorylation and the recognition and docking of the internalization machinery to specific autophosphorylation site(s) [5].

EGFR autophosphorylation sites when activated are the biochemical triggers that start a series of downstream signalling cascades that regulate the effects of this receptor on cell proliferation and cell survival including the activation of the phosphatidylinositol-3 kinase (PI3K)/AKT pathways [5, 11]. Besides promoting cell survival, signalling by the PI3K/AKT pathway also affects mRNA translation through the activation of mTOR (mammalian target of rapamycin) and the subsequent phosphorylation of eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) and S6 kinase 1 (S6K1) [11]. S6 is a ribosomal protein that is a component of the 40S subunit [11]. Hyperphosphorylated 4EBP1 is released from eukaryotic translation initiation factor 4E (eIF4E) resulting in enhanced cap-dependent translation [11]. The phosphorylation of 4EBP1 and S6K1 is stimulated by serum, insulin and growth factors like EGF.

Statins are potent inhibitors of HMG-CoA reductase, the rate limiting enzyme of the mevalonate pathway, that are widely used as hypercholesterolemia treatments [12]. The mevalonate pathway end products are critical for many different cellular functions and include cholesterol, dolichol, ubiquinone, isopentenyladenine, geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP) [13]. Geranylgeranyl transferase and farnesyl transferase use GGPP and FPP, which function as membrane anchors, for post-translational modifications of a wide variety of small GTP-binding proteins that regulate cell proliferation, intracellular trafficking and cell motility [14, 15]. HMG-CoA reductase and EGFR targeted therapies may be linked due to the potential of mevalonate metabolites to affect the function

of the EGFR signalling pathway. For example, FPP and GGPP modified proteins include the ras, rho and rab families that play critical roles in transducing EGFR signals [16, 17].

In this study, we demonstrate the ability of lovastatin to inhibit EGFR dimerization that results in inhibition of ligand activated AKT along with its downstream targets that regulate protein translation initiation. These inhibitory effects of lovastatin on EGFR activity were mediated by its ability to effectively target the function of rhoA and may have clinical implications.

Materials and Methods

Tissue Culture

The SCC9 and SCC25 HNSCC cell lines were obtained from the ATCC (Rockville, MD). The NIH 3T3 cell line was provided by Dr. D. Gray (Ottawa Hospital Research Institute, Ottawa, Canada). The cell lines were maintained in Dulbecco's-MEM (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal). Human recombinant EGF (Sigma, St. Louis, MI) was diluted from a 50μg/ml stock in 10mM acetic acid/0.1% bovine serum albumin (Sigma). Lovastatin (provided by Apotex, Mississauga, Canada), mevalonate, GGPP, FPP, dolichol and ubiquinone (all from Sigma) were utilized as previously described [18]. Gefitinib (provided by AstraZeneca, Macclesfield, England), rapamycin (Sigma), Y-27632 (Calbiochem, San Diego, CA) and sodium orthovanadate (Na_3OV_4) (Sigma) were reconstituted in DMSO (Sigma).

Western Blot Analysis

Total cellular protein was extracted using RIPA buffer containing protease inhibitor cocktail (Sigma). Protein extracts representing 50 to 100μg total protein were separated on a 10% SDS-PAGE gel (unless otherwise stated) and Western blots were performed as previously described [19]**.** The primary antibodies used were specific for phospho-AKT, AKT, phospho-S6K1, S6K1, phospho-4EBP1, 4EBP1, EGFR (Cell Signalling Technology, Danvers, MA), phosphotyrosine (clone PY20), rhoA, rac1, cdc42, cyclinD1 (Santa Cruz Biotechnology, Santa Cruz, CA); EGFR, pEGFR 1068 (Cell Signalling Technology); and actin (Sigma). The secondary antibodies (Amersham Biosciences) were applied at a 1:5000

dilution. The images were acquired using the Gene Gnome Imaging System (Syngene Bio-imaging, Frederick, MD).

Receptor Dimerization and Cell Surface Expression

Cells were washed with ice-cold PBS and incubated on ice for 30 minutes with the cross-linking reagent bis (sulfosuccinimidyl) suberate (3mM in PBS, Pierce) [20, 21]. Dimers were visualized using Western blot analysis as >300kD bands where monomers served as loading control. Pinpoint Cell Surface Protein Isolation Kit (Pierce) was used to isolate proteins expressed on the cell surface. In brief, control and 10μM lovastatin treated SCC9 for 24hrs were stimulated with or without 50ng/ml of EGF for 30min. Cells were then washed with ice-cold PBS and surface proteins were biotinylated and isolated using immobilized avidin and assayed for EGFR expression by Western blot analysis.

Polysome Fractionation

To isolate polysomes, cells were treated with cycloheximide and lysed. Nuclei and cellular debris were removed by centrifugation. 500 μL of cell lysate were layered on a continuous sucrose gradient (10–50% sucrose in 15 mM MgCl2, 15 mM Tris pH 7.4, 0.3 M NaCl). Absorbance of the gradients was measured continuously at 254 nm from top to bottom at a flow rate of 1 ml/min and samples were collected in 12 fractions of 1ml. RNA was extracted by phenol chloroform extraction and an equal volume from each fraction was used to perform real time RT-PCR. Quantitative real time PCR was performed using the QuantiTect SYBR green PCR kit (Qiagen, Mississauga, ON, Canada) with the ABI Prism 7500 system (Applied Biosystems, Foster City, CA) with ABI Prism 7500 SDS Software. Standard curves of each primer set were plotted to determine the relative amount of mRNA in each fraction. The sum of mRNAs from fractions 1-5 or 6-10 were divided by total

mRNAs in all 10 fractions to give the % representation. The primers for gene-specific RT-PCR analysis were as follows: ATF3: TAGGCTGGAAGAGCCAAAG (5') and TTCTCACAGCTGCAAACACC (3'); GAPDH TTGATGTCATCATACTTGGCAGGT (5') and CAG TCAAGGCTGAGAATGGGA (3').

2 Dimensional SDS-PAGE

Following treatment, cells were collected and washed in ice-cold sucrose buffer (10mM BES pH 7.4, 0.3 M sucrose) and lysed in IEF buffer (7M Urea, 2M Thiourea, 4% CHAPS, 65 mM Dithiothreitol and 0.0001% bromophenol blue, all from Sigma) and homogenized by vortexing for 1.5hrs. Proteins were then precipitated by addition of 10 volumes of acetone (Sigma) followed by a 2500 rpm centrifugation for 5min. Pellets were resuspended in IEF buffer plus 0.4% 3-10 ampholytes (Biorad, Mississauga, ON, Canada), and protein concentrations were determined by Bradford Assay (Biorad). IPGs strips (7cm, 3-10 pH range, Biorad) were rehydrated overnight with 50μg of total protein in a total volume of 125μL. Isoelectric focusing was carried out with the following conditions: 250 Volts for 20 min, 4000V for 2hrs for desalting and 10 000 Volts hours at 4000V for focusing. Strips were then equilibrated for 15min in reducing (6M Urea, 375mM Tris-HCl ph 8.8, 20% Glycerol, 2% SDS, 130mM DTT) and alkylating buffers (6M Urea, 375mM Tris-HCl pH 8.8, 20% Glycerol, 2% SDS, 216mM Iodoacetamide) before running the second dimension on a 10% SDS-PAGE gel.

Phalloidin staining and Immunofluorescence

In a six- well tissue culture dish (Fisher), approximately 50,000 cells were used to seed each well containing a 1 cm x1 cm glass cover slip (Fisher). Following treatments, cells were fixed for 15 minutes in 4% PBS buffered paraformaldehyde (Sigma) at 37^0C. The cells

were then permeabilized using 0.1% Triton X-100 (Sigma) in PBS for 15 minutes. To label the actin cytoskeleton, the cells were incubated with rhodamine-conjugated phalloidin (Sigma) (1μg/ml in PBS) for 15min at room temperature. For immunofluorescence, cells were blocked with 3% FBS in PBS for 30min. The primary antibody (EGFR) was added at a 1:50 dilution in 3% FBS in PBS for 1hr followed by 3x PBS washes. The secondary rhodamine labeled antibody (Calbiochem) at a 1:50 dilution in 3% FBS in PBS was then incubated for 1hr. After two washes in PBS, the slides were mounted with a DAPI containing immunofluorescent mounting medium (Vector Laboratories, San Diego, CA). For the EGF and transferrin staining, Alexa 555-conjugated EGF (Molecular Probes) and rhodamine-conjugated transferrin (Molecular Probes) both at 50ng/ml were added to cells 15min prior to fixation as described above. After two PBS washes, the slides were mounted and viewed by immunofluorescence microscopy as above.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay (MTT Assay)

In a 96-well, flat-bottomed plate (Fisher, Mississauga, ON), ~7500 cells/ 150μl of cell suspension were used to seed each well. Cells were pretreated with control media, 10nM rapamycin or 10μM lovastatin for 24hrs, followed by a 48hr treatment of gefitinib and assayed for MTT activity as previously described [19]. Treatments were performed in replicates of six and the means expressed as the percent viability relative to the untreated control (100% viable).

RhoA Activity Assay

The SCC9 and SCC25 cell lines were cultured in serum free medium and treated with 10μM lovastatin for 24hrs with or without 100μM mevalonate or 10μM GGPP. Cells were stimulated with 50ng/ml EGF for 30min to activate rhoA. Cell lysates were either snap

frozen and stored in liquid nitrogen or used directly with the RhoA G-LISA kit (Cytoskeleton, Denver, Co) according to the manufacturer's instructions. The degree of RhoA activation is determined by comparing readings from the activated cell lysates (addition of 0.2mM GTP) versus the non-activated cell lysates (serum starved cultures).

Evaluating the impact of statin use in BR.18 and BR.21. The association between statin use and OS and DSS of patients with advanced NSCLC was evaluated from the databases of two NCIC Clinical Trials Group trials. Using the BR.21 and BR.18 databases, patients on statin therapy were identified. Kaplan Meier Curves, stratified by treatment, were generated to assess the OS and DSS of the statin cohort *versus* the non-statin cohort in both trials. OS was defined as the time from randomization to death from any cause and DSS was defined as date from randomization to date of death from NSCLC and/or related to NSCLC treatment. A Cox regression model was used to assess the association of statin use with OS and DSS adjusted for baseline variables.

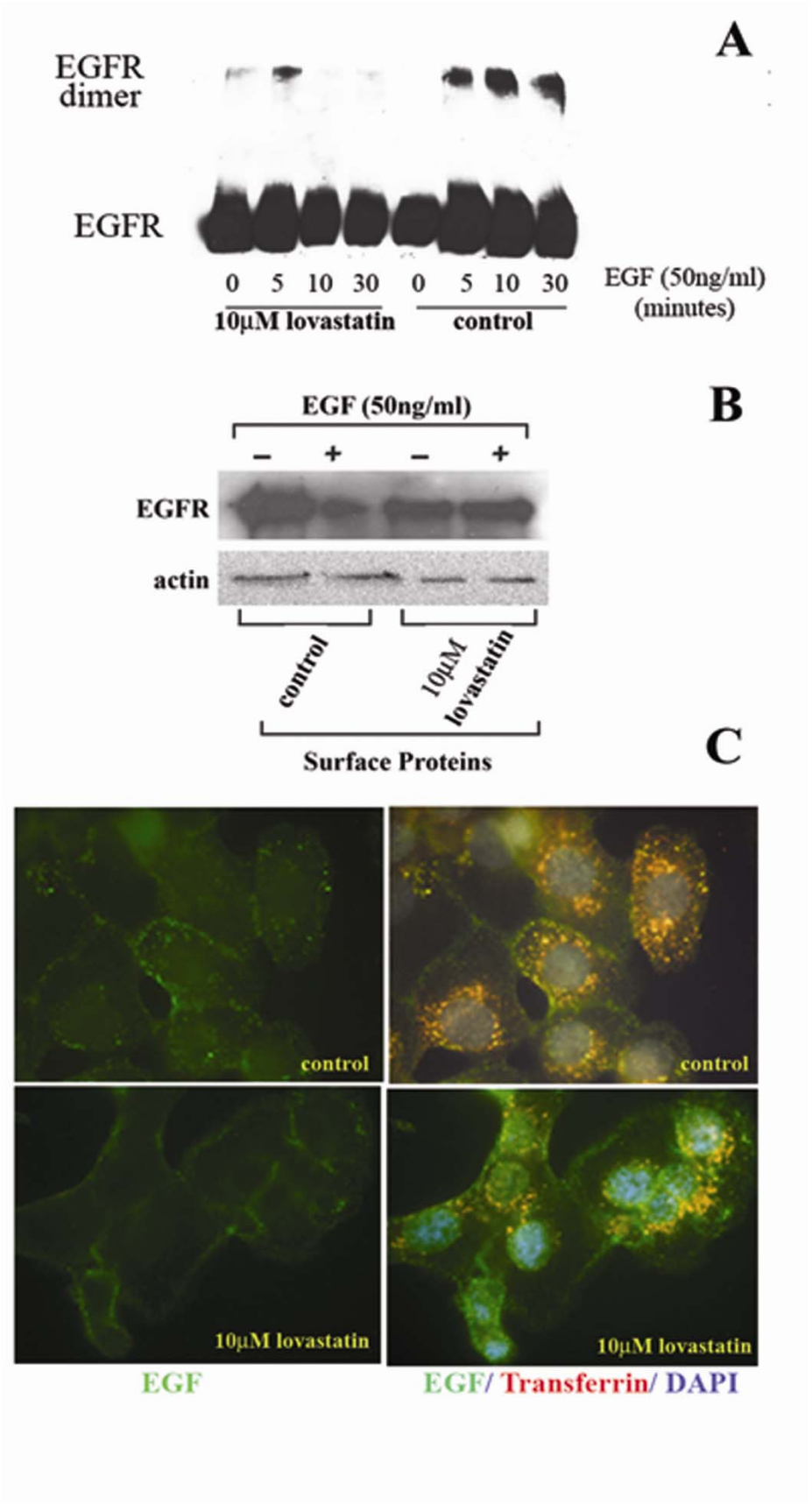
Results

Lovastatin Inhibits EGFR Dimerization and Internalization.

Firstly, we determined the effects of lovastatin treatment on ligand binding induced dimerization of the EGFR by performing cross-linking experiments [21]. SCC9 cells were treated with control and 10μM lovastatin for 24hrs and then stimulated with 50ng/ml of EGF for 0, 5,10 and 30min. Using Western blot analysis dimers were visualized as >300kD bands and the monomers served as loading control. As shown in Figure 1A, lovastatin treatment inhibited ligand stimulated EGFR dimerization in SCC9 cells. To further examine whether lovastatin is also regulating the internalization of the EGFR ligand complex, we performed Pinpoint Cell Surface Protein Isolation for proteins localized on the cell surface. Cell surface proteins were marked by biotinylation and isolated using immobilized avidin, prior to Westerns blot analysis for EGFR expression. As shown in Figure 1B, control cells were found to have relatively high amounts of EGF receptor expressed on the surface of the cell. Stimulation with EGF ligand decreased the levels of EGFR on the cell surface. In cells treated with 10μM lovastatin for 24hrs, although EGFR levels were reduced overall, ligand stimulation did not affect EGFR surface expression on SCC9 cells indicative of inhibition of internalization.

Transferrin receptors are known to undergo constitutive internalization and recycling [22], whereas the receptors for growth factors, like the EGFR, require ligand binding for internalization [22]. To further confirm whether lovastatin treatment can regulate EGFR internalization, we used Alexa 555-conjugated EGF and rhodamine-conjugated transferrin as tagged ligands for their respective receptors. Control and 10μM lovastatin (24hrs) pre-treated SCC9 cells were incubated for 15min with 50nM fluorescently labeled EGF and transferrin,

Chapter 3 Figure 1. Lovastatin inhibits ligand induced EGFR dimerization and internalization in SCC9 cells. A, Control and 10μM lovastatin treated cells for 24hrs were stimulated with 50ng/ml of EGF for 0, 5,10, and 30min. Cells were washed with ice-cold PBS and incubated on ice for 30 minutes with the cross-linking reagent bis (sulfosuccinimidyl) suberate (3 mM in PBS). Dimers were visualized as >300kD bands. Lovastatin treatment significantly decreased the amount of EGFR dimers formed. B, Cell Surface Pinpoint Protein Isolation revealed a decrease in EGFR on the surface of control SCC9 upon EGF stimulation but not with 10μM lovastatin treatment. Actin was readily pulled down in control cells but not in lovastatin treated SCC9 indicating a lack of association of surface proteins with actin in lovastatin treated cells. C, The effect of 10μM lovastatin treatment for 24hrs on EGF internalization in SCC9 cells. Alexa 555-conjugated EGF and rhodamine-conjugated transferrin (50nM each) were added for 15min following lovastatin exposure. In untreated cells, EGF was found on the cell surface and in endosomes (marked by transferrin) in proximity to the cell surface. Lovastatin treatment did not appreciably affect cell surface EGF but EGF was no longer evident in endosomes. Nuclei were counterstained with DAPI. Original magnification, x400.

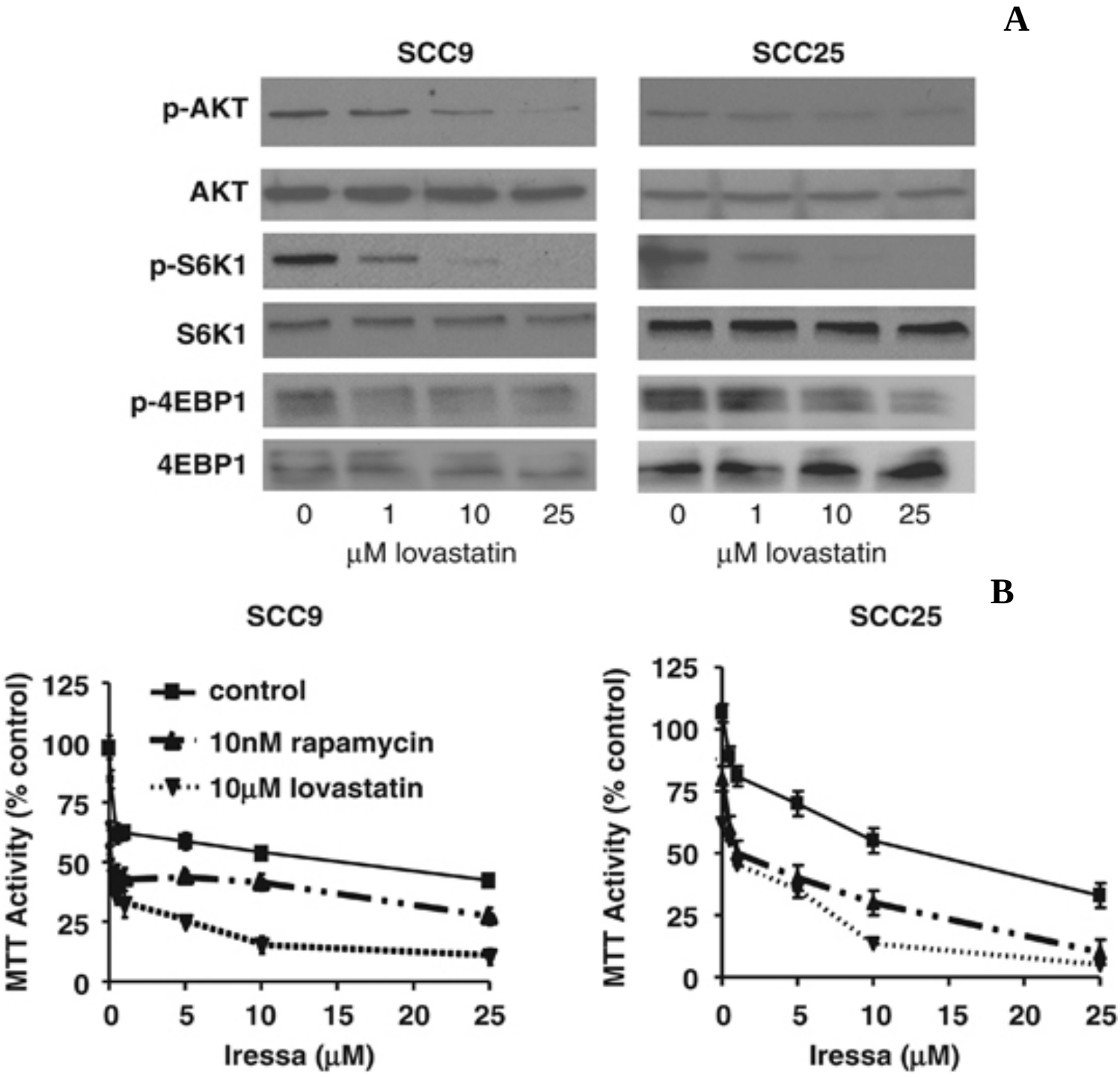

A
EGFR dimer
EGFR
0 5 10 30 0 5 10 30
EGF (50ng/ml) (minutes)
10μM lovastatin
control
B
EGF (50ng/ml)
– + – +
EGFR
actin
control
10μM lovastatin
Surface Proteins
C
control
control
10μM lovastatin
10μM lovastatin
EGF
EGF/ Transferrin/ DAPI

washed, fixed and visualized by fluorescent microscopy. In control SCC9 cells, transferrin and EGF were readily internalized (Figure 1C). In lovastatin treated SCC9 cells, transferrin was readily internalized; however, EGF remained at the cell surface (Figure 1C). These results indicate a lack of internalization of EGF in lovastatin treated SCC9 cells.

Lovastatin inhibits downstream EGF signalling pathways.

Next, we evaluated the effect of lovastatin treatment on the PI3K/AKT pathway signalling cascade triggered by EGFR which plays a role in its cell survival response [9]. Serum starved SCC9 and SCC25 cells were treated with 0, 1, 10 and 25μM lovastatin for 24hrs followed with 50ng/ml EGF stimulation for 30 min. Functional activation of AKT was evaluated by Western blot analysis, employing a phosphospecific antibody. Lovastatin treatments inhibited activation of AKT in a dose dependent manner that was readily detectable at the 1μM dose (Figure 2A), a clinically relevant dose of lovastatin [23]. The effects of AKT on regulation of protein translation are largely regulated by its downstream target mTOR. mTOR phosphorylates S6K1 and active S6K1 stimulates protein synthesis through activation of the S6 ribosomal protein [24]. 4EBP1 directly interacts with eIF4E, which is rate limiting for 40S ribosomal recruitment to mRNAs. Upon phosphorylation by mTOR, 4EBP1 releases eIF4E activating mRNA translation [24]. Lovastatin treatment (same conditions as above) in SCC9 and SCC25 cells inhibited EGF activation of S6K1 and 4EBP1 as evaluated by phosphorylation levels of these proteins (Figure 2A). mTOR has recently emerged as an attractive downstream AKT therapeutic target particularly in combination with EGFR-TKI [25]. In this study, we demonstrate by MTT analysis that both rapamycin (10nM) and lovastatin (10μM) at therapeutically relevant doses [23, 25], augmented the cytotoxic effects of gefitinib in SCC9 and SCC25 cells (Figure 2B). Taken together, these

Chapter 3 Figure 2. Lovastatin in combination with RTK-TKIs in inhibition of phosphorylation status of AKT, S6K1 and 4EBP1. A, Cell lysates were collected after treatments for 24 h with control, 1, 10 and 25 mM lovastatin in serum free medium, followed by 50 ng/ml of EGF stimulation for 30 min. Phosphorylation level of AKT decreased with lovastatin treatment in a dose dependent manner. Expression level of AKT was assayed as the loading control. Phosphorylation levels of S6K1 and 4EBP1 also decreased with lovastatin treatment in a dose dependent manner. Expression levels of S6K1 and 4EBP1 were assayed as the loading control. B, Evaluating the cytotoxic effects of treatment of rapamycin and lovastatin in combination with Iressa (gefitinib) in SCC cell lines employing MTT Assays. Iressa (gefitinib) (0-25nM) was evaluated alone or in combination with10nM rapamycin or 10μM lovastatin. MTT data were normalized to untreated (medium alone) cells (representing 100%) and is typical of 2 independent experiments. In all experiments, error bars indicate standard deviation.


A
SCC9
SCC25
p-AKT
AKT
p-S6K1
S6K1
p-4EBP1
4EBP1
0 1 10 25
μM lovastatin
0 1 10 25
μM lovastatin
B
SCC9
SCC25
MTT Activity (% control)
125
100
75
50
25
0
control
10nM rapamycin
10μM lovastatin
0 5 10 15 20 25
Iressa (μM)
MTT Activity (% control)
125
100
75
50
25
0
0 5 10 15 20 25
Iressa (μM)

results demonstrate that lovastatin treatment can readily inhibit AKT and its downstream targets in SCC cells.

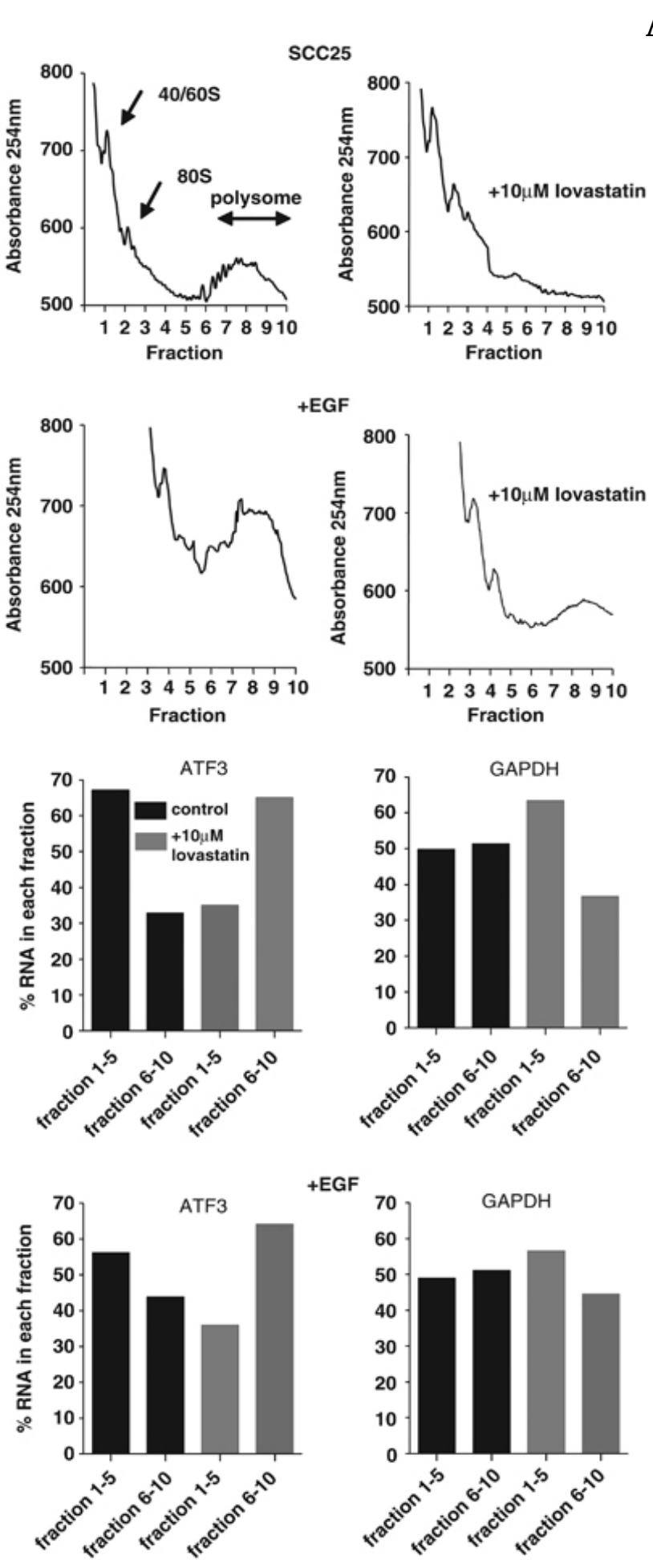
Lovastatin treatment inhibits global protein translation.

In order to assess whether lovastatin treatment of SCC25 cells attenuates global protein translation we fractionated cell lysates by sucrose density gradient centrifugation. In Figure 3A, the optical density profiles of typical sucrose gradients are shown. The top of the gradient contains free mRNAs and ribosomal subunits (40S, 60S, 80S) while the bottom of the gradient contains mRNAs associated with polysomes [26]. Analysis of the optical density profiles demonstrated a reduction in the relative amount of mRNAs associated with polysomal fractions in lovastatin-treated cells thereby indicating an overall attenuation of protein synthesis in lovastatin (10μM, 24hrs) treated SCC25 cells and cells treated with lovastatin in combination with 50ng/ml EGF (Figure 3A). We next performed quantitative RT-PCR analysis to compare the expression profile of (activation of transcription factor) ATF3 and GAPDH (Figure 3B) mRNA in monosome and polysome enriched fractions. It has been shown before that increased ATF3 expression is an indication of attenuation of protein translation during stress [27]. We found that ATF3 mRNA (Figure 3B) shifted toward the heavier polysome fractions in lovastatin treated cells. In contrast, the distribution of GAPDH mRNAs shifted to the lighter monosome fractions (Figure 3B).

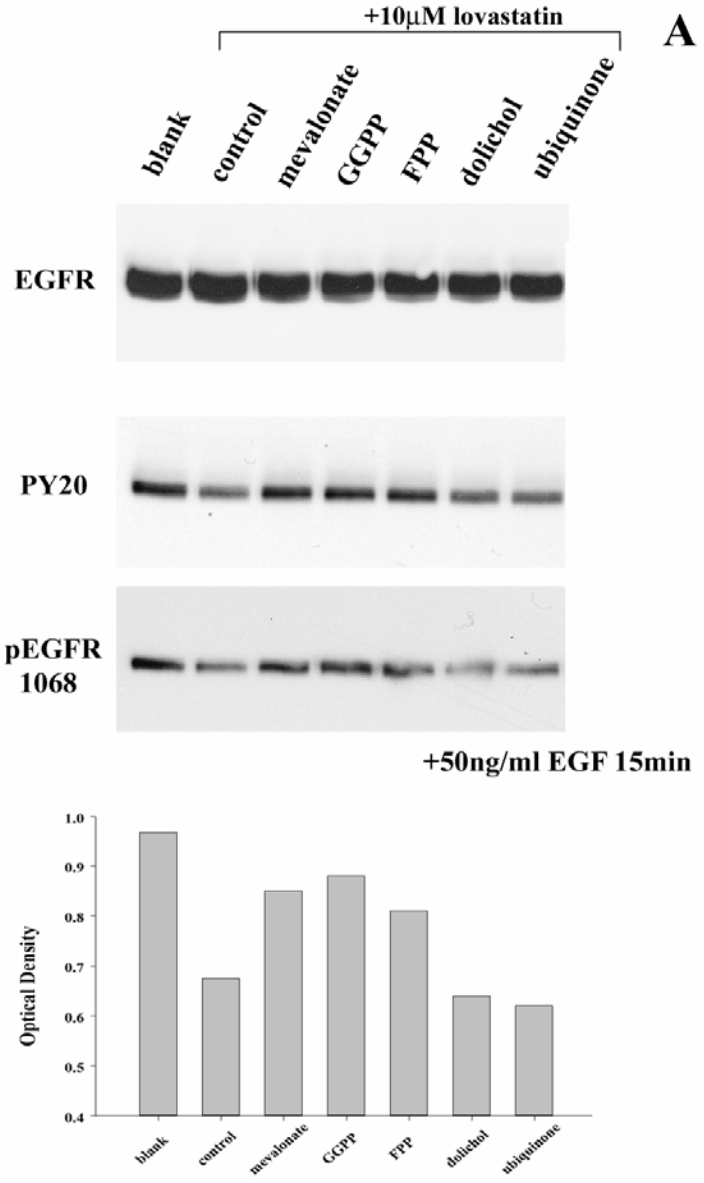
Geranylgeranyl reverses the inhibitory effect of lovastatin on EGFR activation.

Lovastatin (10μM, 24hrs) treated SCC9 cells were co-incubated with 100μM mevalonate, 10μM GGPP, 10μM FPP, 10μM dolichol phosphate or 10μM ubiquinone. The co-administration of mevalonate, GGPP and FPP mitigated the inhibitory effects of lovastatin on EGF induced EGFR activation. Both the pan anti-phosphotyrosine antibody (PY20) and

Chapter 3 Figure 3. Lovastatin inhibits protein translation. A, Representative polysome profiles of SCC25 cell lysates fractionated by sucrose density ultracentrifugation. Cells were incubated in the presence or absence (CTL) of 10μM lovastatin (24hr) in serum-free medium with or without 50ng/ml for 30min EGF stimulation later on. Cells were then treated with cycloheximide and lysed. Nuclei and cellular debris were removed by centrifugation. Cell lysates were layered on a continuous sucrose gradient. Absorbance of the gradients was measured at 254 nm from top to bottom via a peristaltic pump and RNA-containing sucrose gradients were fractioned for a total of 12 fractions. Note the decrease in polysomal RNA in lovastatin treated cells, indicating decreased global protein translation. B, qRT-PCR was performed to detect ATF3 and GAPDH mRNA in monosome (fraction 1-5) or polysome (fraction 6-10) enriched fractions of the sucrose gradient. Percentage quantification demonstrates a shift of ATF3 to the heavier and GAPDH to the lighter polysome fractions of the gradient following lovastatin treatment.


A
SCC25
Absorbance 254nm
800
700
600
500
40/60S
80S
polysome
1 2 3 4 5 6 7 8 9 10
Fraction
+10μM lovastatin
+EGF
B
ATF3
GAPDH
% RNA in each fraction
70
60
50
40
30
20
10
0
control
+10μM lovastatin
fraction 1-5
fraction 6-10

Chapter 3 Figure 4. Geranylgeranyl reverses the inhibitory effect of lovastatin on EGFR activation. A, Mevalonate, farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), dolichol and ubiquinone were co-administered with lovastatin to determine the metabolites of this pathway that could reverse the inhibitory effects of lovastatin of EGFR activity. Western blot analysis of 10 M lovastatin treated SCC9 cells for 24hrs alone and co-incubated with 100 M mevalonate, 10 M GGPP, 10 M FPP, 10 M dolichol phosphate or 10 M ubiquinone were performed. Prior to protein extraction, all cultures were exposed to 50ng EGF for 15min. Total EGFR expression (upper panel) was evaluated as well as total phosphotyrosine levels of EGF stimulated EGFR (PY20) and phosphorylation of the 1068 tyrosine residue of the EGFR. Densitometric analyses of the phosphorylation levels of the 1068 tyrosine residue of the EGFR normalized to the levels of total EGFR were performed. B, Western blot analysis of 10 mM lovastatin-treated SCC9 or SCC25 cells (except lane 1, 2, 5 and 7) for 24 h alone and coincubated with 100 mM mevalonate or 10 mM GGPP was performed. Prior to protein extraction, cells were exposed to 50ng EGF for 30min as indicated. Cells were washed with ice-cold PBS and incubated on ice for 30 minutes with the cross-linking reagent bis (sulfosuccinimidyl) suberate (3 mM in PBS). Dimers were visualized as >300kD bands.


A
+10μM lovastatin
blank
control
mevalonate
GGPP
FPP
dolichol
ubiquinone
EGFR
PY20
pEGFR 1068
+50ng/ml EGF 15min
Optical Density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
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FPP
dolichol
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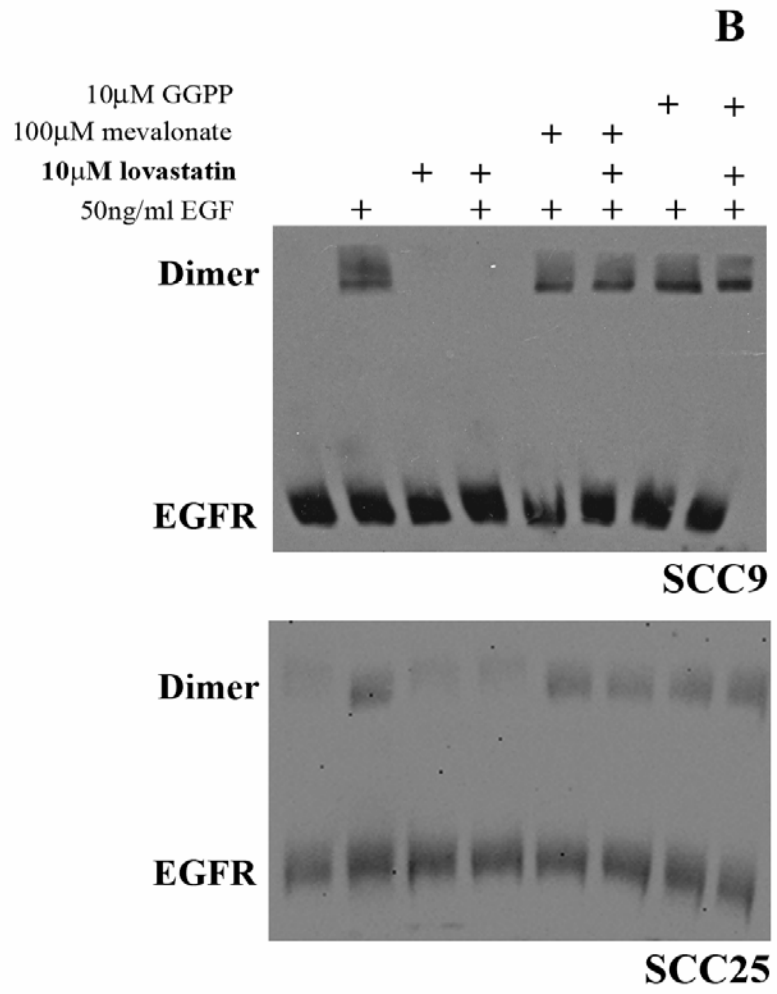

B
10μM GGPP
100μM mevalonate
10μM lovastatin
50ng/ml EGF
Dimer
EGFR
SCC9
Dimer
EGFR
SCC25

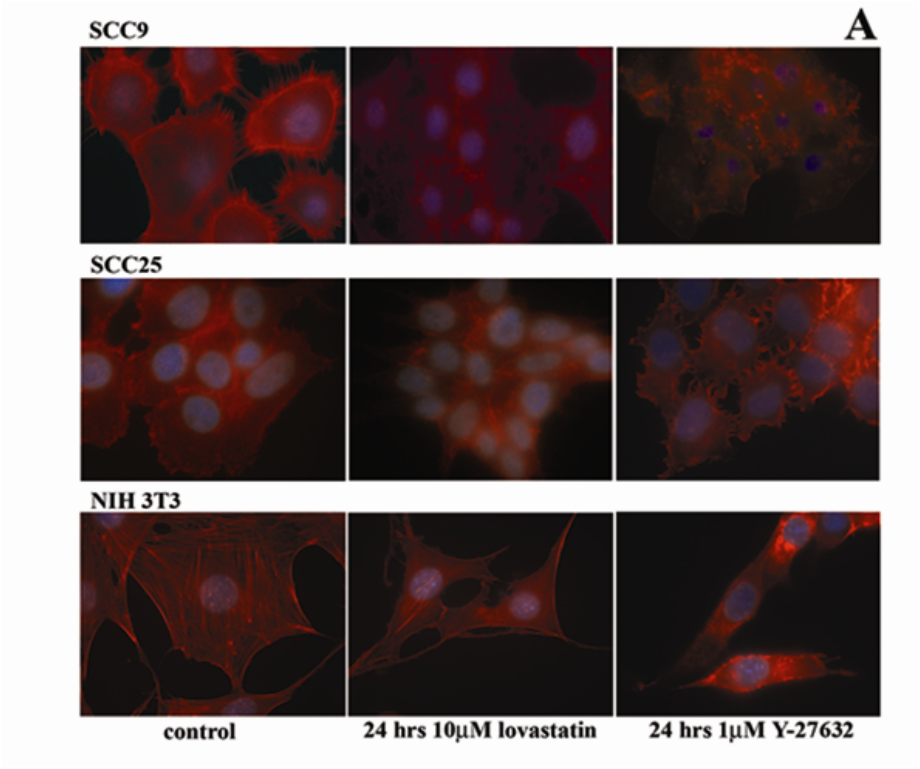
the anti-phosphotyrosine antibody specific for amino acid residue at 1068 of the EGFR showed similar results (Figure 4A). We further examined the effects of adding back mevalonate or GGPP on EGFR dimerization. Mevalonate (100μM) and GGPP (10μM) addition to lovastatin-treated (10μM, 24hrs) SCC9 or SCC25 cells restored EGF induced EGFR dimerization (Figure 4B). These results indicate the ability of lovastatin to inhibit the dimerization/activation of EGFR is through its targeting of the mevalonate pathway and that this effect is likely regulated by geranylgeranylated proteins.

Lovastatin induces cytoskeletal disorganization with increased expression of inactive rhoA

The Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) family, composed of ROCK and the closely related p160ROCK, are downstream targets of rhoA that transducer rho activation into stress fiber formation and assembly of focal adhesions [28]. In this study, we employed a specific inhibitor of the ROCK family kinases, Y-27632 [29]. Actin architecture was visualized with rhodamine-conjugated phalloidin (which has a high affinity for F-actin) following 10μM lovastatin and 1μM Y-27632 treatment for 24hrs in SCC9, SCC25 and NIH3T3 murine fibroblasts. Although the pattern of actin staining differed, stress fiber and focal adhesions were inhibited by both agents and particularly evident in NIH3T3 cells (Figure 5A).

The geranylgeranylated rho proteins regulate actin cytoskeleton and agents that affect actin polymerization have been shown to inhibit EGFR function as well [30]. RhoA, rac1 and cdc42 play significant roles in actin cytoskeleton reorganization [31]. In SCC9 and SCC25 cells, 24hr treatment with 10, 20 and 50μM lovastatin induced significant expression of rhoA and cdc42 but not rac1 by Western blot analysis (Figure 5B). The induction of rhoA and

Chapter 3 Figure 5. Lovastatin induces cytoskeletal disorganization with increased expression of inactive rho family of proteins. A, Immunofluorescence microscopy to examine the effect of lovastatin and Y-27632 on actin cytoskeletal organization in SCC9, SCC25 and NIH3T3 cells. Rhodamine phalloidin staining of solvent control, 10μM lovastatin or 1μM Y-27632 for 24 hours. Nuclei were counterstained with DAPI. Original magnification, x400. B, Western blot analysis of rhoA, rac1 and cdc42 protein expression levels in lovastatin treated SCC9 and SCC25 cell lines for 24hrs. Treatments with 100μM mevalonate or 10μM GGPP with or without 20μM lovastatin were also evaluated. Expression levels of actin were assayed as the loading control. C, Western blot analysis of cyclinD1 expression levels in 10-50μM lovastatin treated SCC9 and SCC25 cell lines for 24hrs. In SCC25 cells, treatments with 100μM mevalonate or 10μM GGPP with or without 20μM lovastatin was also evaluated. Expression levels of actin were assayed as the loading control. D, 2D-gel electrophoresis of SCC9 cells were treated with control and 25μM lovastatin for 24hrs. After isoelectric focusing, the protein extracts were western blotted for expression of rhoA. Equal concentrations of total protein were used for each condition. E, Rho A activation assay. Serum starved SCC9 and SCC25 cells were treated with 10μM lovastatin, 100μM mevalonate and 10μM GGPP alone and in combination as indicated for 24hrs. Cells were stimulated with EGF for 30min as indicated and assayed for rhoA activity employing the RhoA G-LISA kit that quantifies activated GTP loaded rhoA through colorimetric detection of rhoA bound to Rho-GTP-binding protein. In all experiments, error bars indicate standard deviation.


SCC9
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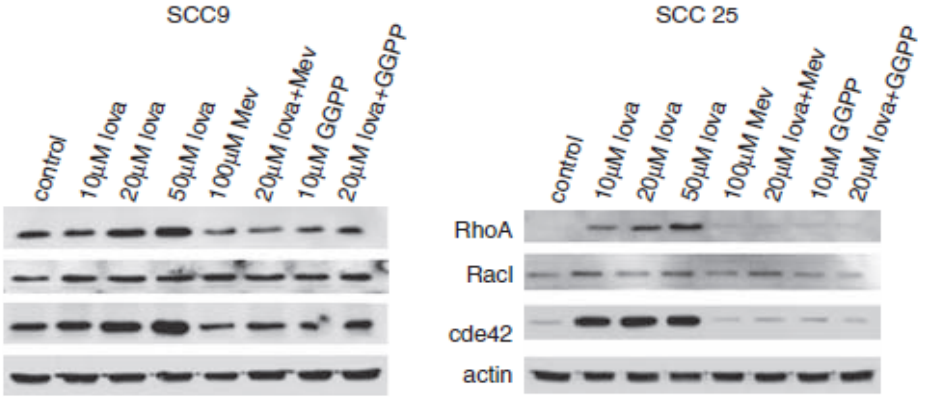
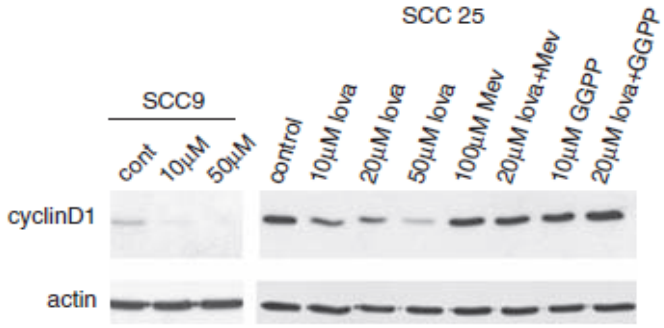
SCC25
NIH 3T3
control
24 hrs 10μM lovastatin
24 hrs 1μM Y-27632

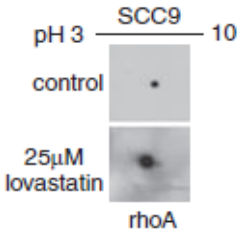
B


SCC9
control
10μM lova
20μM lova
50μM lova
100μM Mev
20μM lova+Mev
10μM GGPP
20μM lova+GGPP
SCC 25
RhoA
Racl
cde42
actin

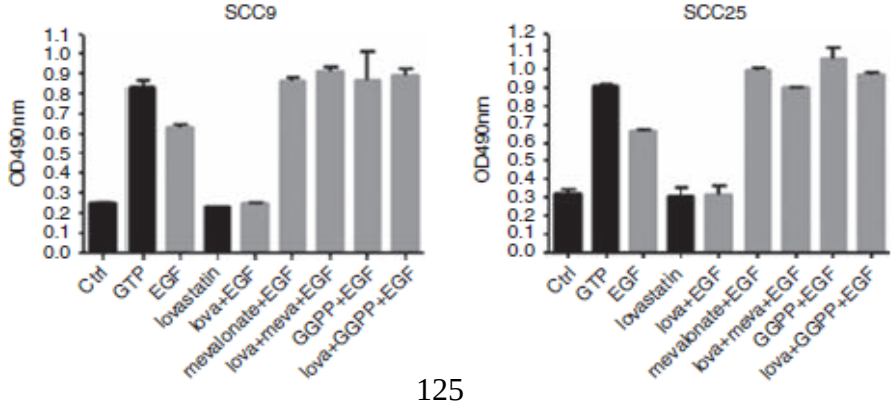
C


SCC 25
SCC9
cont
10μM
50μM
control
10μM lova
20μM lova
50μM lova
100μM Mev
20μM lova+Mev
10μM GGPP
20μM lova+GGPP
cyclinD1
actin

D


pH 3
SCC9
10
control
25μM
lovastatin
rhoA

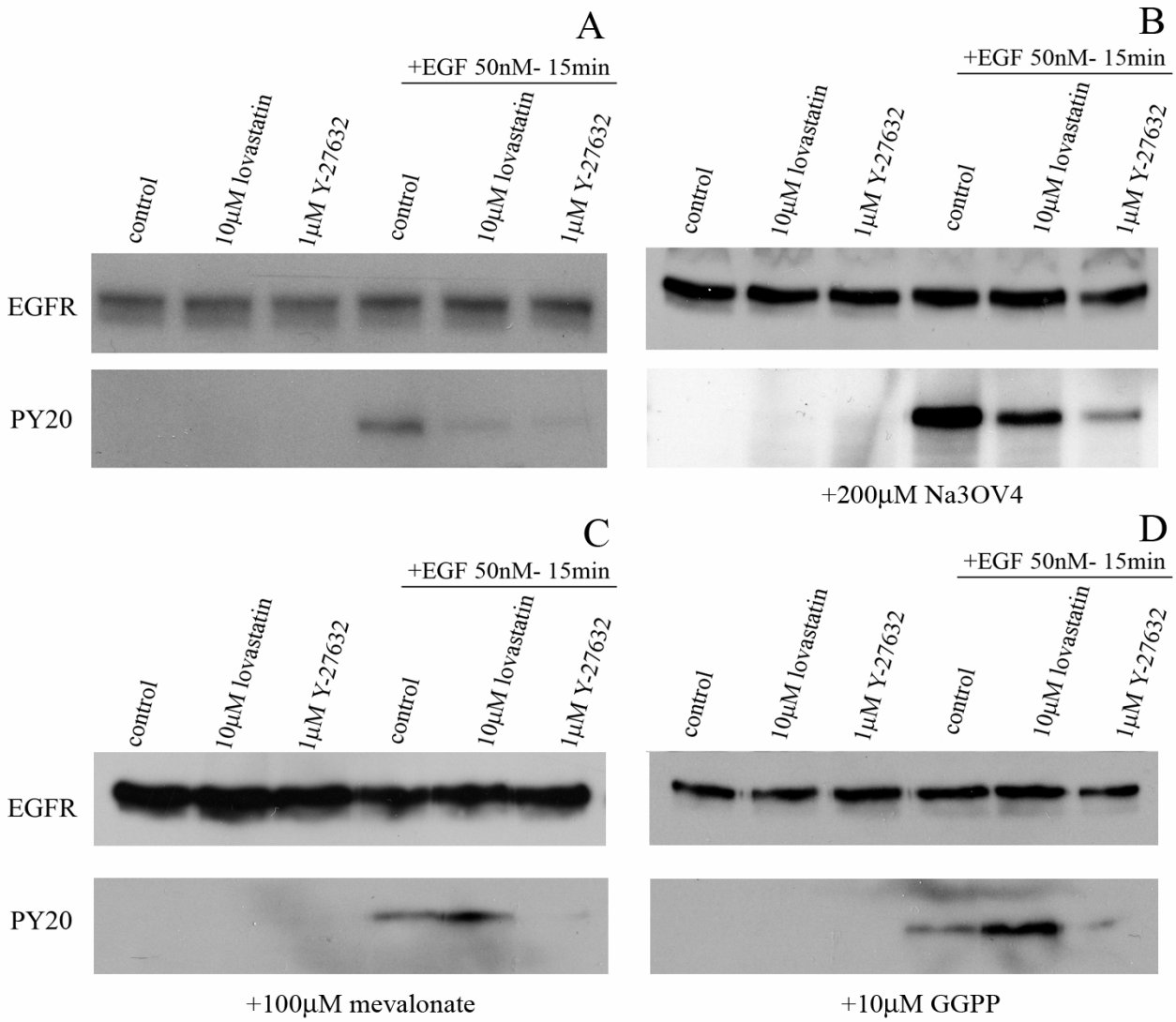
E

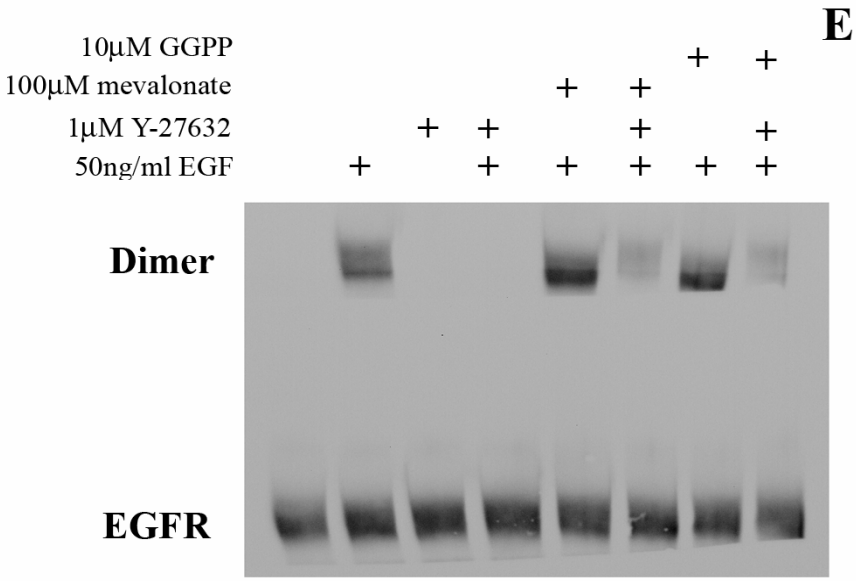

SCC9
SCC25
OD490nm
Ctrl
GTP
EGF
lovastatin
lova+EGF
mevalonate+EGF
lova+meva+EGF
GGPP+EGF
lova+GGPP+EGF

cdc42 expression in these cell lines was reversed by the co-administration of mevalonate and GGPP (Figure 5B). Activation of rho proteins play a role in cell cycle progression through cyclin D1 up-regulation [16]. In SCC9 and SCC25 cell lines, 24hr lovastatin treatment at 10, 20 and 50μM displayed a dose dependant down-regulation of cyclin D1 protein (Figure 5C). This effect was reversed by the co-administration of mevalonate and GGPP. Using 2D gel electrophoresis, we demonstrated that lovastatin-induced rhoA showed a slight shift in isoelectric focusing than the rhoA in non-treated cells (Figure 5D). These results mirror previously published data where rhoA lacked GGPP modification [32]. Furthermore, employing a colorimetric rhoA activation assay, we determined the effect of lovastatin on EGF induced rhoA activation in SCC9 and SCC25 cells. Serum starved cell extracts represent inactive levels of rhoA while 0.2M GTP loaded extracts represent fully active rhoA. As expected EGF stimulation induced rhoA activity to approximately 70% of the GTP loaded activity. Lovastatin (10μM, 24hrs) inhibited EGF induced rhoA activation in both SCC9 and SCC25 cells while co-administration of mevalonate (100μM) and GGPP (10μM) reversed the inhibitory effects of lovastatin (Figure 5E). These results demonstrate that lovastatin-induced rhoA is inactive likely due to the lack of GGPP modification.

Y-27632 inhibits EGF induced EGFR dimerization and activation. To assess the targeting of rhoA/ROCK pathway on EGF induced EGFR activation, we evaluated the effect of Y-27632 treatment. Western blot analysis was employed to compare the effects of 10μM lovastatin treatment for 24hrs and 1μM Y-27632 treatment for 24hrs in SCC9 cells. Both agents had no effect on endogenous EGFR protein levels (Figure 6). However, Y-27632 treatment of SCC9 cells displayed a similar inhibitory effect to lovastatin on EGF induced EGFR activation as demonstrated by decreased levels of phosphotyrosine (PY20)

Chapter 3 Figure 6. Y-27632 inhibits EGF induced EGFR activation. A, Western blot analysis of SCC9 cells treated with solvent control, 10μM lovastatin and 1μM Y-27632 for 24hrs with or without 50nM EGF stimulation for 15min prior to protein extraction. Total EGFR expression (upper panel) was evaluated as well as total phosphotyrosine levels of EGF stimulated EGFR (PY20). Using similar treatments, co-administration of 200μM sodium orthovanadate (Na_3VO_4) (B), 100μM mevalonate (C) and 10μM GGPP (D) were also evaluated. E, SCC9 cells treated with control and 1μM Y-27632 for 24hrs with or without mevalonate and GGPP and were stimulated with 50ng/ml of EGF for 30min as indicated. Cells were washed with ice-cold PBS and incubated on ice for 30 minutes with the cross-linking reagent bis (sulfosuccinimidyl) suberate (3 mM in PBS). Dimers were visualized as >300kD bands. Y-27632 treatment significantly decreased the amount of EGFR dimers formed.


A
+EGF 50nM- 15min
control
10μM lovastatin
1μM Y-27632
control
10μM lovastatin
1μM Y-27632
EGFR
PY20
B
+EGF 50nM- 15min
control
10μM lovastatin
1μM Y-27632
control
10μM lovastatin
1μM Y-27632
+200μM Na3OV4
C
+EGF 50nM- 15min
control
10μM lovastatin
1μM Y-27632
control
10μM lovastatin
1μM Y-27632
EGFR
PY20
+100μM mevalonate
D
+EGF 50nM- 15min
control
10μM lovastatin
1μM Y-27632
control
10μM lovastatin
1μM Y-27632
+10μM GGPP


E
10μM GGPP
100μM mevalonate
1μM Y-27632
50ng/ml EGF
Dimer
EGFR

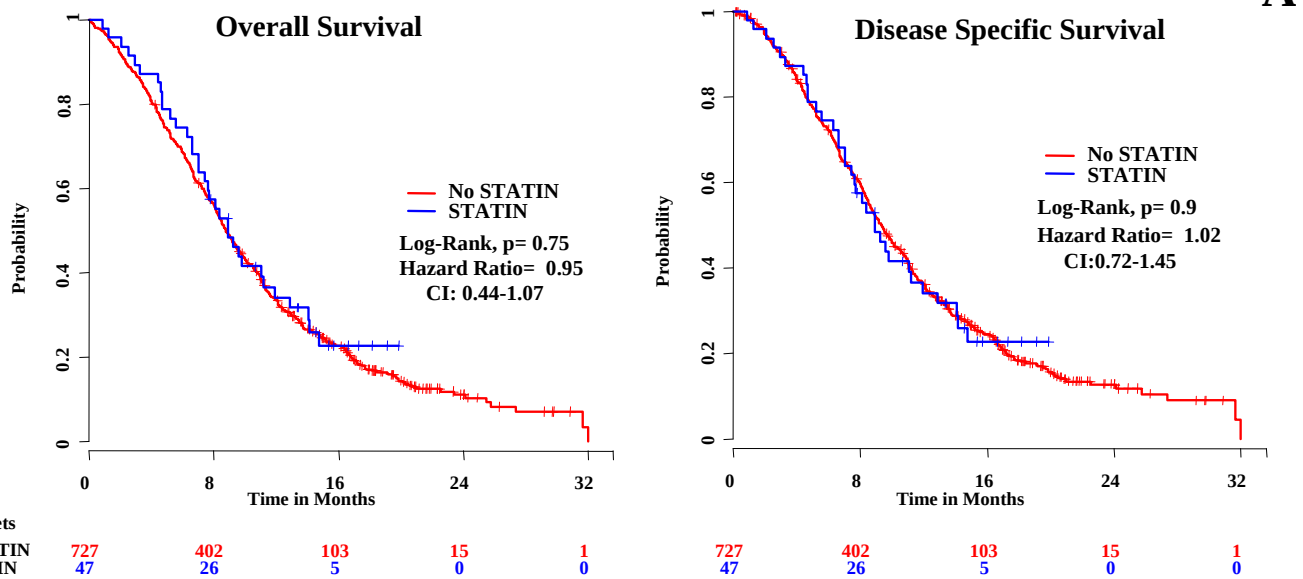
(Figure 6A). Both lovastatin and Y-27632 treatments were unaffected by co-treatment with the phosphatase inhibitor sodium orthovanadate (Na_3VO_4) [33] (Figure 6B). Mevalonate (100μM) and GGPP (10μM) co-administration inhibited the effects of lovastatin on EGF induced EGFR activation but not Y-27632 treated SCC9 cells (Figure 6C and D). These interesting results lead us to further investigate the role of Y-27632 on EGFR dimerization. SCC9 cells treated with control and 1μM Y-27632 for 24hrs were stimulated with 50ng/ml of EGF for 30min as indicated. In SCC9 cells, Y-27632 treatment significantly decreased the EGFR dimer formation and this effect was not rescued by the co-administration of mevalonate or GGPP (Figure 6E). These results suggest a model whereby lovastatin induces actin disorganization through inhibition of geranylgeranylation of rho family of proteins resulting the inhibition of EGFR dimerization, activation and downstream signalling.

Evaluating statin use in patients with advanced NSCLC. In exploratory analyses, the association between statin use and overall survival (OS) and disease specific survival (DSS) in patients with advanced NSCLC was assessed in the NCIC CTG BR.18 [34] and BR.21 [35] clinical trials. BR.18 was a randomized phase III trial of carboplatin and paclitaxel chemotherapy with or without the matrix metalloproteniase inhibitor BMS 275291. BR.21 was a placebo controlled randomized phase III trial of erlotinib in patients with advanced NSCLC after chemotherapy failure. A significant improvement in OS for patients randomized to the erlotinib arm was demonstrated (HR=0.70, log rank $p<0.001$). In BR.18 and BR.21, the number of patients that received statin at or after randomization was 47 and 28 respectively. In BR.18, there was no difference in OS or DSS (Figure 7A) for the statin versus no-statin cohorts (OS: HR=0.95, log rank p=0.75; DSS: HR=1.02, log rank p=0.90). In BR.21, there was a trend for patients in the statin cohort to have an improved OS and DSS

Chapter 3 Figure 7. Analysis of statin use in disease specific survival in patients enrolled in the BR18 (carboplatin/paclitaxel) and BR21 (erlotinib) Phase III clinical trials. Kaplan-Meier curves for OS and DSS comparing statin use to no statin use in the BR18 (A) and BR21 (B) clinical trials. The hazard ratios and log rank test for significance were performed. In the erlotinib treated patients in the BR21 trial, statin use appeared to provide enhanced OS and DSS compared to patients with no statin treatments

BR.18

A

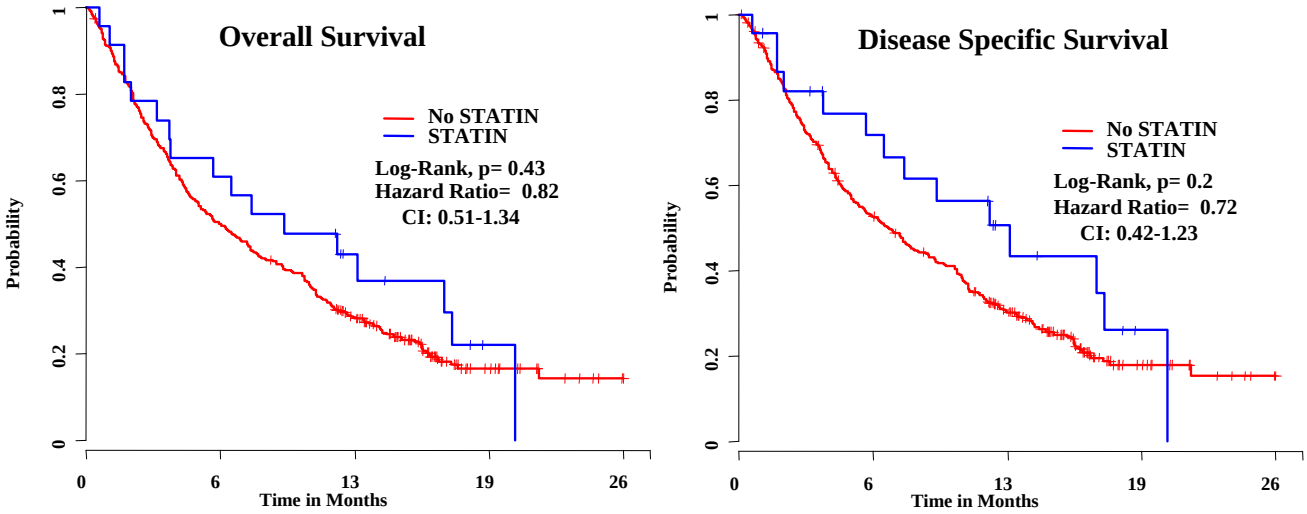


Risk Sets					
No STATIN	727	402	103	15	1
STATIN	47	26	5	0	0

727	402	103	15	1
47	26	5	0	0

BR.21 (Erlotinib Arm)

B



Risk Sets					
No STATIN	465	231	109	15	1
STATIN	23	14	7	1	0

465	231	109	15	1
23	14	7	1	0

(data not shown) compared to the no-statin cohort (OS: HR=0.69, log rank p=0.098; DSS: HR=0.62, log rank p=0.048). To explore this further, the outcome of patients on statins randomized to the erlotinib and placebo arm were evaluated separately. These analyses were limited by the small number of patients on statins in both arms (n=23 and n=5 respectively), however, for patients in the statin cohort randomized to the erlotinib arm, there was a non-significant trend for improvement in OS (HR=0.82, log rank p=0.43) and DSS (HR=0.72, log rank p=0.20) (Figure 7B) compared with patients without statins. However, the statin cohort randomized to the placebo arm of BR21 also had a non-significant improvement, although with only 5 patients the significance of this is uncertain.

Discussion

In this study, we are the first to demonstrate that the inhibitory effects of lovastatin on EGF induced EGFR activation was due to its ability to inhibit receptor dimerization. The inhibitory effects of lovastatin on EGFR dimerization were mediated through targeting of the mevalonate pathway. Furthermore, lovastatin has significant effects on SCC actin architecture and inhibiting rhoA similarly affected EGFR dimerization and activation. Actin organization has been shown to play a role in EGFR localization and activation [30]. This represents a novel mechanism by which lovastatin regulates this important therapeutic target. In a number of malignancies, including SCC, EGFR and its downstream signalling pathways are often deregulated, leading to cell hyper-proliferation, enhanced cell survival and increased metastatic potential [3]. Of particular importance in cancer therapy is the hyper-activation of the PI3K/AKT pathway that regulates cell survival, cellular metabolism and protein translation [11]. In this study, we confirmed that lovastatin treatment inhibits EGF induced AKT activation and its targets that regulate protein translation S6K1 and 4EBP-1 [11]. SCC patients have very limited treatment options when presenting as metastatic disease [2] and inhibitors of EGFR have limited therapeutic efficacy. Employing EGFR targeting agents requires combination-based therapies to enhance their clinical activity.

Our recent work has demonstrated that the depletion of mevalonate metabolites affects the activity of a number of cell signalling cascades including inhibitory effects on EGFR activity and their downstream signalling pathways [19, 27]. Combinations of statins with agents targeting EGFR activity, like gefitinib, induce a potent synergistic cytotoxic response in a variety of responsive tumor types including SCC [19]. Combining these two approaches with each targeting the EGFR through distinct mechanisms represents a novel

combinational therapeutic approach in SCC as well as other cancers where EGFR is involved in their pathogenesis [36]. Rationally designed combination therapies represent a potential strategy to improve their efficacy. Based on the data presented herein, we demonstrated that lovastatin is a potent inhibitor of AKT activation by targeting EGFR in a novel inhibitory mechanism and may represent a novel combinational therapeutic approach in combination with EGFR-TKIs. To evaluate the feasibility of this approach, although not statistically significant, we showed that erlotinib treated patients in the BR21 Phase III trial with statin use showed a trend to enhanced OS and DSS compared to patients with no statin use. While this data is intriguing, due to the small number of patients taking statin therapy, interpretation of these results is limited. Furthermore, it is not possible to determine if statin use has prognostic implications, or whether patients on statin and erlotinib have an incremental benefit compared to erlotinib alone. This requires further evaluation in another independent data set and/or a prospectively designed clinical trial. In fact, based on our studies, we are currently evaluating erlotinib and rosuvastatin in a Phase I/II clinical trial in NSCLC patients at our institute (ClinicalTrials.gov Identifier**:** NCT00966472) to address potential clinical utility of this approach.

In this study, we have demonstrated the ability of lovastatin to inhibit ligand induced receptor dimerization, internalization, autophosphorylation and AKT activation. Depletion of geranylgeranylated proteins due to inhibition of mevalonate pathway end products by lovastatin leads to actin disorganization is likely the mechanisms that regulates lovastatin's inhibition of RTK activity and its downstream effects. The ability of lovastatin to inhibit EGFR dimerization and internalization is intriguing and requires further study to elucidate its mechanism and possible clinical implications.

Acknowledgements

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Conflict of Interest

Authors declare that there are no competing financial interests in relation to the work described.

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CHAPTER 4

Lovastatin inhibits VEGFR and AKT activation: Synergistic cytotoxicity in combination with VEGFR inhibitors

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* These authors made equal contributions to this work.

Key Words: vascular endothelial growth factor receptor, mevalonate pathway, rho GTPases, lovastatin

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Author contributions : TTZ, DT and JD wrote the manuscript. TTZ, DT designed experiments. TTZ, DT (part of Figure 1, 4 and 6) performed experiments. CLA provided reagents.

Abstract

Background:

In a recent study, we demonstrated the ability of lovastatin, a potent inhibitor of mevalonate synthesis, to inhibit the function of the epidermal growth factor receptor (EGFR). Lovastatin attenuated ligand-induced receptor activation and downstream signalling through the PI3K/AKT pathway. Combining lovastatin with gefitinib, a potent EGFR inhibitor, induced synergistic cytotoxicity in a variety of tumor derived cell lines. The vascular endothelial growth factor receptor (VEGFR) and EGFR share similar activation, internalization and downstream signalling characteristics.

Methodology/Principal Findings:

The VEGFRs, particularly VEGFR-2 (KDR, Flt-1), play important roles in regulating tumor angiogenesis by promoting endothelial cell proliferation, survival and migration. As well certain tumors, such as malignant mesothelioma (MM), express both the VEGF ligand and VEGFRs that act in an autocrine loop to directly stimulate tumor cell growth and survival. In this study, we have shown that lovastatin inhibits ligand-induced VEGFR-2 activation through inhibition of receptor internalization and also inhibits VEGF activation of AKT in human umbilical vein endothelial cells (HUVEC) and H28 MM cells employing immunofluorescence and Western blotting. Combinations of lovastatin and a VEGFR-2 inhibitor showed more robust AKT inhibition than either agent alone in the H28 MM cell line. Furthermore, combining 5μM lovastatin treatment, a therapeutically relevant dose, with two different VEGFR-2 inhibitors in HUVEC and the H28 and H2052 mesothelioma derived cell lines demonstrated synergistic cytotoxicity as demonstrated by MTT cell viability and flow cytometric analyses.

Conclusions/Significance:

These results highlight a novel mechanism by which lovastatin can regulate VEGFR-2 function and a potential therapeutic approach for MM through combining statins with VEGFR-2 inhibitors.

Introduction

Angiogenesis is an important physiological process during fetal development and growth as well as in mature tissue remodeling and repair [1]. For cancer expansion and dissemination, both primary lesions and metastatic tumors must develop a new vascular supply in order to survive [1]. Angiogenesis is tightly regulated by balancing the activity of pro- and anti-angiogenic factors [2]. Multiple pathways contribute to tumor angiogenesis including vascular endothelial growth factor (VEGF), fibroblast growth factor, and platelet-derived growth factor [2]. Based on the central role of VEGF in tumor angiogenesis and growth, it has emerged as a promising therapeutic target for angiogenesis inhibition [3]. VEGF, a 35- to 45-kDa dimeric polypeptide, plays a critical role in normal and pathologic angiogenesis [3]. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factors 1 and 2 [4]. The VEGF-A gene, via alternative splicing, yields several isoforms, of which, $VEGF_{165}$ plays a critical role in tumor angiogenesis [3]. Tumor cells secrete VEGF in response to many stimuli including hypoxia, low pH, or cellular stress, which are prevalent in most solid tumors [5].

VEGF exerts its biologic effect through interaction with receptors present on the cell surface. These receptor tyrosine kinases (RTK) include VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1), which are predominantly present on vascular endothelial cells [6]. Both VEGFR-1 and VEGFR-2 have an extracellular ligand binding domain, a transmembrane region, and a tyrosine kinase domain [2,3]. In addition, VEGFR-3 (Flt-4) is expressed on vascular and lymphatic endothelium while the neuropilin receptor is expressed on vascular endothelium and neurons [2,3]. VEGFR-2 is the main receptor responsible for mediating the proangiogenic effects of VEGF in tumor-associated endothelium [7]. VEGF binding to the

extracellular domain of the VEGFR results in dimerization and autophosphorylation of the intracellular tyrosine kinases [8]. This activates multiple downstream proteins that play functional roles in cell survival, proliferation, vascular permeability and stabilization of new blood vessels [8]. For example, VEGF induces endothelial cell proliferation by activating the protein kinase Ras-MEK-ERK pathway [8]. The pro-survival effects of VEGF/VEGFR-2 are mediated by the PI3K/AKT pathway [8]. Recent studies indicate that VEGFR are also expressed by some tumor cells and may represent an additional target [9].

Malignant mesothelioma (MM) is a highly aggressive tumor that arises from the surface serosal cells of the pleura and, less frequently, the peritoneum [10]. A strong link has been established between exposure to asbestos and increased risk for MM [11]. Treatment of MM with surgery, chemotherapy, or radiation therapy is rarely curative and median survival is in the range of 10–17 months [11]. Novel therapies for MM are needed. VEGF up-regulation appears to play an important role in mesothelial cell transformation. High levels of VEGF have been observed in the serum of MM patients and elevated pleural effusion VEGF levels are associated with poor survival in patients with MM [12]. VEGF may also act in a functional autocrine loop capable of directly stimulating the growth of MM cells [9]. MM cell lines express elevated levels of both VEGF and the VEGFR-1 and 2 compared with normal mesothelial cells [9]. VEGF activated these receptors and increased proliferation of all MM cell lines examined [9]. Interestingly, significant vascularization is rarely exhibited in MM suggesting that VEGF may play a key role in MM tumor progression by primarily regulating tumor cell proliferation suggesting VEGF/VEGFR as therapeutic targets in MM [10].

The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate, which is catalyzed by HMG-CoA reductase [13]. The mevalonate pathway produces various end products that are critical for many different cellular functions including cholesterol, dolichol, ubiquinone, isopentenyladenine, geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP) [13]. Geranylgeranyl transferase and farnesyl transferase use GGPP and FPP, respectively, for post-translational modifications of a wide variety of cellular proteins including the Ras, Rab, and Rho families [14,15]. These proteins regulate cell proliferation, intracellular trafficking and cell motility and this post-translational modification functions as a membrane anchor critical for their activity [14,15]. Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products [16] and, thus, may have significant influences on many critical cellular functions.

Malignant cells appear highly dependent on the sustained availability of the end products of the mevalonate pathway [17]. The statin family of drugs are potent inhibitors of HMG-CoA reductase that are widely used as hypercholesterolemia treatments [16]. Mevalonate metabolites are required for the proper function and localization of a number of downstream mediators of the VEGFR-2 signalling cascade [3,18,19,20]. Proteins that require FPP or GGPP posttranslational modifications play critical roles in transducing these signals [3,18,19,20]. In our recent studies, we have demonstrated that lovastatin treatment inhibits ligand-induced activation of EGFR [18,21]. The mechanism by which EGFR inhibition is mediated by lovastatin is novel and suggests a previously unrecognized process controlling EGFR activity.

Due to the potential of lovastatin to target EGFR function and its downstream signalling, we previously evaluated the effects of combining lovastatin with the clinically relevant EGFR tyrosine kinase inhibitor (TKI) gefitinib [22]. The combination of gefitinib and lovastatin demonstrated significant co-operative cytotoxic effects when cells were pretreated with lovastatin for 24hrs. At this time point, lovastatin demonstrated significant inhibition of EGFR function [21]. We demonstrated co-operative cytotoxic effects with this combination that was synergistic due to the induction of a potent apoptotic response [21]. In this study, we evaluated the potential of lovastatin to similarly inhibit VEGFR-2 function. Furthermore, we evaluated the effects of lovastatin on endothelial cell proliferation and survival as well as the effects of combining lovastatin with VEGFR-TKIs on MM tumor cell viability as a potential novel therapeutic approach.

Materials and Methods

Tissue Culture

Human Umbilical Vein Endothelial Cells (HUVEC) (Clonetics, lot 2F1276, Walkersville, MD) were maintained in EGM-2 medium supplemented with 2% fetal bovine serum provided in the EGM-2 Single Quot Kit Supplements and Growth Factors (Lonza, East Rutherford, NJ). The human mesothelioma lines, NCI-H28 and NCI-H2052, were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in HyQ DMEM/High Glucose (HyClone, Logan, Utah) supplemented with 10% fetal bovine serum (Medicorp, Montreal, QC). The cell lines used in this study were exposed to solvent control or lovastatin (provided by Apotex, Mississauga, ON; diluted from a 10mmol/L stock in ethanol), or human recombinant $VEGF_{165}$ (provided by National Cancer Institute, Rockville, MD; reconstituted to a 50mg/ml stock in deionized water) at a concentration of 50ng/ml. The mesothelioma cell lines were exposed to solvent control or VEGFR-2 Inhibitor V, ZM323881, or VEGFR-TKI III, KRN633 (Calbiochem; both reconstituted to a 1mmol/L stock in DMSO). The siRNA oligonucleotides used in this study were purchased from Dharmacon (Boulder, CO). siControl: siGENOME non-targeting siRNA, siVEGFR-2: siGENOME SMARTpool human KDR. Transfection procedures were performed with DharmaFECT-4 reagent (Dharmacon) in both H28 and H2052 cells according to the manufacturer's protocols. Cells were grown on 6-well plates or 96-well plates and transfected with 50nM of the siRNAs. After two days incubation, cells were treated with medium or 10μM lovastatin for another 48hrs. The cytotoxic effects of lovastatin remained consistent in all three cell lines throughout the course of these experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay (MTT Assay)

In a 96-well, flat-bottomed plate (Fisher, Mississauga, ON), ~7500 cells/ 150µl of cell suspension were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following a 48- or 72-hr treatment of lovastatin, ZM323881, KRN633, or a combination of lovastatin and a VEGFR-TKI, 42µL of a 5mg/ml solution in PBS of the MTT substrate (Sigma) was added and incubated for up to one hr at 37°C. The resulting blue-brown formazan precipitate formed was solubilized by the addition of 84µL of a 0.01M HCl/10%SDS (Sigma) solution and incubated for 8hrs at 37°C. The plates were then analyzed on a Dynex Technologies MRX Microplate Reader at 570nm using the Revel software (Dynex Technologies, Chantilly, VA) to determine the absorbance of the samples. Treatments were performed in replicates of six and the means expressed as the percent viability relative to the untreated control (100% viable). Statistical analysis: Combination Index (C.I.) was determined by the method of Chou and Talalay as previously described [23]. P values were determined by standard paired T-test evaluations.

Cell Viability Assay

In a 6-well flat-bottomed plate (Fisher), ~500000 HUVEC were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following 72hr treatment using solvent control or lovastatin in the presence or absence of 50ng/ml $VEGF_{165,}$ the cells were trypsinized and collected. The number of viable cells in 500µl of each sample was subsequently counted on the Beckman Coulter Vi-Cell-XR Cell Viability Analyzer (Mississauga, ON). Treatments were performed in triplicates. Data were normalized to the untreated control.

Propidium Iodide Flow Cytometry

In 10-cm plates (Fisher), ~3.5 x 10^5 mesothelioma cells or ~5 x 10^5 HUVEC were used to seed each plate. The plates were incubated overnight to allow for cell attachment and recovery. The HUVEC were treated with solvent control or lovastatin, in the presence or absence of 50ng/ml $VEGF_{165}$ for 72hrs. The mesothelioma cells were treated with solvent control or lovastatin. Following a 24-hour pre-treatment with lovastatin alone, solvent control or VEGFR Inhibitor (KRN633 or ZM323881) was added for an additional 48hrs. After the desired treatment length, the media, PBS wash and trypsinized cells were collected in the same 50mL conical tube. The collected cells were fixed with 80% ethanol and incubated at -20ºC for a minimum of 24hrs. The cells were washed once then resuspended in staining buffer containing 50μg/ml propidium iodide (Sigma) and 100μg/ml RNaseA (Invitrogen, Carlsbad, CA). Ten thousand cells were evaluated using the Beckman Coulter Epics XL Flow Cytometer and the percentage of cells in pre-G_1 phase was determined using the ModFit LT program (Verity Software House, Topsham, ME).

Western Blot Analysis

Total cellular protein was extracted using a buffer that consisted of 50mM Tris-HCl pH 7.5, 150mM NaCl, 0.25% sodium deoxycholate (Sigma), 1% IgePal, 0.1% SDS (Sigma), 1mM EDTA, 5mM sodium fluoride (Sigma), 1mM sodium orthovanadate (Sigma), and protease inhibitor cocktail (Sigma; diluted from a 10x stock). Approximately 100μL of extraction buffer was used per plate. Total protein was quantified with the BCA Protein Assay Reagents (Pierce, Nepean, ON) using bovine serum albumin (Sigma) for the standard. Protein extracts representing 50 to 100μg total protein were separated on SDS-PAGE gel using the BioRad Mini Protean 3 System (Bio-Rad Laboratories, Hercules, CA) and electro-

blotted onto Hybond P PVDF membranes (Amersham, Piscataway, NJ). Membranes were blocked in 5% skim milk powder in PBS/0.02% Tween (Sigma) for an hour at room temperature. Primary antibody, diluted in 5% skim milk powder in PBST, was incubated with the membrane overnight at 4°C. The primary antibodies used were specific for VEGFR-2, RhoA, cdc42, cyclinD1 (Santa Cruz Biotechnologies, Santa Cruz, CA); phospho-AKT, AKT, phospho-S6K1, S6K1, phospho-4EBP1, 4EBP1 (Cell Signalling Technology, Danvers, MA); and actin (Sigma). The peroxidase-conjugated AffiniPure Goat Anti-mouse/rabbit IgG (Jackson ImmunoResearch, West Grove, PA) secondary antibodies were applied at a 1:5000 dilution and the peroxidase-labeled Affinity Purified Antibody to goat IgG (KPL) secondary antibody was applied at a 1:1000 dilution in 5% skim milk powder in PBST and incubated for a minimum of an hour at room temperature then processed for detection with the Supersignal West Pico Chemiluminescent Substrate (Pierce), using the Gene GNOME Imager and Genesnap Imaging Software (Syngene, Frederick, MD). After the desired exposure was obtained, the membrane was stained with Ponceau Red (Fisher) to ensure equal loading of the samples. Membranes were stripped using Restore Western Stripping Buffer (Pierce, Nepean, ON) to allow for a second probing.

Pinpoint Cell Surface Protein Labeling

The Pinpoint Cell Surface Protein Isolation Kit (Pierce) was used to identify and isolate cell surface proteins following the manufacturer's instructions. In brief, control or 24hrs lovastatin treated HUVEC cells were stimulated with or without 50ng/ml of VEGF for 30min. Cells were then washed with ice-cold PBS and surface proteins were biotinylated and isolated using immobilized avidin, prior to Western blot analysis of VEGFR-2 and actin levels as described above.

Phalloidin Staining/ Immunofluorescence

In a 6-well flat-bottomed plate (Fisher), glass cover slips (Fisher) were placed into each well and ~250000 cells were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following a 24hr treatment of solvent control or lovastatin in serum-free media, the HUVEC cells were treated with recombinant human$VEGF_{165}$ for 30 min prior to fixation. The cells were subsequently washed with PBS then fixed with 4% paraformaldehyde (Sigma) buffered in PBS for 15 min at 37°C and stored in PBS at 4°C. To visualize actin cytoskeletal architecture, 100µL of a 1ng phalloidin-rhodamine conjugate in PBS was used to treat each cover slip containing the attached HUVEC cells for 15 min in the dark. Prior to immunofluorescence staining, the cells were permeabilized with PBS+0.2%Triton X-100 (Sigma) for 15 min. The cells were blocked for 30 min with PBS+3%FBS then incubated with the VEGFR-2 antibody at a dilution of 1:50 in PBS+3%FBS for an hr. The cells were then blocked with PBS+5% chicken serum (Sigma) for 30 min. Following the second blocking, the cells were incubated with Alexa Fluor 488 chicken anti-mouse IgG (Molecular Probes, Carlsbad, CA) at a working dilution of 10µg/ml in the dark for an hr. The cells were then mounted to a microslide with DAPI mounting medium (Vector Laboratories, Burlingame, CA) and analyzed under fluorescent microscopy using the Axiovision software (Allied High Tech Products, Rancho Dominguez, CA).

Rho A Activation Assay

The HUVEC and H28 cell lines were cultured in serum free medium treated with 10µM lovastatin for 24hrs with or without 100µM mevalonate or 10µM GGPP. Cells were stimulated with 50ng/ml EGF for 30min to activate rhoA. Cell lysates were either snap frozen and stored in liquid nitrogen or used directly with the RhoA G-LISA kit (Cytoskeleton,

Denver, Co) according to the manufacturer's instructions. This assay is based on the principle that a Rho-GTP-binding protein is linked to the 96-well plates. The active GTP-bound Rho in the cell lysates binds to the wells, while the inactive GDP-bound Rho is removed during the washing steps. The bound active RhoA is detected with a RhoA specific antibody and quantified by absorbance. The degree of RhoA activation is determined by comparing readings from the activated cell lysates (addition of 0.2mM GTP) versus the non-activated cell lysates (serum starved cultures).

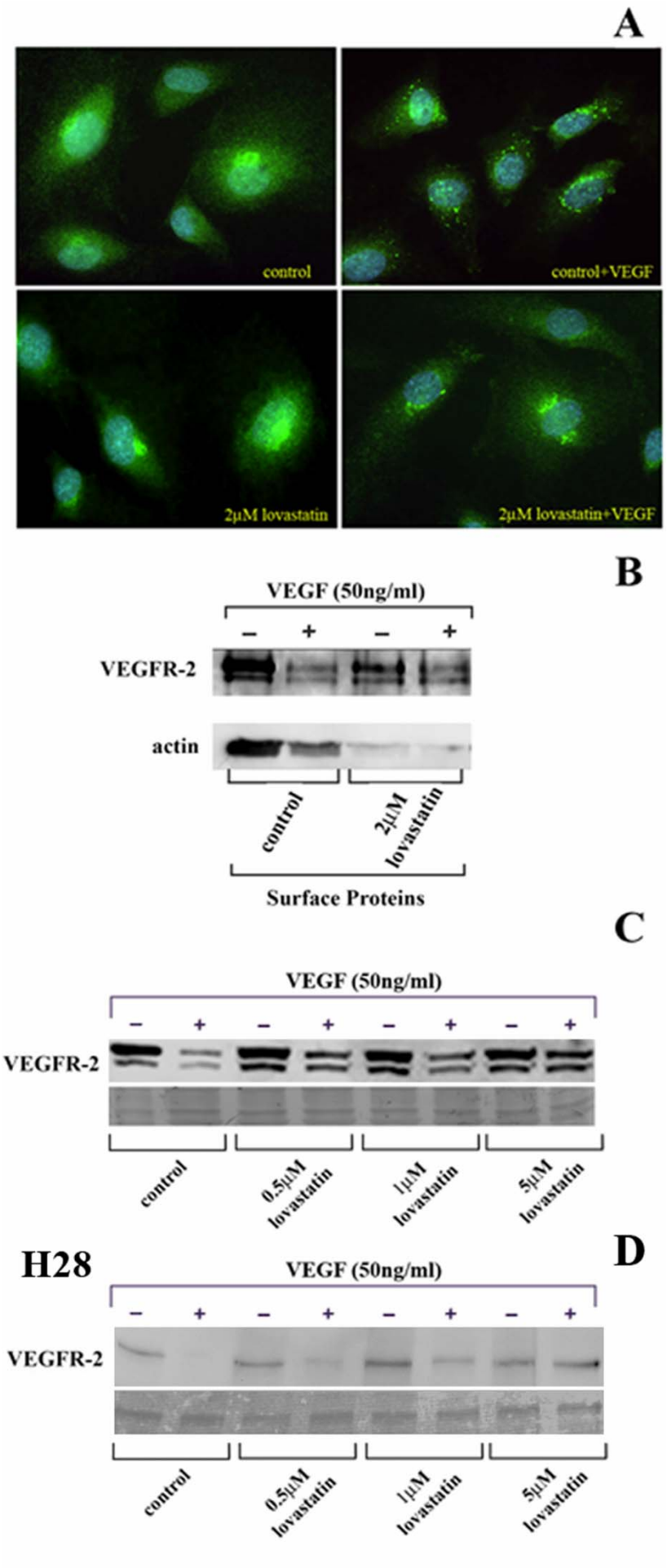
Results

Lovastatin inhibits internalization and degradation of the VEGFR-2

Previous studies have demonstrated that ligand binding to VEGFR-2 leads to receptor dimerization and autophosphorylation [8]. Autophosphorylation leads to the activation of its downstream signalling cascades and receptor internalization and degradation in lysosomes [8]. In this study, we evaluated the effect of lovastatin on VEGFR-2 internalization and degradation in VEGF treated HUVEC cells. Localization of VEGFR-2 was visualized by immunofluorescence staining. HUVEC cells were exposed to solvent control with or without treatment of 50ng/ml $VEGF_{165}$ for 30min. In un-stimulated HUVEC cells, VEGFR-2 showed a dispersed staining pattern on the cell surface. With the addition of $VEGF_{165}$, however, VEGFR-2 showed a distinct punctate intracellular staining pattern indicating efficient internalization of this receptor [24] in HUVEC (Figure 1A). Treatment of HUVEC with 2μM lovastatin for 24hrs showed a similar diffuse surface-staining pattern for VEGFR-2 as control cells. Addition of 50ng/ml of $VEGF_{165}$ for 30min in lovastatin treated cells significantly reduced the punctuate intracellular staining pattern shown in control $VEGF_{165}$ treated cells but displayed a similar diffuse staining pattern to control un-stimulated cells (Figure 1A).

To further examine whether lovastatin is regulating the internalization of the VEGFR ligand complex, we performed the Pinpoint Cell Surface Protein Isolation method that specifically labels and isolates proteins found on the cell surface. Cell surface proteins were biotinylated and isolated using immobilized avidin, prior to Western blotting with the VEGFR-2 antibody. As shown in Figure 1B, untreated HUVEC were found to have significant levels of VEGFR-2 expressed on the cell surface. As expected, stimulation with $VEGF_{165}$ at 50ng/ml for 30min decreased the levels of VEGFR-2 on the cell surface (Figure

Chapter 4 Figure 1. Lovastatin treatment inhibits VEGFR-2 internalization. A, VEGFR-2 internalization in HUVEC was evaluated by immunofluorescence. HUVEC were treated with solvent control or 2μM lovastatin for 24hrs in serum-free medium followed by 30 min of stimulation with $VEGF_{165}$. Immunofluorescence staining of HUVEC revealed a punctate intracellular staining pattern upon $VEGF_{165}$ ligand binding in the control but not in cells treated with 2μM lovastatin. The data is typical of 3 independent experiments. B, Cell Surface Pinpoint Protein Isolation revealed a decrease in VEGFR-2 on the surface of control HUVEC upon VEGF stimulation but not with 2μM lovastatin treatment. Actin was readily pulled down in control cells but not in lovastatin treated HUVEC indicating a lack of association of surface proteins with actin in lovastatin treated cells. C and D, Western blot analysis reveals that VEGFR-2 receptor levels decrease with 30 min of stimulation with $VEGF_{165}$ stimulation in control HUVEC and H28 cells respectively. Lovastatin treatments of 0.5, 1 and 5μM inhibited VEGFR degradation in a dose dependent manner. The data is typical of at least 3 independent experiments and the membranes were stained with Ponceau Red to visualize total protein loading


A
control
control+VEGF
2μM lovastatin
2μM lovastatin+VEGF
B
VEGF (50ng/ml)
– + – +
VEGFR-2
actin
control
2μM lovastatin
Surface Proteins
C
VEGF (50ng/ml)
– + – + – + – +
VEGFR-2
control
0.5μM lovastatin
1μM lovastatin
5μM lovastatin
H28
D
VEGF (50ng/ml)
– + – + – + – +
VEGFR-2
control
0.5μM lovastatin
1μM lovastatin
5μM lovastatin

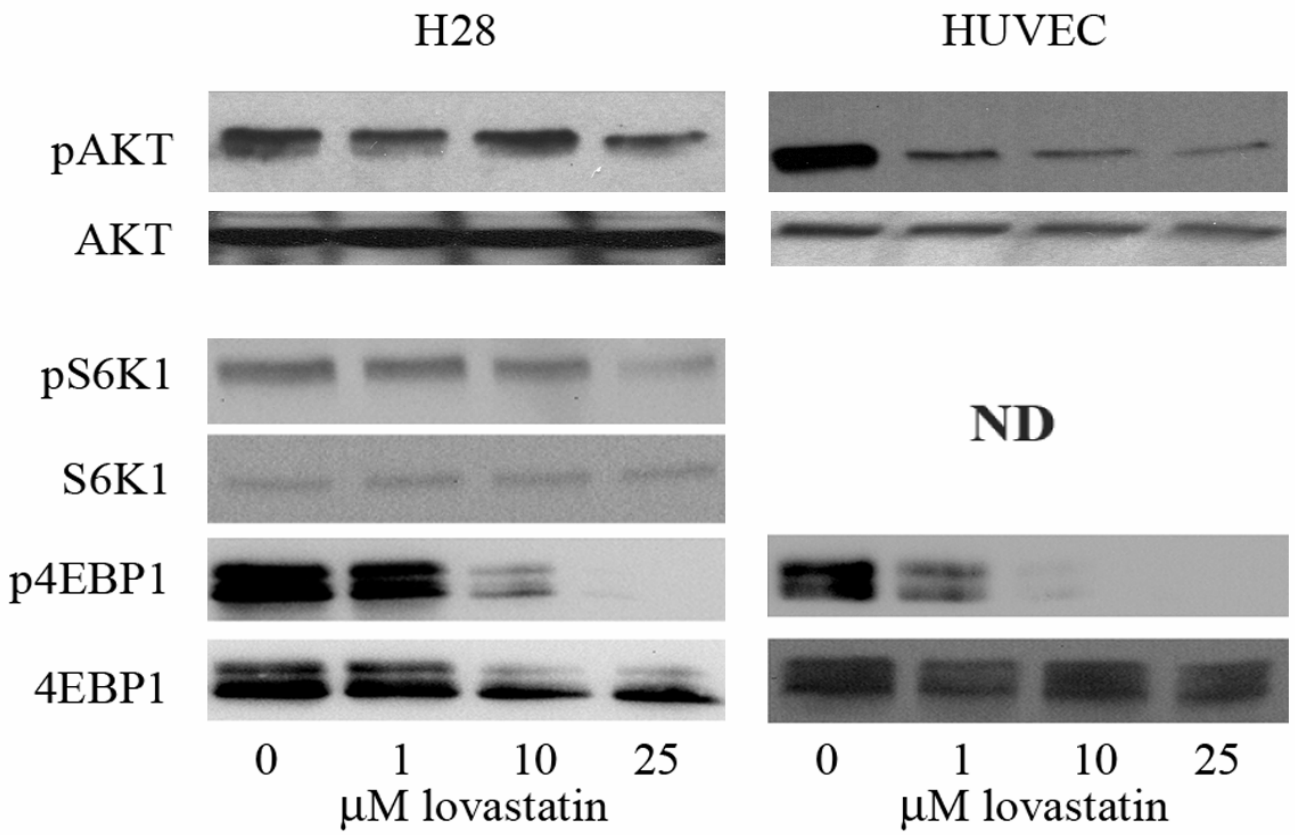
1B). In 2μM lovastatin treated cells for 24hrs, lower levels of surface expression of VEGFR were evident. This decrease may be the result of the inhibition of intracellular transport that is regulated in part by the geranylgeranylated rab protein family. Ligand stimulation did not affect VEGFR-2 surface expression in lovastatin treated cells indicative of inhibition of internalization. In untreated cells, actin was readily detected in the avidin pull downs while lovastatin treated cells had significantly lower levels (Figure 1B). These results suggest that in lovastatin treated HUVEC, surface protein binding of actin was inhibited. These results correspond well with recent studies that demonstrate a role for the actin cytoskeleton in the multi-step process of receptor internalization [25,26].

Internalization of ligand bound VEGFR-2 often leads to its degradation in lysosomes as a way to attenuate its signal. To determine the effect of lovastatin on VEGFR-2 degradation, we performed Western blot analyses of total cellular protein extracted from $VEGF_{165}$ stimulated HUVEC and H28 MM cells with or without lovastatin treatments. In HUVEC, the basal levels of VEGFR-2 were unchanged with or without 0.5, 1 and 5μM lovastatin treatments for 24hrs (Figures 1C and D). Control HUVEC cells stimulated with 50ng/ml $VEGF_{165}$ for 30min demonstrated a significant decrease in VEGFR-2 protein levels indicating efficient degradation of ligand bound VEGFR-2 in these cells (Figures 1C and D). Treatment of HUVEC with 0.5, 1 and 5μM lovastatin for 24hrs attenuated the effect of $VEGF_{165}$ addition on VEGFR-2 degradation as the levels of VEGFR-2 were significantly elevated in lovastatin-treated in comparison to control cells (Figures 1C and D). Ponceau Red staining of the membranes confirmed equal loading between samples and the area of the blot shown corresponds to the area where VEGFR-2 migrated. These results indicate that

lovastatin treatment inhibits ligand-induced internalization and degradation of VEGFR-2 in HUVEC and H28 MM cells.

Based on lovastatin's ability to inhibit ligand-induced internalization of VEGFR-2, we further evaluated the effect of lovastatin treatment on the signalling cascades triggered by VEGFR-2 activation. The PI3K/AKT signalling pathway plays a significant role in cell survival responses mediated by VEGFR-2 [3]. Ligand bound VEGFR-2 activates PI3K that phosphorylates the phospholipid PIP2 resulting in the accumulation of PIP3 that in turn activates AKT [27]. Serum starved H28 MM derived cell line and HUVEC cells were treated with 0, 1, 10 and 25μM lovastatin for 24hrs followed with 50ng/ml $VEGF_{165}$ stimulation for 30min. The functional activation of this pathway was evaluated by Western blot analysis, employing phospho-specific antibody recognizing the active form and control antibody for total AKT. Lovastatin treatment inhibited activation of AKT in a dose dependent manner that was readily detectable at the 1μM dose in HUVEC but was less efficient in inhibiting AKT activation in H28 cells (Figure 2). There are a wide variety of AKT targets that regulate its effects on protein translation, proliferation and cell survival. These targets include ribosomal S6 kinase (S6K1) and eukaryotic translation initiation factor 4E (eIF4E) that regulate translation [28]. We evaluated the effects of lovastatin on ligand-induced activation of these proteins in our 2 model cell lines. Western blot analysis determined the effects of 0, 1, 10 and 25μM lovastatin treatment for 24hrs with 30min 50ng/ml VEGF addition on these AKT targets. Lovastatin treatment significantly inhibited phosphorylation of S6K1 (not detected in HUVEC) and 4EBP1 in a dose dependent manner (Figure 2). Activated phosphorylated AKT, S6K1 and 4EBP1 were not detected in serum starved control cells (data not shown). These results demonstrate the ability of lovastatin to readily inhibit VEGF induced AKT

Chapter 4 Figure 2. Lovastatin inhibits VEGF induced activation of AKT and its downstream targets. Cell lysates from HUVEC and H28 cells were collected following control, 1, 10 and 25μM 24hr lovastatin treatments in serum-free medium with 50ng/ml 30min $VEGF_{165}$ stimulation. Phosphorylation level of AKT decreased with lovastatin treatment in a dose dependent manner. Expression level of total AKT was assayed as the loading control. Phosphorylation levels of S6K1 and 4EBP1 also decreased with lovastatin treatment in a dose dependent manner. Phosphorylated S6K1 in HUVEC cells was not detectable (ND). Expression levels of total S6K1 and 4EBP1 were assayed as the loading control.

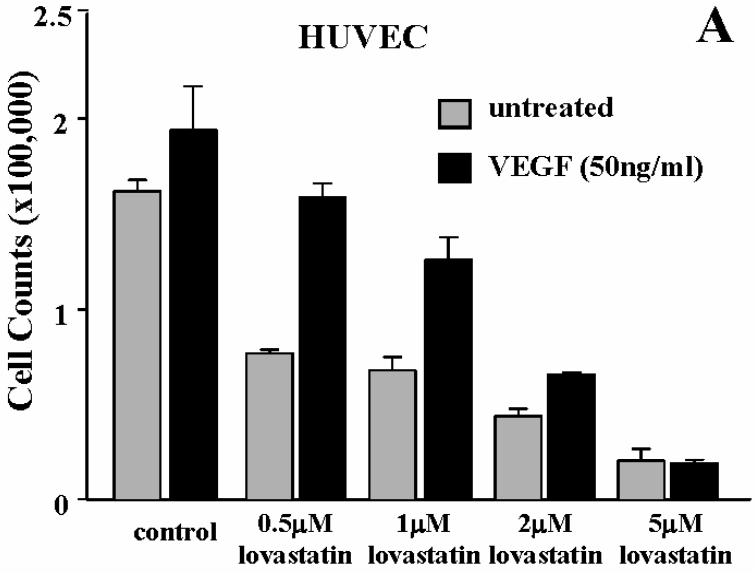

H28
HUVEC
pAKT
AKT
pS6K1
S6K1
p4EBP1
4EBP1
ND
0
1
10
25
µM lovastatin
0
1
10
25
µM lovastatin

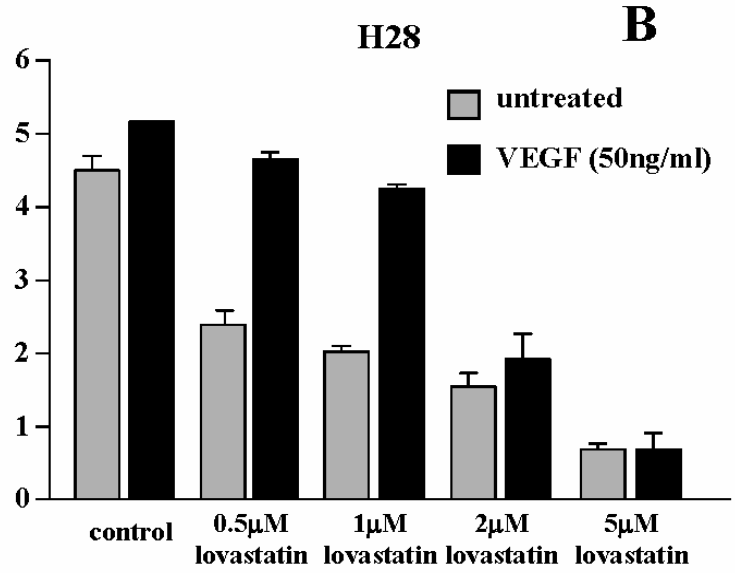
activation in these cell lines.

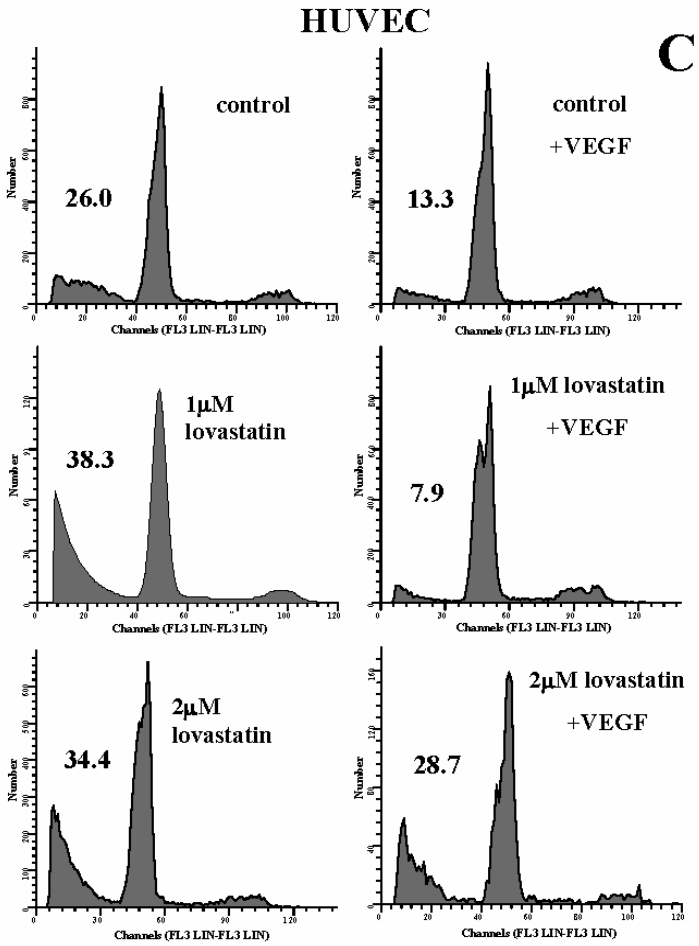
Lovastatin induces cytotoxicity of HUVEC and MM Cells

Due to the regulation of cell viability by the AKT pathway, we evaluated the effects of lovastatin treatment on HUVEC and H28 cell viability. Cell viability assays based on trypan blue exclusion cell counts of HUVEC and H28 cells were evaluated at 72hrs. The effect on cell viability of exogenous addition of $VEGF_{165}$ was included in this study to determine the role of this pathway in regulating lovastatin-induced cytotoxicity. Treatment with lovastatin alone at 0.5, 1, 2 and 5μM concentrations resulted in a dose-dependant decrease in the percentage of viable cells (Figures 3A and B). $VEGF_{165}$ proliferative effects were observed in control cells (Figures 3A and B). The addition of $VEGF_{165}$ to lovastatin treated cells inhibited lovastatin induced cytotoxicity at the low 0.5 and 1μM lovastatin doses but this compensatory effect was reduced or eliminated at the higher 2 and 5μM lovastatin treated cells (Figures 3A and B). The percentage of apoptotic HUVEC 72hrs (Figure 3B) post-treatment was assessed using propidium iodide flow cytometry to study the effects of lovastatin in inducing apoptosis. The control cells showed a sub-G1 peak in the DNA histogram that is characteristic of apoptotic cells representing approximately 26% of cells analyzed, while addition of $VEGF_{165}$ resulted in a reduction of apoptotic cells to approximately 13%, highlighting the role of VEGF in promoting HUVEC cell survival. At a dose of 1μM and 2μM, lovastatin induced significant apoptosis above the levels of that observed in the control cells. However, for the 1μM lovastatin concentration, $VEGF_{165}$ was still able to able to diminish the apoptotic effects of lovastatin on HUVEC but with the higher 2μM lovastatin dose, addition of $VEGF_{165}$ had no significant affect on the induction of

Chapter 4 Figure 3. VEGF can partially rescue the cytotoxic and apoptotic effects of lovastatin. A and B, HUVEC and H28 cell proliferation was measured with a cell viability assay following either control or 0.5-5μM 72hr lovastatin treatments with or without 50ng/ml $VEGF_{165}$. $VEGF_{165}$ stimulated control cells to proliferate, however, higher doses of lovastatin inhibited the proliferative effects of $VEGF_{165}$. The data were normalized to untreated (media alone) cells (representing 100%) and are representative of 4 independent experiments. C, Apoptosis was measured using flow cytometric analysis of HUVEC following either control or 1 and 2μM 72hr lovastatin treatments with or without 50ng/ml $VEGF_{165}$. Results demonstrated that lovastatin was preventing the apoptotic inhibitory effects of $VEGF_{165}$ at higher doses (2μM). The data is typical of 2 independent experiments. In all experiments, error bars indicate standard deviation.


HUVEC
A
Cell Counts (x100,000)
2.5
2
1
0
untreated
VEGF (50ng/ml)
control
0.5μM lovastatin
1μM lovastatin
2μM lovastatin
5μM lovastatin


H28
B
6
5
4
3
2
1
0
untreated
VEGF (50ng/ml)
control
0.5μM lovastatin
1μM lovastatin
2μM lovastatin
5μM lovastatin

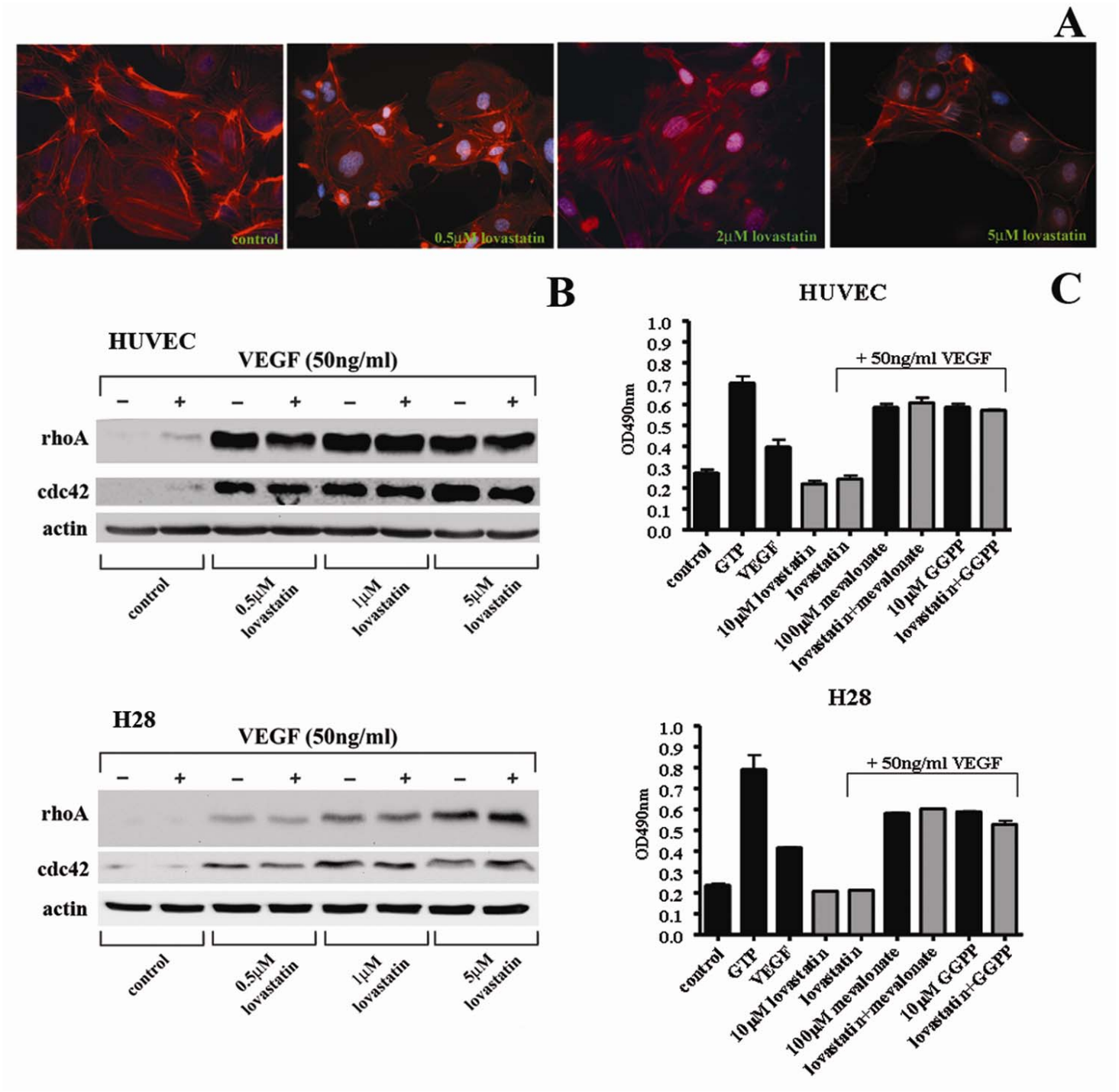

HUVEC
C
control
26.0
control
+VEGF
13.3
1μM lovastatin
38.3
1μM lovastatin
+VEGF
7.9
2μM lovastatin
34.4
2μM lovastatin
+VEGF
28.7
Number
Channels (FL3 LIN-FL3 LIN)

apoptosis (Figure 3B). The cell viability and flow cytometric analyses show the ability of lovastatin to induce a potent apoptotic response in HUVEC that at lower doses can be rescued by VEGF but not at the higher doses relevant for use of lovastatin as an anti-cancer therapeutic [29,30].

Lovastatin affects cytoskeleton organization and RhoA Activity

Actin cytoskeletal organization is known to play a significant role in the internalization and intracellular trafficking of RTK including VEGFRs. RhoA and cdc42 regulate actin cytoskeleton architecture and are activated by VEGF to control cell shape and motility [24]. RhoA and cdc42 are GGPP modified proteins whose function can be inhibited by lovastatin treatment [15]. Lovastatin induced dramatic changes in the actin cytoskeletal organization of HUVEC. Treatment with 0.5, 2 and 5μM lovastatin for 24hrs, resulted in a significant reduction of F-actin fibers stained with rhodamine-conjugated phalloidin and these fibers appeared disorganized (Figure 4A). In HUVEC and H28 MM cells, treatment with 0.5, 1 and 5μM lovastatin for 24hrs induced a dramatic up-regulation of both rhoA and cdc42 protein levels (Figure 4B). Cyclin D1 is a regulator of cell cycle progression and is up-regulated by a wide variety of cellular signalling pathways including rhoA activation [31]. The significant increase of rhoA protein levels did not result in up-regulation cyclinD1 protein levels but were reduced with lovastatin treatment of HUVEC and H28 cells (data not shown). Furthermore, employing a colorimetric rhoA activation assay, we determined the effect of lovastatin on $VEGF_{165}$ induced rhoA activation in HUVEC and H28 cells. Serum starved cell extract represent inactive levels of rhoA while 0.2M GTP loaded extract represents fully active rhoA. As expected VEGF stimulation induced rhoA activity to approximately 60% of the GTP loaded activity. Lovastatin (10μM, 24hrs) inhibited $VEGF_{165}$

Chapter 4 Figure 4. Lovastatin treatment results in actin disorganization and inhibits VEGF induced rhoA activation. A, Actin cytoskeletal organization was visualized using rhodamine-conjugated phalloidin following 24hr 0.5, 2 and 5μM lovastatin treatments of HUVEC. Staining revealed a lovastatin induced decrease in F-actin fibers along with a disorganized pattern. The data is typical of 3 independent experiments. B, Western blot analysis of various downstream targets of the VEGF receptor in HUVEC and H28 cells. Cell lysates were collected following 24hr lovastatin treatment in serum-free medium and either control or 30 min $VEGF_{165}$ stimulation. Total levels of RhoA and Cdc42 increase with increasing concentrations of lovastatin irrespective of $VEGF_{165}$ stimulation. C, Rho A activation assays. Serum starved HUVEC and H28 cells were treated with 10μM lovastatin, 100μM mevalonate and 10μM GGPP alone and in combination as indicated for 24hrs. Cells were stimulated with VEGF for 30min as indicated and assayed for rhoA activity employing the RhoA G-LISA kit that quantifies activated GTP loaded rhoA through colorimetric detection of rhoA bound to Rho-GTP-binding protein. In all experiments, error bars indicate standard deviation.

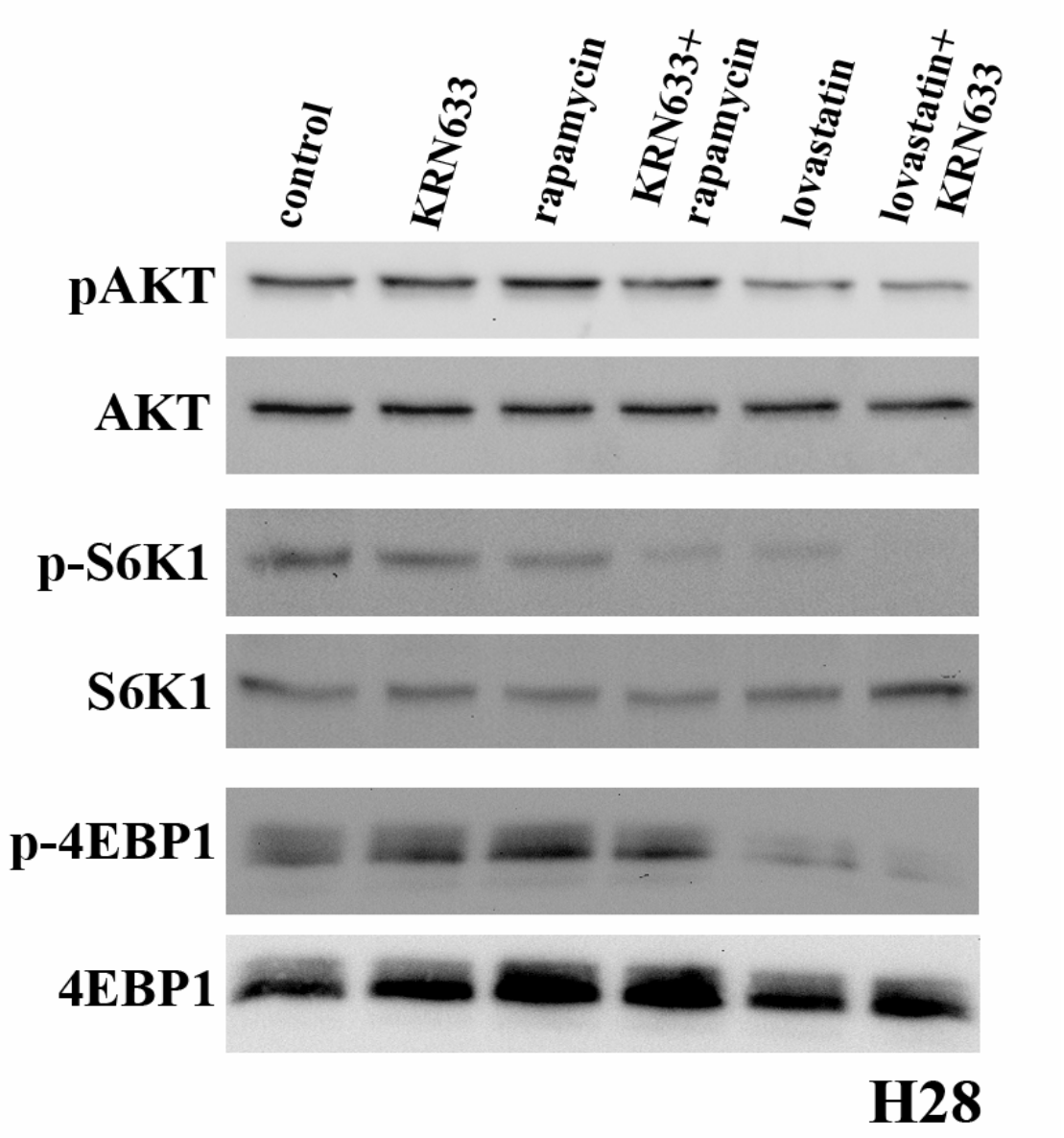

A
control
0.5μM lovastatin
2μM lovastatin
5μM lovastatin
B
HUVEC
VEGF (50ng/ml)
– + – + – + – +
rhoA
cdc42
actin
control
0.5μM lovastatin
1μM lovastatin
5μM lovastatin
H28
VEGF (50ng/ml)
– + – + – + – +
rhoA
cdc42
actin
control
0.5μM lovastatin
1μM lovastatin
5μM lovastatin
C
HUVEC
OD490nm
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
+ 50ng/ml VEGF
control
GTP
VEGF
10μM lovastatin
lovastatin
100μM mevalonate
lovastatin+mevalonate
10μM GGPP
lovastatin+GGPP
H28
OD490nm
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0
+ 50ng/ml VEGF
control
GTP
VEGF
10μM lovastatin
lovastatin
100μM mevalonate
lovastatin+mevalonate
10μM GGPP
lovastatin+GGPP

induced rhoA activation in both HUVEC and H28 cells while co-administration of mevalonate (100μM) and GGPP (10μM) reversed the inhibitory effects of lovastatin (Figure 4C). These results demonstrate that lovastatin-induced rhoA is inactive likely due to the lack of GGPP modification.

Inhibition of the VEGFR augments lovastatin-induced apoptosis

Our previous studies have demonstrated that the combination of lovastatin and EGFR-TKI have resulted in synergistic cytotoxicity in a variety of human cancer derived cell lines [21]. Other studies have demonstrated the utility of combining EGFR-TKI with downstream inhibitors of the AKT pathway including rapamycin. Mammalian target of rapamycin (mTOR) plays a central role in regulating AKT driven translation initiation by regulating S6K1 and 4EBP1 activity [32]. Rapamycin has limited clinical activity due to a feedback loop that activates AKT and acquired resistance [32] suggesting that lovastatin may represent a novel therapeutic approach to target this pathway and enhance RTK-TKI activity. In this study, we evaluated the ability of rapamycin or lovastatin to augment the effects of the VEGFR-2 inhibitor KRN633. The H28 MM cell line had a relatively weak response to lovastatin-induced AKT inhibition. H28 cells express both VEGF and VEGFR-2. By Western blot analysis of activated AKT and its downstream targets S6K1 and 4EBP1, KRN633 and rapamycin treatments alone had minimal effects on the activation of these proteins. The combination of these agents showed enhanced inhibition of this pathway (Figure 5). In contrast, lovastatin treatment alone inhibited AKT, S6K1 and 4EPB1 phosphorylation and the combination of lovastatin and KRN633 induced a dramatic inhibition of the AKT pathway in this MM derived cell line (Figure 5).

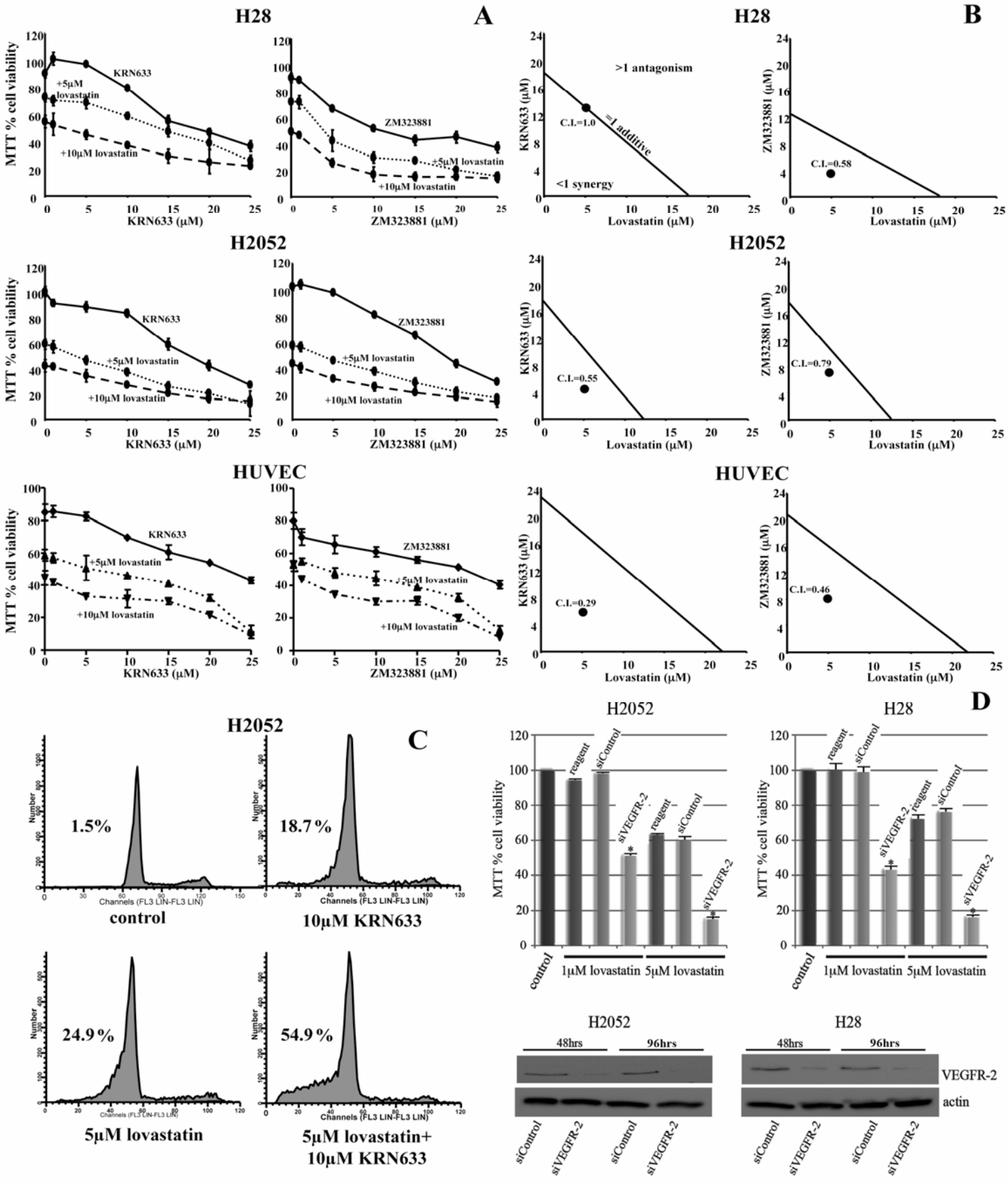
Chapter 4 Figure 5. Lovastatin in combination with VEGFR-2 TKIs inhibits ligand induced activation of AKT, S6K1 and 4EBP1. Control cells were serum starved for 24hr followed by 2hr treatments with either 10μM KRN633, 10nM rapamycin, 5μM lovastatin or their combinations. All cells were then lysed after stimulation with 50ng/ml $VEGF_{165}$ for 30min. Results demonstrated that lovastatin in combination with KRN633 induced the most significant decrease in phosphorylation status of all three proteins in H28 cells. Expression levels of total AKT, S6K1 and 4EBP1 were assayed as loading controls.


control
KRN633
rapamycin
KRN633+ rapamycin
lovastatin
lovastatin+ KRN633
pAKT
AKT
p-S6K1
S6K1
p-4EBP1
4EBP1
H28

We further evaluated the combination of lovastatin and VEGFR-2 TKI on tumor cell cytotoxicity in HUVEC and MM cells. Utilizing MTT analysis and propidium iodide flow cytometry, we investigated the effects of combining two different VEGFR-TKIs with lovastatin on the viability of the H28 and H2052 MM derived cell lines and HUVEC. KRN633 inhibits VEGFR 1, 2 and 3 with similar kinetics while ZM323881 is highly selective for VEGFR-2 [33,34]. With both MM derived cell lines and in HUVEC, increases in the concentration of the VEGFR-TKIs, KRN633 and ZM323881, resulted in a dose dependent decrease of MTT activity (Figure 6A). The pre-treatment of either 5μM or 10μM lovastatin for 24hrs prior to the addition of 0-25μM concentrations of the VEGFR-TKIs for 48hrs resulted in co-operative cytotoxicity in both MM cell lines and HUVEC treated with either VEGFR-TKI (Figure 6A). The use of the Combination Index (CI) isobologram method of analysis [23] allowed for the determination of the effects of the combination of the lovastatin and VEGFR-TKIs (Figure 6B). CI values of <1, 1, and >1 are indicative of synergism, additive effect, and antagonism, respectively. The H28 MM cell line at the therapeutically relevant 5μM dose of lovastatin resulted in a CI value of 0.58 for the combinatorial treatment of lovastatin and ZM323881, but the combination of lovastatin and KRN633 obtained a CI value of 1 (Figure 6B). The H2052 MM cell line and HUVEC had CI values of less than one for both VEGFR-TKIs. These results indicate that combining lovastatin with VEGFR-TKIs consistently induced synergistic cytotoxicity in MM and HUVEC cells.

To determine if this combination based approach resulted in enhanced apoptosis, we assessed MM cells treated with 5μM or 10μM of the VEGFR-TKIs alone or in combination with 5 μM lovastatin using the same experimental conditions as above. In both cell lines,

Chapter 4 Figure 6. Combining lovastatin with VEGFR-2 TKIs induces synergistic cytotoxicity in MM cells and HUVEC. A, Evaluating the cytotoxic effects of treatment of lovastatin in combination with VEGFR-TKI on MM cell lines H28 and H2052 and HUVEC employing MTT Assays. The VEGFR-2-TKIs KRN633 and ZM323881 at doses of 1-25μM were evaluated alone or in combination with 5 and 10μM lovastatin. MTT data were normalized to untreated (media alone) cells (representing 100%) and is typical of 2 independent experiments. B, Isobologram analyses of the combination of 5μM lovastatin and VEGF receptor inhibitor. LD50 values were determined for 72hr lovastatin and 48hr VEGF receptor inhibitor treatments. LD50 values are represented on the axes. The concentration of VEGF receptor inhibitor that demonstrated LD50 with 5μM lovastatin was plotted. Combination-Index (CI) was determined with CI<1, CI=1, and CI>1 as synergism, additive effect, and antagonism, respectively [34]. C, Apoptosis was measured using flow cytometry analysis of H2052 MM cells following either control or combinations of 10μM KRN633 with 5μM lovastatin treatments. The percentage of apoptotic cells is shown in the upper left quadrant of each histogram. Results demonstrated that lovastatin in combination with KRN633 induced a potent apoptotic response in these cells. The data is typical of 2 independent experiments. D, H28 or H2052 cells were transiently transfected with 50nM control (siControl) or VEGFR-2 (siVEGFR-2) siRNA oligonucleotides for 48hrs. Cells were then treated with medium or 1 and 5μM lovastatin with fresh 50nM siRNAs for an additional 48hrs and analyzed for cell viability using the MTT assay. * $P<0.001$ comparing siControl to siVEGFR-2 in lovastatin treated cells as determined by paired T-test analysis. Total protein extracts in H2052 and H28 cells were analyzed by Western blotting for VEGFR-2 and actin following 48 and 96hrs treatments with 50nM of siControl and siVEGFR-2 siRNAs.


A
H28
MTT % cell viability
KRN633
+5μM lovastatin
+10μM lovastatin
KRN633 (μM)
ZM323881
ZM323881 (μM)
B
>1 antagonism
=1 additive
<1 synergy
C.I.=1.0
C.I.=0.58
KRN633 (μM)
ZM323881 (μM)
Lovastatin (μM)
H2052
C.I.=0.55
C.I.=0.79
HUVEC
C.I.=0.29
C.I.=0.46
C
H2052
1.5%
18.7%
control
10μM KRN633
24.9%
54.9%
5μM lovastatin
5μM lovastatin+
10μM KRN633
Number
Channels (FL3 LIN-FL3 LIN)
D
H2052
H28
reagent
siControl
siVEGFR-2
control
1μM lovastatin
5μM lovastatin
48hrs
96hrs
VEGFR-2
actin

with both VEGFR-TKIs tested, the combination of 5μM lovastatin with 5μM and 10μM of the VEGFR-TKIs induced a more potent apoptotic response than either agent alone. Representative results for the H2052 cell line using the inhibitor KRN633 are shown (Figure 6C) and demonstrate a significant increase in apoptosis of the cells when the treatments were combined. Lovastatin treatment (5μM) induced an apoptotic response that was significantly enhanced in combination with 10μM KRN633 treatments (Figure 6C). Thus, the synergistic cytotoxicity observed with the combination of lovastatin and VEGFR-TKIs in MM cells is accompanied by a potent apoptotic response.

To further demonstrate the role of VEGFR-2 as a target of these VEGFR-TKIs in the synergistic cytotoxicity observed in combination with lovastatin in MM cells, we specifically targeted the expression of VEGFR-2 employing short inhibitory RNA sequences (siRNAs). Employing the MTT cell viability assay, we demonstrated that while the siControl treatments (50nM 48hrs, followed by 48hrs lovastatin treatment) had no effect on lovastatin treatments (1 and 5μM) compared to reagent alone, siVEGFR-2 (50nM 48hrs, followed by 48hrs lovastatin treatment) significantly enhanced lovastatin-induced cytotoxicity in H2052 and H28 MM cells (Figure 6D). Western blot analysis confirmed the specificity of the siRNAs employed as siVEGFR-2 but not siControl targeted VEGFR-2 expression at 48 and 96hr treatments (Figure 6D).

Discussion

In our previous study, we demonstrated that the targeting of HMG-CoA reductase, which results in mevalonate depletion [16], can inhibit the function of the EGFR [21]. Furthermore, combining lovastatin with gefitinib, an EGFR-TKI, induced apoptotic and cytotoxic effects that were synergistic. This was demonstrated in several types of tumor cell lines and potentially involved the PI3K/AKT pathway [21]. The mechanisms regulating the inhibitory effects of lovastatin on EGFR function and the synergistic cytotoxicity in combination with gefitinib are currently not known. These findings suggest that mevalonate pathway inhibitors and receptor TKI may represent a novel combinational therapeutic approach in a variety of human cancers. The VEGFR and the EGFR are both members of RTK family that share similar activation, internalization and downstream signalling characteristics [3,35]. Therefore, targeting the mevalonate pathway may have similar inhibitory effects on VEGFR and may also enhance the activity of VEGFR-TKI. VEGFR, particularly VEGFR-2, play important roles in regulating angiogenesis by promoting endothelial cell proliferation, survival and migration [7]. VEGF and VEGFR are also expressed by some tumor cells, like MM, acting in a functional autocrine loop capable of directly stimulating the growth and survival of MM cells [9].

In this study, we have shown lovastatin does indeed inhibit ligand-induced VEGFR-2 activation through inhibition of receptor internalization resulting in diminished AKT activation in HUVEC and MM cells. Lovastatin treatment re-organized the actin cytoskeleton, inhibited proliferation and induced apoptosis of HUVEC at therapeutically relevant doses (<5μM) [30] despite addition of exogenous VEGF. AKT activation, which mediates cell survival, along with its downstream targets S6K1 and 4EBP1 were significantly inhibited by

lovastatin treatment. Combining lovastatin with VEGFR-TKIs also induced synergistic cytotoxicity of HUVEC cells. Due to their role in promoting tumor neovascularization, inhibiting the function of VEGF and VEGFR has been the focus of a number of therapeutic approaches [1]. The limited clinical responses associated with these agents have been associated with their ability to promote disease stabilization and rarely induce tumor regression [1,36]. Thus, agents that can co-operate and enhance the activity of VEGFR-TKI, like lovastatin, may increase their therapeutic activity.

MM is a highly aggressive tumor that is rarely curative and median survival is in the range of 10–17 months [11], therefore, novel therapies for MM are needed. Elevated levels of circulating and serousal VEGF in MM patients and the expression of VEGF and VEGFR on MM cells that can drive their proliferation and enhance their survival [9] has led to the evaluation of VEGFR targeted therapies. Bevacizumab, a monoclonal antibody against the VEGF, which is approved for the treatment of colon cancer, in combination with chemotherapy, failed to significantly affect outcome to chemotherapy treatment alone [37]. Various VEGFR-TKI employed a single agents also failed to demonstrate clinical utility in MM patients [37]. As like HUVEC, MM cells also depend on VEGFR signalling, we also examined the effect of lovastatin alone and in combination with VEGFR-2 TKI on MM cell viability. Combining 5μM lovastatin treatments with two VEGFR-2 inhibitors in the H28 and H2052 mesothelioma derived cell lines demonstrated synergistic cytotoxicity through the induction of a potent apoptotic response. These results highlight a novel mechanism regulating VEGFR-2 function and a potential novel therapeutic approach for MM.

Inhibition of HMG-CoA reductase has been evaluated as an anti-cancer therapeutic approach owing to its ability to inhibit tumor cell proliferation, induce tumor specific

apoptosis and inhibit cell motility and metastasis in several tumor models [38,39,40,41]. A number of Phase I Clinical trials evaluating the efficacy of high doses of lovastatin failed to demonstrate significant anti-tumor activity [30]. The tumor types evaluated in these studies did not include those that we identified as being highly sensitive to lovastatin-induced apoptosis, including head and neck squamous cell carcinomas and cervical carcinomas [39]. As a result, a Phase I clinical evaluation of lovastatin in recurrent head and neck squamous cell carcinomas and cervical carcinoma patients was undertaken by our group. Although no tumor regressions were observed, 23% of patients exhibited stable disease [29]. Taken together, the most effective use of lovastatin and VEGFR-TKI would be as part of a combined modality approach.

Due to the potential for mevalonate metabolite depletion to functionally alter the VEGFR signalling pathway, HMG-CoA reductase and VEGFR targeted therapies may be associated. This study has shown that the combination of lovastatin with two VEGFR-TKIs induced significant co-operative cytotoxicity in both MM cell lines tested. More detailed isobologram analysis demonstrated that this enhanced cytotoxic response was synergistic. These results suggest the potential of combining these two therapeutic approaches. The inhibition of mevalonate synthesis and the depletion of one or more mevalonate metabolites is the mechanism regulating this phenomenon. The combination of statins and VEGFR-TKI represents an attractive therapeutic approach as clinical trials have shown a different spectrum of toxicities with these agents [30,36]. In a recent manuscript, we have demonstrated similar inhibition of EGFR function by lovastatin in squamous cell carcinoma cells [42]. While in vivo murine tumor models evaluating the efficacy of statins have been employed, differences in drug metabolism between species and lack of target validation in

many studies suggests the potential of off target effects playing a role in statin response [43,44,45]. To circumvent these issues, we evaluated the BR.21 NCIC-CTG Phase III clinical trial of the EGFR-TKI inhibitor tarceva as a single agent in non-small cell lung carcinoma patients [46]. In this trial, patients on erlotinib that were also taking statins to treat hypercholesterolemia had a trend to better outcomes than patients on erlotinib alone [42]. These studies have led to a Phase I/II clinical trial at our institute combining rosuvastatin and erlotinib that is currently accruing patients (ClinicalTrials.gov Identifier**:** NCT00966472). Similar data for statin usage in VEGFR-TKI treated MM patients were not available due to the lack of a sufficient patient population for analysis. The ability of lovastatin to inhibit both EGFR and VEGFR function is intriguing and requires further study to elucidate its underlying mechanism. This suggests the potential for HMG-CoA reductase inhibition to affect the activity of a number of RTK potentially through a similar, novel and as yet uncharacterized mechanism.

Ethical Statement

Not applicable with respect to this study.

Acknowledgements

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CHAPTER 5

General discussion

Statins are potent inhibitors of HMG-CoA reductase, the rate limiting enzyme of the mevalonate pathway [1], that are widely used as hypercholesterolemia treatments [2]. The mevalonate pathway produces a variety of end products that are critical for many different cellular pathways [1, 3, 4], thus, targeting this pathway can affect multiple signalling pathways. We have previously shown that lovastatin can induce tumor specific apoptosis especially in SCC and that 23% of recurrent SCC patients treated with lovastatin as a single agent showed disease stabilization in our Phase I clinical trial [5]. Subsequently, our laboratory demonstrated that lovastatin in combination with gefitinib, a potent inhibitor of the EGFR showed co-operative cytotoxicity when combined [6]. Furthermore, the pro-apoptotic and cytotoxic effects of these agents were found to be synergistic and to be manifested in several types of tumor cell lines including SCC, NSCLC [6] and glioblastoma (Chapter 2). I was able to expand upon these important findings and demonstrated that lovastatin, through its ability to disrupt the actin cytoskeleton, inhibited EGFR dimerization and activation (Chapter 3). This novel mechanism targeting this receptor has clinical implications as lovastatin treatment combined with gefitinib showed co-operative inhibitory effects on EGFR activation and downstream signalling [6, 7]. The RTK family of proteins share similar features with respect to activation, internalization and downstream signalling effectors [8]. I further demonstrated that lovastatin can inhibit the VEGFR-2 in endothelial cells and mesotheliomas, where VEGF and its receptor are co-expressed, driving their proliferation, and inducing synergistic cytotoxicity in mesothelioma cells in combination with VEGFR-2 tyrosine kinase inhibitors (Chapter 4). These findings suggest that statins may augment the effects of a variety of RTK inhibitors in a similar fashion representing a novel combinational therapeutic approach in a wide repertoire of human cancers. More

importantly, based on this work, we initiated a Phase I/II study evaluating high-dose rosuvastatin and the EGFR inhibitor tarceva in SCC and NSCLC patients at our institute. This clinical evaluation will provide insights to the efficacy of this approach and provide invaluable data that will play a role in developing novel therapeutic strategies. The objective of my Ph.D. project was to identify the mechanism of statin induced inhibition of RTK function and synergy with RTK inhibitors.

My hypothesis was that targeting the mevalonate pathway will inhibit the activity of RTKs and act co-operatively with known inhibitors of these receptors. The mechanism by which statins affect RTK function will likely be regulated by inhibiting the function of prenylated rho family proteins.

1. Lovastatin-The magic drug?

Our laboratory has played a key role in identifying this novel application of statin-based therapies. Our previous studies have demonstrated that a subset of human cancers, including neuroblastoma, acute myeloid leukemias, paediatric solid malignancies, and SCC, are susceptible to lovastatin-induced apoptosis within therapeutically achievable levels (<5μM) [9-11]. Similar results were demonstrated by others in medulloblastoma, mesothelioma and glioblastomas [12, 13]. These results rekindled the interest of HMG-CoA reductase inhibitors and resulted in the clinical evaluation of lovastatin in a Phase I trial in recurrent SCC by our clinical trial group. Although no tumor regressions were observed, 23% of patients exhibited stable disease further implicating the feasibility of this approach [5]. These results suggested that the most effective use of lovastatin would be as part of a combined modality approach. As a result, our laboratory identified the ability of lovastatin to induce synergistic tumor cell cytotoxicity through the induction of a potent apoptotic response in combination with

gefitinib, a clinically relevant EGFR-TKI. This was the first demonstration of the ability of lovastatin to potentiate the cytotoxic effects of EGFR inhibitors. This synergy was demonstrated in a variety of NSCLC, SCC and colon carcinoma cell lines [6]. Furthermore, I identified the ability of lovastatin treatment to inhibit ligand induced EGFR and AKT activation in glioblastoma cells (Chapter 2 Figure 4 & 5). I have also shown that lovastatin treatment of SCC cells inhibits ligand-induced dimerization and internalization of the EGFR (Chapter 3 Figure 1). Subsequently, AKT activation that mediates cell survival was significantly inhibited with lovastatin treatment (Chapter 3 Figure 2).

Previous reports have shown that hyper-activation of the PI3K/AKT pathway can inhibit the sensitivity of gefitinib and tarceva [14]. For example, in glioblastomas, co-expression of the constitutively active mutant EGFRvIII and PTEN, a negative regulator of PI3K activity is associated with clinical response [14]. Loss of PTEN appears to act as a resistance factor. Inhibition of downstream targets of the PI3K/AKT pathway, like the mammalian target of rapamycin (mTOR) with rapamycin, enhanced gefitinib activity in these PTEN deficient cells [15]. Similarly, I evaluated the effects of lovastatin on gefitinib activity in an isogenic U87MG glioblastoma cell line model with exogenously expressed EGFRvIII and PTEN in relevant combinations. I demonstrated that lovastatin in combination with gefitinib induced synergistic cytotoxicity irrespective of EGFRvIII or PTEN status (Chapter 2 Figure 6). The identification of the ability of lovastatin and gefitinib to induce synergistic cytotoxicity as well as lovastatin's inhibitory effects on EGFR and AKT activation were novel findings with therapeutic implications (hypothesis confirmed). Inhibiting receptor tyrosine kinase function has been the focus of a number of therapeutic approaches [16-19]. Combination treatments of

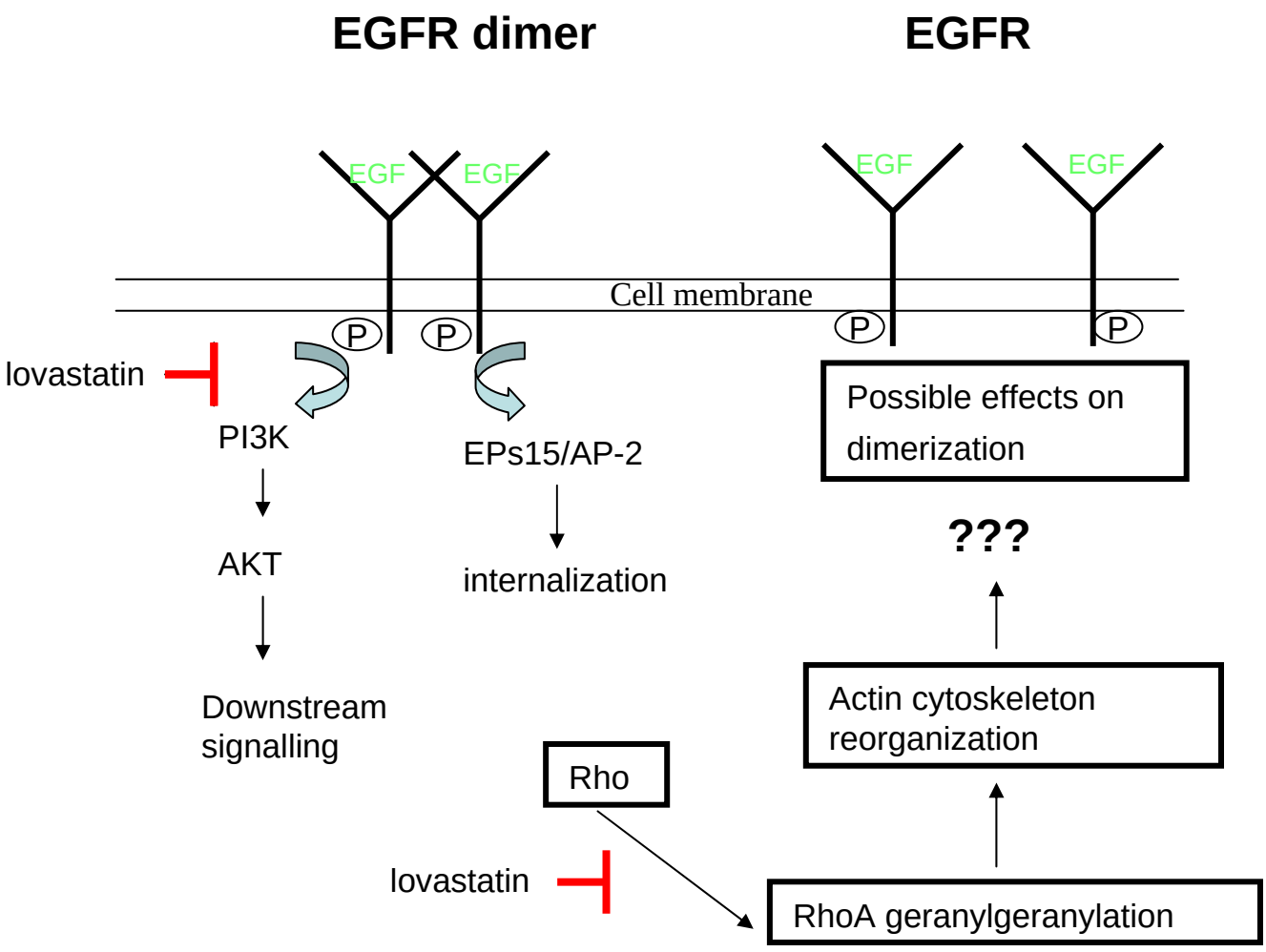
mevalonate pathway inhibitors and receptor tyrosine kinase inhibitors may represent a novel therapeutic approach.

Inhibiting HMG-CoA reductase has the potential to inhibit tumor cell proliferation, cell motility, metastasis and induce tumor-specific apoptosis in several tumor models [9-11, 20-29]. What are the underlying mechanisms of these effects? Others have shown that HMG-CoA reductase inhibitors exert their anti-proliferative effects by blocking the transition of G1-S phase in tumor cells [11, 21]. This effect can be reversed with the addition of mevalonate [21]. The effect of HMG-CoA reductase inhibitors on G1-S arrest is likely due to an increase in two cyclin-dependent kinase inhibitors, p21 and p27 [30-32].

2. The model of lovastatin's action on RTK

Despite the large amount of evidence demonstrating the interaction of the EGFR with the cytoskeleton, the molecular basis of this interaction under physiological conditions remains unknown. In chapter 3, I delineated the mechanism of lovastatin-induced inhibition of EGFR activation with a particular emphasis on the rho family of proteins in regulating this phenomenon (hypothesis confirmed). Our working model is that lovastatin treatment reduced cellular pools of GGPP that affects the geranylgeranylation and proper membrane localization of the rho family of proteins, resulting in cytoskeletal disorganization and subsequent receptor dimerization, activation and downstream signalling inhibition (Chapter 5 Figure 1). GGPP modification is essential for a wide variety of proteins. Prenylated proteins like Ras and Rho are post-translationally modified at or near the carboxyl terminus by formation of cysteine thioethers with the isoprenoid lipid substrates FPP or GGPP [3, 4]. Prenylation is a common post-translational modification that acts as a membrane anchor for Ras and Rho, regulating actin cytoskeleton and cellular trafficking, respectively [3, 4, 33].

Chapter 5 Figure 1. Proposed model of inhibition on EGFR dimerization, activation and internalization by lovastatin. Lovastatin exerts its inhibitory effects possibly through targeting rho protein.


EGFR dimer
EGFR
EGF
EGF
EGF
EGF
Cell membrane
P
P
P
P
lovastatin
PI3K
AKT
Downstream signalling
EPs15/AP-2
internalization
Possible effects on dimerization
???
Actin cytoskeleton reorganization
Rho
lovastatin
RhoA geranylgeranylation

Supporting this model, I determined that lovastatin treatment inhibits ligand-induced EGFR dimerization in SCC cells by performing cross-linking experiments and using Western blot analysis to visualize EGFR-dimer formation (Chapter 3 Figure 1). The effects of lovastatin on EGFR dimerization were reversed by the addition of mevalonate and GGPP that acts as a protein membrane anchor for rho and rab family proteins that regulate actin cytoskeleton and cellular trafficking, respectively (Chapter 3 Figure 4). Lovastatin treatment induced actin cytoskeletal disorganization, likely through inhibition of rhoA function, as Y-27632 [34] (an inhibitor of the downstream rhoA effector ROCK) also showed similar inhibitory effects on actin cytoskeletal disorganization and EGFR dimerization (Chapter 3 Figure 5 & 6). Although controversial, actin organization has been shown to play a role in EGFR localization and activation [35]. The ability of lovastatin to inhibit EGFR dimerization is a novel exploitable mechanism regulating this therapeutically relevant target that results in inhibition of RTK-induced AKT activation. There are multiple mechanisms by which the cytoskeleton may influence EGFR activation:

The cytoskeleton may stabilize the receptor, such as in the maintenance of receptor dimer formation. Using a co-immunoprecipitation method, I showed that F-actin is readily pulled down with EGFR in control cells, but not in lovastatin-treated cells (Appendix A). Importantly, this data further confirms the direct interactions between EGFR and actin. EGFR family members have two putative actin-binding domains (ABD) containing two phosphorylation sites, T992 (in ABD1) and T1148 (in ABD2) [36]. I propose that these C-terminus binding sites for actin are involved in the formation of higher order receptor oligomers and/or receptor clustering after ligand activation of the kinase domain.

The cytoskeleton may form a matrix that facilitates the interaction of downstream signalling proteins by sequestering substrates and keeping them in close contact with the receptor. It is well known that the binding of EGF to its receptor triggers a conformational change, resulting in receptor dimerization, tyrosine kinase activation and downstream signalling. The EGFR signal transduction process involves a series of downstream signalling molecules that themselves interact with cytoskeleton-associated proteins [35]. Others have shown that pT992 in ABD1 and pT1148 in ABD2 provide binding sites for different signalling molecules. For example, phospholipase C (PLC) interacts with pT992, Src homology 2 domain containing protein (SHC) with pT1148, and Protein tyrosine phosphates 1B (PTP1B) with both [36]. On the other hand, several components involved in EGF-induced signal transduction such as PLC, phosphatidylinositol-kinase, phosphatidylinositol-4-phosphate kinase and diacylglycerol-kinase are also associated with the cytoskeleton [37]. Several experiments in this thesis indicate that lovastatin induces cytoskeletal disorganization and inhibition of downstream signalling (through AKT) (Chapter 3 Figure 2 and 5). Therefore, actin cytoskeleton reorganization may be required for recruiting downstream signalling molecules after EGFR activation by providing a scaffold for signalling intermediates.

The cytoskeleton may also regulate receptor internalization, thus modulating receptor activity, as the actin cytoskeleton is crucial to endocytosis [38-40]. EGFR complexes and downstream signalling molecules that associate with the actin cytoskeleton are involved in receptor endocytosis [38, 39]. For example, EGF stimulation leads to phosphorylation of epidermal growth factor receptor substrate 15 (Eps15 is present at clathrin-coated pits and is involved in receptor-mediated endocytosis of EGF) and recruitment of the EPs15/AP-2 (AP-

2 functions at the plasma membrane to internalize cargo in clathrin-mediated endocytosis) complex to the EGFR [41]. The interaction of Eps15 with the AP-2 complex is required for EGFR endocytosis [42]. Several point mutations in the actin-binding domain of the EGFR can slow the rate of receptor internalization and consequently, reduce the clearance rate of activated EGFR from the cell surface, thereby prolonging receptor signalling [43]. I found that lovastatin treatment induces cytoskeleton disorganization and inhibits RTK internalization (Chapter 3 Figure 1 & 5). The disorganization of the cytoskeleton may have the same effects as mutations in actin-binding domains of the EGFR presented by others.

Understanding the relationship between statin-induced inhibition of RTK activity and cytoskeleton may uncover more tumor specific inhibitors of this pathway. *In vitro* studies suggest that the EGFR can associate directly with the cytoskeleton [44]. Co-localization of F-actin and the EGFR has been observed with both immunofluorescence and immunoelectron microscopy [44]. To address whether EGFR binds to actin directly, I immunoprecipitated EGFR and determine if actin is bound to the receptor and whether statin treatment affects this interaction. Actin can bind readily to many substrates and is a well-known background contaminant of many co-immunoprecipitation experiments. Therefore, I evaluated a number of EGFR antibodies with various bead preparations to optimize the EGFR/actin co-immunoprecipitation to ensure physiological binding of these proteins. In appendix A, I show the results of my optimized protocol and demonstrate actin binding to the EGFR in SCC9 cells. Moreover, this binding is shown to be reduced in the presence of lovastatin. As I have indicated before, two actin-binding domains have been described in the C-terminal cytoplasmic domain of EGFR that encompass the tyrosine 992 residue and the tyrosine 1148 residue [36]. These actin-binding domains provide binding sites for several signalling

molecules including PLCγ, Src, and Shc that can interact with F-actin [36]. For future experiments, I propose to delete both of these actin-binding domains, as has been previously described [36], either singly or in combination to determine their effects on actin binding to EGFR in COS-7 cells that do not express endogenous EGFR. For these modified EGFR proteins, one can assess their effects on both actin binding and ligand-induced dimerization with the co-immunoprecipitation and cross-linking methods that are described previously in this thesis. In addition, one can also evaluate the binding/activation of PLCγ, Src, and Shc to these modified EGFR proteins.

Besides immunoprecipitation based methodologies, protein-protein interaction can also be visualized in vivo by fluorescence resonance energy transfer (FRET) [36, 45, 46]. In brief, one can evaluate the effect of lovastatin and Y-27632 on EGFR and actin binding where overlapping green fluorescent protein variants are fused to these host proteins. The GFP mutants with longer wavelengths; CFP (cyan fluorescent protein) with excitation and emission peaks of 436 and 476 nm and YFP (yellow fluorescent protein) with excitation and emission peaks of 516 and 529 nm can be used [46]. Optimal conditions occur when all the donors are paired with an acceptor, as any unpaired protein adds noise to the signal. Additionally, if the distance or orientation between the pairs are unfavorable, FRET may not occur, even if the two proteins form a complex [46]. To perform FRET analysis, our lab has obtained EGFR-CFP and actin-YFP expression constructs to transfect into COS-7 cells which do not express endogenous EGFR. Previous FRET analyses using this combination have demonstrated association of these proteins in a complex after ligand stimulation of the EGF receptor [36].

3. Mevalonate pathway enzymes and RTK

Biochemical add-back approaches have been used to identify the mevalonate metabolites that mediate the mechanism of lovastatin's action on tumor cell growth and cell survival [47]. Previous studies have also shown that apoptosis induced by HMG-CoA reductase inhibitor is mediated through depletion of geranylgeranylated proteins (mevalonate pathway metabolites) [29, 48]. In one study by Xia et al 2001, add-back experiments of downstream products in the mevalonate pathway were conducted on statin-sensitive human acute myelocytic leukemia (AML) cells (pre-treated with lovastatin). Lovastatin-induced apoptosis was abrogated by addition of mevalonate and GGPP, which allowed for continued generation of geranylgeranylated proteins [29]. However, supplementing cells with other products in the mevalonate pathway such as cholesterol, squalene, lanosterol, desmosterol, dolichol, dolichol phosphate, ubiquinone, and isopentenyladenine, did not show any effect in lovastatin-induced apoptosis in AML cells [29]. Using this approach, I found that the cytotoxic and apoptotic effects of lovastatin (Chapter 2 Figure 3), and its inhibitory effects on EGFR dimerization (Chapter 3 Figure 4 & 6) were reversed by the addition of mevalonate or GGPP. These results indirectly demonstrate that the reversal of lovastatin-induced RTK inhibition in cells by GGPP is due to the replenishment of the intracellular pool of GGPP that is depleted by lovastatin treatment.

In the future, one can specifically target the downstream mevalonate pathway enzyme, geranylgeranyl diphosphate synthase that generates GGPP in cells (Chapter 1 Figure 9) [49]. Digeranyl biphosphonate (a cell-permeable specific inhibitor of geranylgeranyl diphosphate synthase) that targets the function of this enzyme can be employed [49]. The effects of targeting geranylgeranyl diphosphate on the cytotoxicity of the RTK-TKIs can be determined. Further evaluation can be done on the effects of targeting this enzyme on RTK and AKT

activity. Digeranyl biphosphonate can be tested alone and in combination with relevant RTK-TKIs in SCC, NSCLC and mesothelioma-derived cell lines. In addition, one should also target downstream enzymes of the mevalonate pathway that directly modify rho proteins, i.e. the geranylgeranyl transferase I (GGTaseI) (Chapter 1 Figure 9). A number of well established inhibitors of GGTaseI are available including GGTI-2147 and GGTI-298 that can be utilized [50]. While as single agents, these GGTase inhibitors have been shown to inhibit tumor cell growth without significant apoptosis induction like lovastatin [50, 51]. One can evaluate GGTaseI's potential alone or in combination with statins to induce cytotoxicity and apoptosis in tumor cell models listed above.

In Chapter 3, I demonstrated a novel mechanism where statins can affect EGFR function through inhibition of EGFR dimerization (Chapter 3 Figure 1). This effect was regulated by lovastatin's ability to deregulate actin architecture and confirms previous reports that actin plays a role in EGFR activity [36, 52]. Furthermore, an analysis of the Phase III BR21 study of tarceva as a single agent in NSCLC (kindly performed by the NCIC-CTG) demonstrated a trend to better survival outcome in patients taking statins at hypercholesterolemia doses with tarceva than patients on tarceva alone (Chapter 3 Figure 7). Taken together, our results were the basis for a Phase I/II study at our institute to clinically evaluate rosuvastatin and tarceva in NSCLC. We believe therapeutic efficacy would be enhanced by uncovering the mechanism of RTK inhibition by statins and that through which statins and RTK-TKIs induce synergistic cytotoxicity. The future experiments I proposed in this thesis may uncover novel mechanisms that could lead to better therapeutic strategies.

4. Other RTK and RTK inhibitors that synergize with lovastatin

The VEGFR-2 and EGFR share similar activation, internalization and downstream signalling characteristics [53, 54]. Therefore, I evaluated the effect of lovastatin on VEGFR-2 activation, its downstream signalling and cytotoxic effects alone and in combination with VEGFR-TKI in relevant cell line models. Similar to my work with the EGFR, lovastatin inhibits ligand-induced VEGFR-2 activation through inhibition of receptor internalization and also inhibits AKT activation in H28 and HUVEC (Chapter 4 Figure 1 & 2). Lovastatin also induces Rho inactivation and actin disorganization in these two cell lines (Chapter 4 Figure 4). In addition, combining 5μM lovastatin treatments with two VEGFR-2 TKIs in the HUVEC or H28 and H2052 mesothelioma derived cell lines demonstrated synergistic cytotoxicity through the induction of a potent apoptotic response (Chapter 4 Figure 6). These results highlight a novel function of lovastatin regulating other RTK function and uncover a novel therapeutic approach combining statins with VEGFR2-TKI.

Several pre-clinical studies have provided evidence for either direct or indirect angiogenic effects of EGFR signalling [55-60]. Although activation of EGFR on tumor cells can increase VEGF production and contribute towards angiogenesis, many of the mechanisms for increasing tumor growth related to activation of EGF or VEGF receptors are distinct [61-65]. Therefore it is reasonable that cancer cells treated chronically with EGFR inhibitor therapy may escape from growth inhibition by using alternative survival pathways such as VEGF. For this reason simultaneous blockade of these receptor families would be expected to result in a more pronounced inhibition of tumor cell proliferation and survival, and would thus prove to be a valuable therapeutic strategy. I have demonstrated lovastatin's ability to combine molecular therapies targeting several pathways such as anti-angiogenic therapy (VEGFR-2 inhibition) with EGFR inhibitor therapy. It is intriguing that lovastatin

can have a broader therapeutic effect through inhibition of VEGFR-2 and its downstream signalling in addition to EGFR inhibition. In cancer cells, altered control of angiogenesis could be a mechanism responsible for resistance to EGFR inhibitors in vivo [61-65]. Treatment with lovastatin alone or in combination with VEGFR-2 TKI would be expected to minimize resistance and constitute a strong rational approach to cancer treatment.

5. Future perspectives

Over the past two decades, efforts have been made to better understand the cellular processes related to malignancy. During this time, our understanding about the structure and function of ErbB receptors, and their role in human cancer has increased substantially. However, only recently has it become clear that selectively targeting these receptors can have therapeutic value. Since the idea of targeting EGFR as an option for cancer treatment was first proposed, a large number of EGFR inhibitors have been developed and their respective anti-tumor activities are being investigated. As we have seen, the ErbB signalling network is complex, where the involvement of numerous ligands and multiple dimerization partners creates a substantial challenge to establishing the crucial signalling pathways involved in tumorigenesis [66-69]. In Appendix A, I also observed that ErbB2 is pulled down with EGFR in control cells, but not in lovastatin-treated cells. Many basic questions about RTK still have to be addressed: What are other dimerisation partners of ErbB family receptors with EGFR in lovastatin sensitive cell lines? Does dimerization between EGFR and ErbB2 regulate the activation of these receptors in lovastatin-sensitive cell lines? Does ErbB2 EGFR heterodimer activate different signalling pathways and induce stronger signal transduction compare to EGFR EGFR homodimer in lovastatin sensitive cell lines? Does lovastatin inhibit ErbB2 the same way as EGFR? In addition to the communication between EGFR and ErbB2,

cross-talk has also been described between EGFR and other cell surface receptors such as cell adhesion molecules (integrins) and G-protein coupled receptors (GPCR) [70-72]. Integrins are activated by interaction with extracellular matrix proteins (ECM) and induce EGFR activation through activation of EGFR intracellular protein tyrosine kinases. Ligand-activated GPCR can also induce EGFR activation in the same manner [70-72]. Another interesting question to ask would be whether lovastatin has any effects on other cell surface receptors and how does it impact transactivation between receptors. A better understanding of these various mechanisms of EGFR and other receptors may lead to ways to enhance the effectiveness of anti-EGFR drugs (lovastatin) in cancer therapy.

A number of VEGFR-2 tyrosine kinase inhibitors have been developed including CP-547632, CGP79787, SU11248, AZD2171 and ABT-869 [73, 74]. As single agents, these drugs have also shown limited clinical activity and are also likely to require combined modality treatments to improve their efficacy [73, 74]. ABT-869 is a potent, ATP-competitive small molecule that inhibits VEGFR-2 tyrosine kinase activity that also shows potent activity against VEGFR-1 and VEGFR-3 [74]. ABT-869 also targets the function of the platelet derived growth fact receptor β (PDGFR-β), another relevant target for inhibiting angiogenesis [74]. VEGF-stimulated proliferation and VEGFR-2 phosphorylation of human vascular endothelial cells are inhibited by ABT-869 [74]. In *in vivo* studies, inhibition of VEGFR-2 signalling by ABT-869 reduced microvessel density and dose-dependently inhibited the growth of various human tumor xenografts (including NSCLC) [74]. In a recent Phase I study, ABT-869 administration showed evidence of antiangiogenic activity and limited but promising clinical activity [74]. Many of these RTK inhibitors have shown similar limited clinical activity as single agents and will likely require combination based

therapeutic approaches to increase their efficacy. As I have discussed before, it is highly unlikely that a given tumor will be dependent on just one signalling pathway for its growth and survival. There is strong pre-clinical evidence that crosstalk exists between activated EGFR pathways and angiogenesis in tumors [55-60]. Clinical studies showed that combined therapies with angiogenesis inhibitors and EGFR inhibitors are promising in a number of tumor types [75]. Therefore, strategies to inhibit multiple signalling pathways or multiple steps in the same pathway will be required to enhance their clinical activity. In the future, different combinations of lovastatin with VEGF receptor TKI (i.e. ABT-869) should be carried out and identification of an optimal combination may provide more efficacious therapeutic approaches.

6. Summary

Together, the work detailed in this thesis has added significantly to our understanding of how lovastatin inhibits EGFR/VEGFR-2 activation and builds a strong argument for the role that RhoA might play in lovastatin induced cytoskeleton disorganization and RTK dimerization inhibition. Expanding these studies together with clinical trials will provide invaluable data that will play a role in developing novel therapeutic strategies.

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Appendix A

Scc 9 cells were treated with control and 10μM lovastatin for 24h, with or without 50ng/ml of EGF for 30min. Protein A agarose beads were washed twice with PBS and restored to a 50% slurry. The beads were then added to pre-clear the cell lysate and incubated at 4°C for 30 minutes followed with centrifugation. 50μl of anti rabbit EGFR antibody was added to 500 μl of cell lysate. The mixture was gently rocked overnight at 4°C. The immunocomplex was captured by adding 50 μl of protein A agarose bead slurry and gently rocked for 2 hours at 4°C. The beads were collected by centrifugation and washed 3 times with ice cold PBS, then resuspended in sample buffer. The immunocomplexes were dissociated by boiling the beads for 5 minutes. SDS-PAGE was performed with the supernatant. Rabbit IgG and beads served as negative controls. EGFR blot served as loading control. ErbB2, actin, Grb2 were readily pulled down with anti-EGFR antibody in control treatment, lovastatin treatment inhibited the amount of proteins associated with EGFR to a certain extent.

Appendix A:
HER2, Actin and Grb2 are co-immunoprecipitated with EGFR