GGTI-298 IN COMBINATION WITH EGFR INHIBITORS: EVALUATING A NOVEL THERAPY IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS

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ABSTRACT

Overall survival of the metastatic forms of epithelial derived cancers, especially head and neck squamous cell carcinomas (HNSCC), has not significantly improved even with the application of aggressive combined modality approaches incorporating radiation and chemotherapy. Cumulative evidence implicates the epidermal growth factor receptor (EGFR) as an important therapeutic target in HNSCC. We have previously demonstrated that the combination of lovastatin, a potent inhibitor of the mevalonate pathway, with EGFR tyrosine kinase inhibitors induced robust synergistic cytotoxicity. However, the use of high dose statins in our clinical trial was associated with significant toxicities including higher than anticipated rate of muscle pathologies. Our goal was to uncover novel downstream targets of the mevalonate pathway that may enhance the efficacy or limit toxicities of this novel combination therapeutic approach. In this study we have demonstrated that GGTI-298, an inhibitor of protein geranylgeranylation, through its ability to disrupt the actin cytoskeleton, inhibits EGFR dimerization and cellular trafficking. This novel mechanism targeting the EGFR has clinical implications as GGTI-298 in combination with tarceva, a clinically relevant EGFR inhibitor, showed enhanced cytotoxicity and inhibitory effects on EGFR activation and its downstream signaling.

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DEDICATIONS

To my beautiful angel in heaven, my mother. I know you are looking down on me and proud of all my accomplishments…

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LIST OF ABBREVIATIONS

Akt (PKB)	Protein kinase B
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promote
Cdc42	Cell division control protein 42 homolog
CDK4	cyclin D1-cyclin-dependent kinase 4
CMF-PBS	calcium and magnesium free phosphate buffered saline
CR1(2)	Cysteine-rich domain 1(2)
DAG	1,2-diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
deltaEF1	Delta-crystallin enhancer binding factor 1
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III
EMT	Epithelial-to-mesenchymal transition
ErbB1(2,3,4)	Eukaryotic ribosome biogenesis protein 1(2,3,4)
ERK	Extracellular signal regulated kinases
FBS	Fetal bovine serum
FPP	Farnesyl pyrophosphate
FTase	Farnesyl transferase
FTIs	Farnesyl transferase inhibitors
GDP	Guanosine diphosphate
GGPP	Geranylgeranyl pyrophosphate
GGTase	Geranylgeranyl transferase
GGTIs	Geranylgeranyl transferase inhibitors
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphatases
H-ras	Harvey rat sarcoma viral oncogene homolog
HER1(2,3,4)	Human epidermal growth factor receptor
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
ICMT	isoprenyl–cysteine carboxyl methyltransferase
IgG	Immunoglobulin G
IP_3	Inositol 1,3,5-triphosphate
JAK	Janus kinase
JNK	c-Jun NH_2-terminal kinase

K-ras	Kirsten rat sarcoma viral oncogene homolog
L1(2)	Homologous large domain 1(2)
MEK (MAPK)	Mitogen activated protein kinases
MTT	3-(4,5-Dimethylthiazol-2-yl)- 2,5-Diphenyltetrazolium bromide
NSCLC	Non-small cell lung cancer
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI(3,4,5)P	Phosphatidylinositol 3,4,5-triphosphate
PI3K	Phosphoinositide 3-kinases
PIC	Protease inhibitor cocktail
PKC	Protein kinase C
PLCγ	Phospholypase Cγ
pRb	phospho-Retinoblastoma protein
PTB	Phosphotyrosine binding domain
PtdIns(4,5)-P_2	Phosphatidylinositol 4,5-diphosphate
PTEN	Phosphatase and tensin homolog
Rab	Rat sarcoma-related proteins in brain
Rac	Ras-related C3 botulinum toxin
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
Rce-1	Ras Converting Enzyme 1
Rho	Ras homolog gene family
RIPA	Radio immuno precipitation assay
RTKs	Receptor tyrosine kinases
SCC	Squamous cell carcinoma
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH2	Sarcoma (Src) Homology 2
Sos	Son of sevenless
STAT	Signal transducer and activator of transcription
TKIs	Tyrosine kinase inhibitors
TKs	Tyrosine kinases
TGF-α	Transforming growth factor- α
tRNAs	Transfer ribonucleic acid

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Chapter 1: INTRODUCTION

1.1 Head and Neck Cancers

1.1.1 Epidemiology and etiology

Cancer of the head and neck is a major global health issue, being the sixth most common form of cancer worldwide [1] with an annual incidence of approximately 650,000 cases and over 350,000 deaths [2]. Within Canada there will be an expected 4,300 new cases of head and neck cancers with approximately 1,610 deaths this year [3]. Head and neck cancer is a heterogeneous disease and it includes cancers arising from different anatomical sites of the aerodigestive tract: lip, oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, nose, and paranasal sinuses [4]. Head and neck squamous cell carcinoma (HNSCC) represents roughly 90% of head and neck tumors with an overall 5-year survival rate of 50% [5]. The most important risk factors of head and neck cancers that have been identified are tobacco and heavy alcohol consumption [6]. However, while the rate of tobacco use has declined, the incidence of HNSCC has not due to the recent emergence of human papillomavirus (HPV) as an etiologic factor for head and neck cancers especially oropharyngeal cancer. Oropharynx cancer is associated with oncogenic HPV infection in approximately 80% of cases and is more common compared to oral cavity cancers where approximately 20% are HPV related as well as larynx cancers, which lack detectable HPV infection [7]. The response to therapy and survival of HPV-positive HNSCC patients is enhanced in comparison to HPV-negative HNSCC patients [8].

The prognosis for patients with HNSCC is generally determined by the stage at presentation. The stage is determined by the extent of the tumor, the presence of lymph-node metastases and distant metastases [9]. Due to the heterogeneous aspect of HNSCC, accurate prognostication, treatment planning, and identification of the causative cancer genes are difficult [10]. Treatment of HNSCC has mainly consisted of a combination of surgery and radiation, or a combination of radiation and chemotherapy depending on the site and stage of the disease and the health status of the patient. Roughly, 30-40% of patients are at stage I/II when they are diagnosed and these patients can be treated with either surgery or radiotherapy alone. Although patients with early stage of disease present with excellent prognosis, there is still a risk of recurrence, and secondary primary tumor, hence close monitoring is required [11].

1.1.2 Challenges and recent advances in HNSCC treatment

The major challenges in the therapeutic management of HNSCC is to achieve cure or disease stabilization while preserving tissue function and patient survival [12]. Many acute toxicities associated with radiotherapy and chemotherapy occur during or shortly after treatment including: mucositis, radiation dermatitis, xerostomia, dysphagia, regional alopecia, hoarseness of voice, transient ear discomfort, and weight loss. Patients who are treated with chemoradiation therapy have incidence of mucositis as high as 80-90% [13]. The clinical use of cisplatin, the platinum-based and widely used chemotherapeutic drug, is diminishing due to its cumulative and irreversible toxicities such as nephrotoxicity and neurotoxicity [14]. These current conventional modalities have been moderately successful, however, they lack specificity for the tumor cells and they often cause unacceptably high toxicity without survival advantage [5] [15]. Consequently, new effective and selective

treatments, with limited side effects, are needed in order to improve treatment and survival in patients with HNSCC. In 2006, cetuximab (Erbitux), an agent that targets the epidermal growth factor receptor, became the first new treatment of HNSCC to receive approval in the US in 45 years and was the start of a new treatment strategy in HNSCC known as targeted therapy [16].

1.2 Receptor Tyrosine Kinases

1.2.1 EGFR and its binding ligand

Communication between individual cells is essential for the regulation and coordination of complex cellular processes such as growth, differentiation, migration and apoptosis. The receptor tyrosine kinases (RTKs) are primary mediators of such processes that sustain the communication between signals outside the cell surface and the internal compartments [17] [18, 19]. The epidermal growth factor receptor (EGFR) is one of the most studied RTK that is expressed in some normal epithelial tissues, including the skin, hair follicles, and the gastrointestinal tract [20-22]. EGFR is a 170KDa transmembrane protein belonging to the ErbB/HER-family of RTKs, which includes four members: ErbB1 (EGFR/HER1), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors are anchored in the cytoplasmic membrane and share a similar structure composed of an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and an intracellular tyrosine kinase domain [23, 24]. There are six known ligands that bind to the EGFR, including EGF itself and transforming growth factor-a (TGF-α) [17]. Stanley Cohen of Vanderbilt University initially discovered EGFR in 1962. Cohen, along with Rita Levi-Montalcini, received a Nobel Prize in 1986 for their discovery of EGF. EGFR is ubiquitously distributed on normal epithelial tissues; its overexpression has been linked with advanced

tumor stage, resistance to standard therapies (chemotherapy and radiation), and poor patient prognosis in some tumors [25].

1.2.2 EGFR structure, dimerization, and activation

The EGFR gene encodes a protein containing 1186 amino acids, with an extracellular ligand-binding region (~620 residues), a single transmembrane domain (~23 residues), and an intracellular tyrosine kinase domain (~260 residues) that is flanked by juxtamembrane (~40 residues) and C-terminal (~232 residues) regulatory regions [17]. The extracellular domain of the EGFR is divided into four subdomains: I (L1), II (CR1), III (L2), and IV (CR2) with domains I and III participating in ligand binding [26] (Figure 1.1). In the absence of ligand, specific intramolecular interactions between domains II and IV maintain the extracellular region of receptor in an autoinhibited or tethered conformation [27, 28]. Under normal conditions, EGF ligand will bind simultaneously to both domains I and III, drawing them closer to one another and will induce a conformational change. This causes EGFR to undergo a more extended conformation, due to the severing of intramolecular bonds acting upon domains II and IV. During this process, domain II, a cystine-rich domain becomes exposed and drives the formation of a homodimer between two EGF receptors or a heterodimer between an EGFR and another member of the ErbB family. Following dimerization, there is activation of the intrinsic tyrosine kinase domain [29, 30].

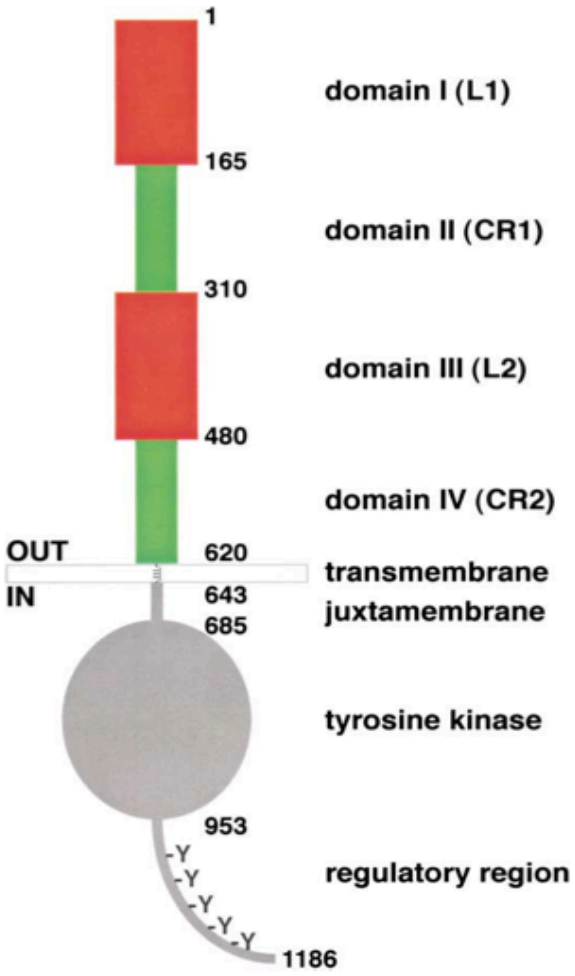


Figure 1.1 Domain organization of EGFR (I) The extracellular domains: (1) domain I: L1; (2) domain II: CR1; (3) domain III: L2; (4) domain IV: CR2. (II) Transmembrane domains. (III) The intracellular domains: (1) juxtamembrane domain; (2) tyrosine kinase domain; (3) regulatory region domain. Adapted from Burgess, A. W., 2003 [17].

1.3 Signal Transduction via EGFR

1.3.1 Physical association between EGFR and signalling proteins

Dimerization of the receptor brings the C-terminus of each receptor into close proximity, allowing trans-phosphorylation of the receptors to occur on key tyrosine residues within the C-terminus region of EGFR and, as a result, provides specific docking sites for cytoplasmic proteins containing Src homology 2 (SH2) and phosphotyrosine binding domains (PTB) [31]. These proteins bind to specific phosphotyrosine residues and initiate intracellular signaling via several pathways [23]. Ligand binding to the EGFR leads to the activation of a number of cell signaling pathways such as Ras/Raf/mitogen activated extracellular regulated kinase (MEK)/extracellular regulated kinase (ERK), phosphoinositide 3-kinases (PI3K)/AKT, Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and Phospholypase Cγ (PLCγ)/protein kinase C (PKC) mediated pathways [31, 32] [33] (Figure 1.2).

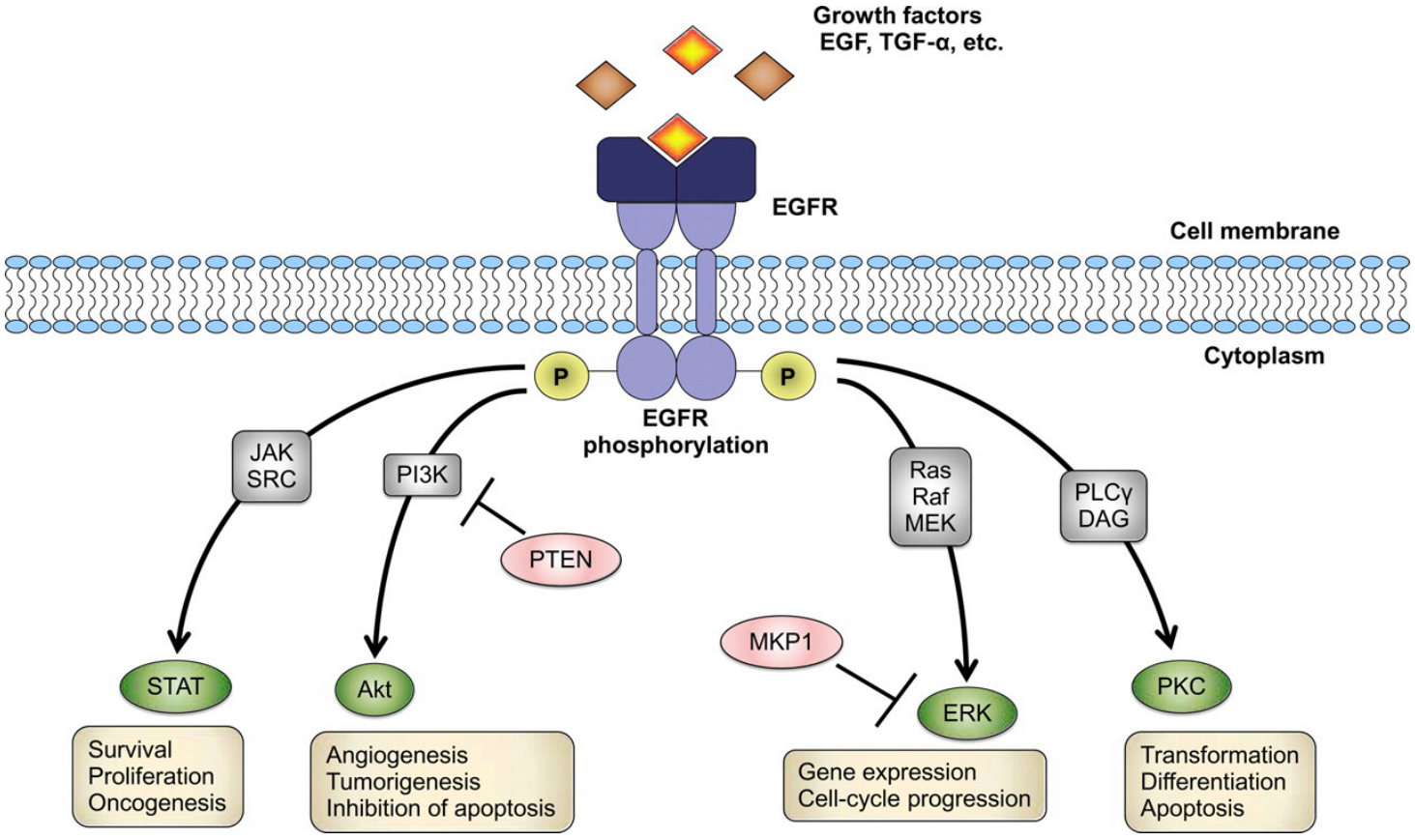


Figure 1.2 The EGFR signaling pathway The figure shows signaling pathways that are activated by EGFR. Important signaling pathways regulated by EGFR are represented with important functions highlighted in the boxes. The signaling cascade shows only the important components of the EGFR pathway. Examples of inhibitory signals are also shown, such as PTEN (phosphatase and tensin homologue), which can dephosphorylate PI3K, and MKP1 (MAPK phosphatase 1), which can dephosphorylate ERK1/2 signaling pathways. Adapted from Lee, J., 2011 [34].

1.3.2 Major signaling pathways activated by EGFR

Raf/MEK/ERK pathway This signaling pathway can be activated by a variety of factors such as growth factors, stress and inflammatory stimulus and G protein-coupled receptor (GPCR) activation and is an important route in regulating cell proliferation and survival [31, 35, 36]. The adaptor protein Grb2, is one of the many signaling molecules involved in this pathway which is constitutively bound to the guanyl nucleotide exchange factor-Son of sevenless (Sos). Grb2 and Sos are normally localized in the cytosol [32, 37]. Following EGFR activation, autophosphorylation at numerous sites on the C-terminus allows Grb2 to bind directly to the Y1068 and Y1086 docking sites of EGFR via its SH2 domain [38, 39]. Subsequently, Grb2 and Sos are recruited to the plasma membrane and brought into proximity with the guanine nucleotide-binding protein Ras [40]. Sos, through conformational modification, accelerates the exchange of GDP for GTP and transforms Ras into its active conformation (Ras-GTP) [40]. This activation allows Ras to recruit the serine/threonine kinase Raf to the plasma membrane where it can be phosphorylated and activated. As a result, MEK and ERK are subsequently phosphorylated and activated [40]. In turn, active ERK translocates to the nucleus where it regulates the expression of many downstream genes such as transcription factors involved in cell proliferation [41, 42]. Abnormal activation of this pathway occurs in many human malignancies due to mutations in Ras and Raf rendering it resistant to conventional therapies (chemotherapy) [43]. Targeting the Raf/MEK/ERK pathway is important in therapeutic intervention.

PI3K/AKT pathway This pathway is involved in cell growth, apoptosis resistance, invasion, and migration [44, 45]. Phosphatidylinositol 3-kinase (PI3K) is a dimeric enzyme containing a p85 adapter subunit, responsible for anchorage on specific docking sites of EGFR through

its SH2 domain. Binding of p85 to EGFR recruits the p110 kinase that generates the secondary messenger phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P), responsible for the phosphorylation and activation of the protein serine/threonine kinase AKT [44, 46, 47]. Activation of AKT promotes survival, proliferation and prevents apoptosis by phosphorylating and inactivating several proteins such as BAD, caspase-9, Forkhead transcription factor, and Raf-1 [47, 48]. In many cancers, deletion of phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K/AKT signaling pathway, causes abnormal activation of PI3K [46, 47].

JAK/STAT pathway Inactive STATs are coupled with the intracellular portion of EGFR in its resting state. When EGFR is activated, the STATs become activated, and translocate to the nucleus [49]. Activation of STATs leads to the expression of genes involved in proliferation and differentiation. Continuous activation of STAT proteins has been identified in numerous primary cancers and tumor-derived cell lines [50].

Phospholipase Cγ Activated PLCγ hydrolyzes phosphatidylinositol 4,5-diphosphate (PtdIns(4,5)-P_2) to give inositol 1,3,5-triphosphate (IP_3), which mediates intracellular calcium release, and 1,2-diacylglycerol (DAG), a cofactor of protein kinase C (PKC) activation [51, 52]. Protein kinase C activation results in MAPK and c-Jun NH_2-terminal kinase (JNK) activation [53, 54]. Enhanced activation of this pathway is involved in invasion and metastasis [55].

These signaling pathways activate various transcription factors, which in turn, activate the expression of genes that induce cellular responses such as proliferation, invasion, migration, adhesion, differentiation, and apoptosis [35, 56] [37].

1.3.3 EGFR endocytosis

During signal termination, activated EGFR complexes migrate from the caveolae/raft component of the cell membrane to the bulk membrane component and are endocytosed in clathrin-coated pits [57]. Two distinct processes determine the fate of the internalized receptors. The first process is regulated by a ubiquitin ligase, Cbl. After the activation of EGFR by its ligand, Cbl is rapidly recruited to the EGFR and mediates its ubiquitination [58, 59]. Recruitment of Cbl to the receptor-ligand complexes in early endosomes, targets the receptors for lysosomal degradation by promoting receptor ubiquitination. In the absence of Cbl, receptors are instead recycled to the plasma membrane [60]. The second process that determines the fate of the internalized receptor is the stability of the receptor-ligand complex in the mildly acidic endosomal environment. Activated EGFR homodimers are relatively stable and remain bound to Cbl, which results in their endocytic sorting to the lysosomes for degradation. On the other hand, EGFR–HER2 heterodimers are less stable and uncouple in early endosomes, causing Cbl to dissociate from the receptor-ligand complex. These receptors travel through the recycling pathway that returns them to the plasma membrane [61]. In addition to receptor endocytosis, EGFR undergoes metabolic turnover with a half-life of roughly 10-14 hours in fibroblasts and epithelial cells [62, 63], and of approximately 20-48 hours in transformed cells [63, 64]. On average, a receptor will cycle dozens of times through the endocytic pathway during its life span with a low probability of degradation.

1.4 Role of EGFR in Cancer

1.4.1 EGFR expression in human carcinomas

The complexity of the signal transduction pathways induced by EGFR activation and their importance in cell growth and survival, highlights the potential role of EGFR alterations

in the development and maintenance of some pathological diseases such as cancer. The discovery that dysregulation of the EGFR signaling pathway might contribute to malignant transformation was initially made in the early 1980s where studies showed that EGFR is the cellular homolog of the avian erythroblastosis virus v-erbB oncogene, which encodes a truncated protein preserving the transmembrane domain and the domain involved in cell proliferation similar to that of the EGFR [65, 66].

Many cell types express EGFR, including epithelial and mesenchymal lineages [67], with levels ranging between 40 000 and 100 000 receptors per cell [68]. An over activated EGFR can transform a normal cell into a malignant cell by providing constant signals for cell proliferation, survival, angiogenesis and metastasis, which are the basic properties of cancer [69]. The levels of EGFR expression are increased in many malignant cells. This increased expression results from a variety of mechanisms such as: receptor overexpression, overproduction of growth factors, ligand-independent receptor activation, cross-talk with heterologous receptors, activating mutations, alterations in the dimerization process, limited or enhanced endocytosis of activated receptor, deficiency of specific phosphatases deactivating the phosphorylated EGFR tyrosine residues, and limited turnover [70-72].

EGFR is highly expressed in a variety of tumors including non-small cell lung cancer (NSCLC), breast, HNSCC, gastric, colorectal, esophageal, prostate, bladder, renal, pancreatic, and ovarian cancers [73-77]. High EGFR expression has been associated with advanced tumor stage, resistance to standard therapies (hormonal therapy, chemotherapy, and radiation) [78-80] and poor patient prognosis [75, 81, 82]. From 74 studies in these cancer types, 70% showed a correlation between high EGFR expression and a reduction in recurrence-free survival or overall survival rates [25]. In a study of 140 patients with head

and neck cancers, high levels of EGFR correlated with a risk of relapse and death [73]. The 5-year survival rate for patients with EGFR-negative tumors was 81%, while patients with EGFR-positive tumors had a survival rate of 25%. Likewise, the 5-year relapse-free survival rate for patients with EGFR-negative tumors was 77% compared with 24% for patients with EGFR-positive tumors [73]. In other studies, high expression of both EGFR and its related ligand also appeared to be a strong predictive tool in some cancer types. For example, a study of 40 patients with esophageal cancer, showed a 69% 5-year survival rate for patients with low levels of both EGFR and EGF, in contrast to a 14% 5-year survival rate for patients with high levels of the same receptor and ligand [83]. These results indicate that high levels of EGFR and its ligand correlate with aggressive growth behavior in some tumors that cannot be fully controlled by conventional treatments. These cancers are appropriate candidates for EGFR targeted therapies.

1.4.2 EGFR expression in HNSCC

Worldwide, HNSCC is the sixth most common form of cancer, with approximately 600,000 patients newly diagnosed each year [84]. Over 50% of the newly diagnosed patients do not achieve complete remission, and approximately 10% relapse with metastasis to distant organs [85]. EGFR is highly expressed in a majority of epithelial malignancies including HNSCC [86]. Studies have shown that approximately 90% of HNSCCs express elevated EGFR levels compared with levels in normal mucosa from patients without cancer [87]. EGFR overexpression in HNSCC correlates with a more aggressive disease, resistance to both chemotherapy and radiotherapy, and a poor prognosis [88-90]. Due to its critical role in cell survival and proliferation, EGFR has been a target of anticancer treatments [91].

1.5 Targeting Receptor Tyrosine Kinases

1.5.1 EGFR Tyrosine Kinase as a target for anticancer therapy

EGFR contributes to a number of processes important in cancer development and progression, including cell proliferation, apoptosis, angiogenesis, and metastasis. The critical role that EGFR plays in many tumors including HNSCC, colorectal cancer, glioblastoma, and NSCLC [92], has led to an extensive search for specific EGFR inhibitors and their downstream targets. Two therapeutic approaches have been shown most promising and are currently being used to inhibit the EGFR in clinical studies. One approach involves the use of monoclonal antibodies, such as cetuximab and panitumumab, which compete for the extracellular domain of receptor tyrosine kinases by blocking ligand binding and inhibiting receptor activation [93-95]. The other therapeutic approach involves small molecule tyrosine kinase inhibitors (TKIs) that inhibit the phosphorylation of intracellular tyrosine residues located on transmembrane receptor TKs by the blockage of ATP-binding sites [96, 97]. In order to specifically inhibit EGFR kinase activity, many natural and synthetic compounds that compete with ATP for EGFR were screened. Numerous identified compounds that effectively inhibit EGFR kinase activity differ in their abilities to bind to the ATP-binding pocket, either reversibly or irreversibly, or in their capabilities to inhibit other members of the ErbB family of receptors [98]. Two TKIs of the EGFR are now in clinical development and have been evaluated in HNSCC: Gefitinib (ZD1839, Iressa) and Erlotinib (OSI-774, formerly known as CP-358, 774, Tarceva) [99].

Erlotinib (tarceva) is an oral low-molecular weight quinazoline-based agent that reversibly inhibits the tyrosine kinase activity of EGFR and has selectivity more than 1000

times greater than other tyrosine kinase inhibitors [12, 70] and it has been approved by the Food and Drug Administration since November 2004 [100]. Studies in human cancer cells found that erlotinib inhibits epidermal growth factor-dependent cell proliferation at nanomolar concentrations and induces both G_1 cell-cycle arrest and apoptosis [21]. The maximal tolerated dose of erlotinib on an extended daily schedule was 150 mg/daily. The most recurrent adverse effects seen with erlotinib are an acneiform skin rash and diarrhea. Headache, mucositis, hyperbilirubinemia, neutropenia, and anemia have also been reported [101, 102]. The antitumor activity of erlotinib as single-agent therapy has been observed in heavily pretreated patients with advanced head and neck cancer, ovarian cancer and NSCLC [103]. In skin and tumor biopsy specimens, phosphorylated forms of EGFR, AKT, and ERK were lower after treatment with erlotinib [98]. Moreover, erlotinib selectively inhibited molecular effectors involved in the invasion of human glioblastoma cell lines expressing the EGFRvIII mutant. EGFR-specific monoclonal antibodies such as cetuximab cannot recognize this mutant receptor, displaying the advantages of using EGFR-TKIs over monoclonal antibodies [98]. In mouse xenograft models, erlotinib in combination with cisplatin chemotherapy produced increased antitumor activity over that of cisplatin alone, without any increased toxicity [34]. A Phase II trial of erlotinib in recurrent or metastatic HNSCC has been reported with a response data from 78 patients, which included 10 patients (13%) with partial responses, 23 (29%) with stable disease and 45 (58%) with progressive disease [104]. In addition to the monotherapeutic approach, the effects of combining erloitinb with chemotherapeutics are being evaluated in patients with various solid tumors including HNSCC [34].

1.5.2 Resistance of HNSCC to EGFR inhibitors

HNSCC cells have the ability to undergo diverse signaling pathways for growth advantage, cell survival and evasion of apoptosis. Despite the high levels of EGFR expression within the tumor, clinical data show that many patients are resistant to EGFR inhibitor treatment, underlining that simple EGFR expression is not a reliable predictor of response to therapy. EGFR TKIs as a monotherapy have had limited results in patients with HNSCC. A Phase II trial of gefitinib in 52 patients with recurrent or metastatic HNSCC showed an overall response rate of 11% [105]. Similarly, a study of erlotinib in 115 patients with recurrent and/or metastatic HNSCC showed a response rate of 4% [104].

K-ras (Kirsten rat sarcoma viral oncogene homolog) mutations predict resistance to EGFR inhibitors. Clinical trials of NSCLC patients with K-ras mutations not only fail to benefit from erlotinib in combination with chemotherapy, but experience decreased survival and time-to-progression compared with chemotherapy treatment alone in the first-line metastatic setting [106]. However, K-ras mutations rarely occur in HNSCC. H-ras (Harvey rat sarcoma viral oncogene homolog) mutations are more common in HNSCC and may play an important role in resistance to EGFR-targeted therapies [107, 108].

Secondary or acquired resistance usually occurs after prolonged treatment. Several mechanisms contribute to the development of resistance including epithelial-mesenchymal transition, the development of secondary mutations in EGFR, activation of alternative pathways, and constitutive activation of downstream pathways. Epithelial-to-mesenchymal transition (EMT) is characterized by morphology and motility changes and is indicated by increased expression of vimentin, and by decreased expression of E-cadherin and claudins 4 and 7 [109]. Recently, cortactin, a cytoskeletal protein that regulates actin assembly,

receptor-mediated endocytosis, and epithelial-to-mesenchymal phenotypic conversion of cells, has been associated with gefitinib resistance and invasive phenotype in HNSCC [110, 111]. In addition, the E-cadherin repressor, delta-crystallin enhancer binding factor 1 (deltaEF1) was recently identified as a regulator of mesenchymal phenotype and correlated with erlotinib resistance in HNSCC *in vitro* [111].

Inherent cell signaling is a significant factor in response to therapy. Up-regulation of cyclin D1 in HNSCC cell lines has been specifically associated with resistance to gefitinib. Upregulation of cyclin D1 results in the activation of cyclin-dependent kinase 4 (CDK4), which hyperphosphorylates retinoblastoma protein (pRb) [112]. Retinoblastoma protein is a tumor suppressor and is inactivated by phosphorylation [113]. Additionally, mutations or decreased expression of PTEN, regulator of PI3K/AKT signaling, have shown resistance to EGFR inhibitors. Loss of PTEN uncouples the EGFR from its downstream signaling pathway leading to constitutive activation of AKT, which causes the cell to survive independently of EGFR [114]. One way to overcome resistance to EGFR-targeted therapy is the use of combination therapies with different mechanisms of action to target the network of pathways involved in HNSCC pathogenesis.

1.6 The Mevalonate Pathway

1.6.1 The mevalonate pathway metabolites

The mevalonate pathway first step results in the synthesis of 3-hydroxy-3-methylglutaryl-CoA (HMG)-CoA from acetyl-CoA through acetoacetyl-CoA. 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the rate-limiting enzyme of the pathway, catalyzes the conversion of HMG-CoA to mevalonic acid. [115]. The mevalonate pathway produces

various end products that are critical for functioning in both normal and cancerous cells. These products include cholesterol, dolichol, ubiquinone, isopentenyladenine, geranylgeranyl pyrophosphate, and farnesyl pyrophosphate [115]. Cholesterol serves as a precursor for the synthesis of steroid hormones and bile acid and is essential in maintaining cellular membrane structure and integrity [115]. Dolichol is a carrier molecule of oligosaccharides in N-linked protein glycosylation used for the production of glycoproteins [115]. Ubiquinone is involved in mitochondrial respiration, while isopentenyladenine is an important substrate for the modification of certain tRNAs [115]. Geranylgeranyl transferase (GGTase) and farnesyl transferase (FTase) use geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), respectively, for post-translational modifications of various cellular proteins. These proteins include Ras, and many small GTP-binding proteins, such as members of the Rab, Rac, and Rho families that regulate cell proliferation, intracellular trafficking, and cell motility [116, 117] (Figure 1.3).

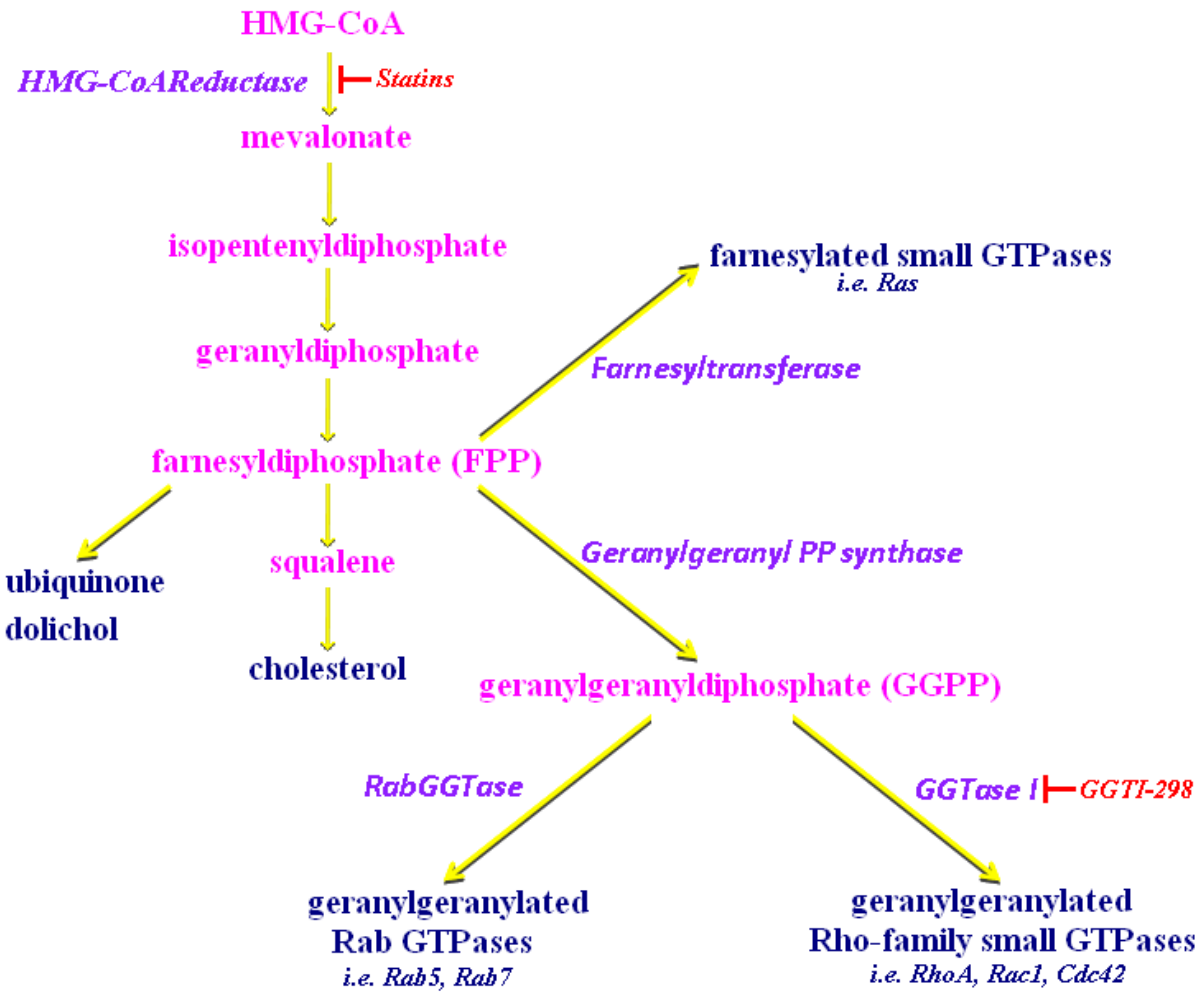


Figure 1.3 Schematic diagram of the mevalonate pathway Inhibition of HMG-CoA reductase by statins has multiple downstream effects, including inhibition of cholesterol biosynthesis and of isoprenoid intermediates such as FPP and GGPP. GGTI-298 is an inhibitor of GGTaseI and prevents protein prenylation of the Rho-family GTPases.

1.6.2 The statin family

Malignant cells appear highly dependent on the sustained availability of the end products of the mevalonate pathway [118]. Deregulated or elevated activity of HMG-CoA reductase has been shown in a range of different tumors [118]. The statin family of drugs, widely used as hypercholesterolemia treatments, is a potent inhibitor of HMG-CoA reductase [119]. Some studies have shown that statins can reduce cancer incidence by 28–33% [119, 120]. Indeed, recent analyses have shown that statin treatment can directly block tumor cell growth, invasion, and metastatic potential both *in vitro* and *in vivo* [118] [121, 122]. Statins bind to the active site of HMG-CoA reductase with up to a 1,000 times higher affinity than its natural substrate [119]. Due to their diverse effects on various aspects of carcinogenesis, several clinical trials have been undertaken to assess the potential clinical benefit of HMG-CoA reductase inhibitors. Naturally or chemically synthesized, statins share a common side chain that exists either in a closed ring (inactive, lactone pro-drug) or an open ring (active, acid) form [123, 124].

1.6.3 Role of mevalonate metabolites in the transduction of EGFR-Mediated Signaling

Mevalonate pathway metabolites are a critical requirement for the function or the appropriate expression/localization of RTKs. Depletion of mevalonate metabolites through inhibition of HMG-CoA reductase has the potential to inhibit both the EGFR and its downstream signaling cascades [115, 125, 126]. The mevalonate metabolites dolichol and cholesterol are required for the proper functioning of EGFR. Dolichol is involved in the N-linked glycosylation of numerous receptor tyrosine kinases, including EGFR, which allows them to properly change conformation and bind their specific ligands [126, 127]. The ligand-binding domain of EGFR is glycosylated, and this modification is necessary for efficient

ligand binding and activation of EGFR [127]. EGFR activation is also regulated by membrane cholesterol composition [128]. Proteins that require farnesyl and geranylgeranyl post-translational modifications include the Ras, Rho, and Rab families. Once activated, these proteins can then regulate RTK activity on cell proliferation, cell survival, intracellular trafficking and cell motility [129, 130] (Figure 1.4).

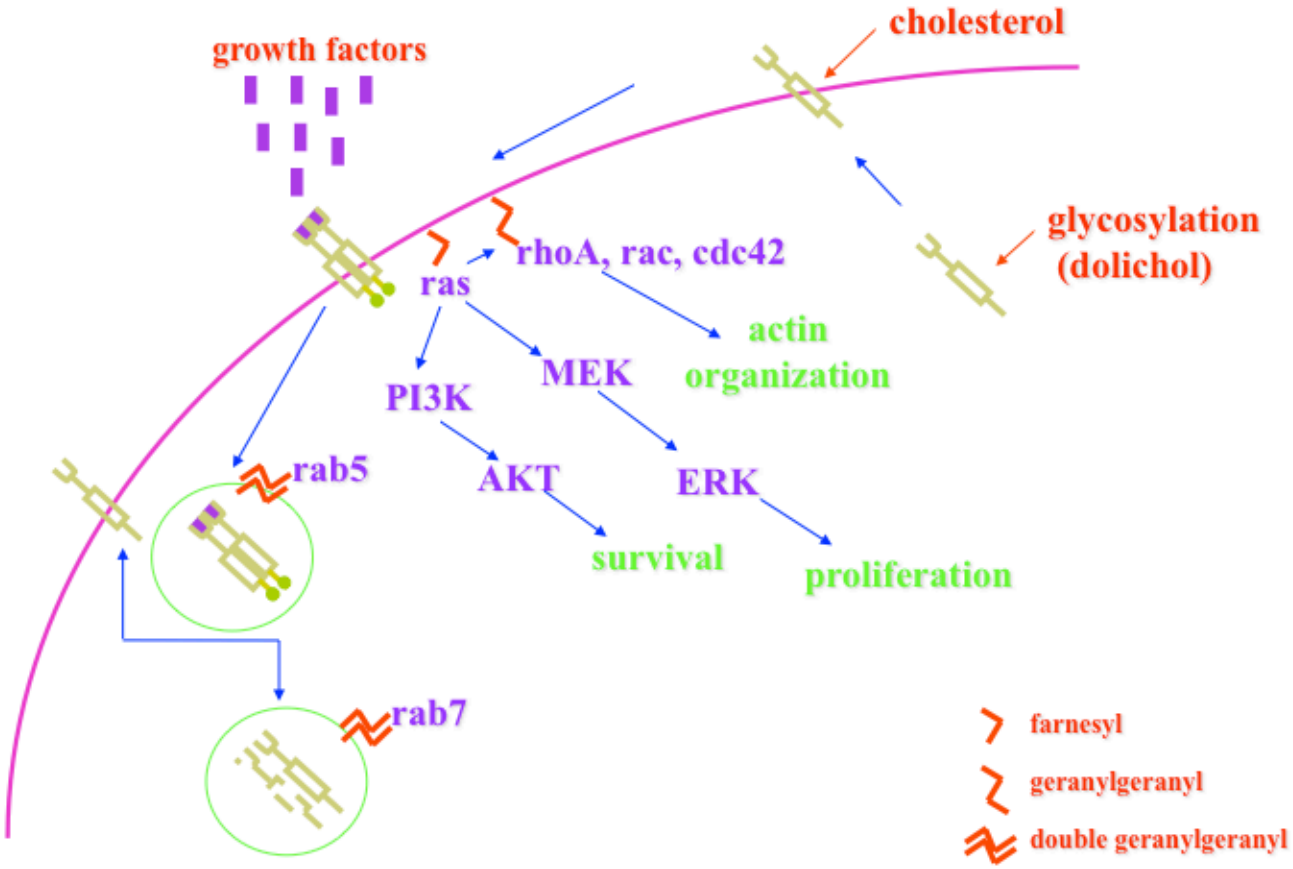


Figure 1.4 Role of mevalonate metabolites in RTK activation and downstream signaling. Ras activates several pathways, of which the mitogen-activated protein (MAP) kinase cascade involved in cell growth and division and the AKT pathway that inhibits apoptosis. Rho proteins regulate actin cytoskeletal organization. Rab GTPases regulate many steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion.

1.6.4 Lovastatin in combination with an EGFR inhibitor

HMG-CoA reductase and EGFR-targeted therapies may be linked because of the potential of mevalonate metabolites to affect the function of the EGFR signaling pathway. Lovastatin, one of the first-generation statins, is a specific and irreversible competitive inhibitor of HMG-CoA reductase [119]. It has been extensively used for the treatment of hypercholesterolemia and its active open ring form can effectively block the mevalonate pathway by binding to the active site of HMG-CoA reductase with greater affinity than the natural substrate [119]. It has been shown that lovastatin treatment of HNSCC cells *in vitro* inhibited ligand-induced activation of EGFR [131]. A recent study in our laboratory showed the ability of lovastatin to inhibit ligand-induced EGFR dimerization, internalization, and autophosphorylation [132]. Furthermore, lovastatin showed significant effects on HNSCC actin architecture, and inhibiting rhoA similarly affected EGFR dimerization and activation [132]. Actin organization has been shown to have a role in EGFR localization and activation [133]. In many malignancies, including HNSCC, EGFR and its downstream signaling pathways are often deregulated, leading to cell hyperproliferation, enhanced cell survival and increased metastatic potential [25]. Of particular importance in cancer therapy is the hyperactivation of the PI3K/AKT pathway that regulates cell survival, cellular metabolism and protein translation [134]. In the previously mentioned study, lovastatin inhibited EGF-induced AKT activation and its downstream targets [132].

HNSCC patients have very limited treatment options when presenting with metastatic disease [135] and inhibitors of EGFR have limited therapeutic efficacy. In order to enhance their clinical activity, EGFR inhibitors require combination-based therapies. Our recent studies have shown that the depletion of mevalonate metabolites affects the activity of a

number of cell signaling cascades, including inhibitory effects on EGFR activity and their downstream signaling pathways [131, 136]. The combination of gefitinib and lovastatin was evaluated *in vitro* in a total of 16 HNSCC, NSCLC, and colon carcinomas cell lines and showed a potent synergistic cytotoxic response [131]. The combination of statins and EGFR inhibitors, with each targeting the EGFR through distinct mechanisms, represents a novel combinational therapeutic approach in SCC, as well as in other cancers in which EGFR is involved in their pathogenesis [137].

1.6.5 Statin associated myopathy

Several hypotheses have been proposed for the pathogenic mechanisms that account for the spectrum of muscle dysfunction related to HMG-CoA reductase inhibitors. These have focused on this toxicity to uncover the effects of HMG-CoA reductase inhibitors on ubiquinone, membrane function, calcium regulation, and drug interactions. Ubiquinone is one of the end products of the mevalonate pathway and, therefore, can be depleted by treatment with statins.

Based on our current studies, our high dose, single agent, Phase I statin trials in HNSCC and acute myeloid leukemia, have shown durable responses (on study for greater than 6 months) in 6 of the 40 patients treated (15%) [138, 139]. In our rosuvastatin/tarceva combination study (ClinicalTrials.gov Identifier: NCT00966472), 20% of the 24 NSCLC and HNSCC patients treated showed durable stable disease. In these patient populations with aggressive refractory disease, overall survival is generally less than 6 months, thus, we believe that statins demonstrated therapeutic benefits in a subset of these patients. However in 13/40 patients treated (32.5%) in our single agent lovastatin phase I trials and 9/24 patients treated (40%) in our combination phase I trial, significant muscle toxicities were observed at

a higher rate than at cholesterol lowering doses of statins (0.5-5%) [140] limiting the potential therapeutic benefit of this approach. To address this toxicity limitation, we are currently evaluating differing treatment schedules of the rosuvastatin/tarceva combination as well as evaluating the valuable patient tumor and serum samples collected in this trial to potentially identify markers of both toxicity and response.

The goal is to identify agents that can recapitulate statins ability to induce tumor specific apoptosis by replacing statins in this therapeutic treatment and uncovering novel strategies that will lead to more refine and effective therapeutic approaches.

1.7 Downstream Targets of the Mevalonate Pathway

1.7.1 Protein prenylation

Studies in the early 1980s identified that membrane localization of the small G proteins was critical to their function, and that this localization was dependent on a post-translational modification that resulted in attachment of a lipidated hydrophobic domain that plays a role in membrane attachment [141]. Prenylation is a common feature of many members of the Ras and Rho family of small guanosine triphosphatases (GTPases). The enzymes farnesyltransferase (FTase), geranylgeranyltransferase (GGTase I) and Rab GGTase (GGTase II) catalyze the addition of farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) derived from the mevalonate pathway [142, 143]. FTase uses FPP (C15) as a prenyl donor to transfer a farnesyl group to the C-terminal CAAX motif (C is cysteine, A is usually an aliphatic residue, and X is any amino acid) of their target proteins (e.g. Ras GTPases) while GGTase uses GGPP (C20) as a prenyl donor to transfer a geranylgeranyl moiety to their target proteins (e.g. Rho GTPases) [142]. Rab proteins contain

a CC or CXC C-terminal sequence and their prenylation is catalyzed by GGTase II. Rab proteins are bound by the Rab escort protein over these more-conserved regions and then presented to the Rab GGTase. Rab GGTase often transfers 2 geranylgeranyl groups to the C-terminal cysteines of Rab proteins [142]. Following prenylation, proteins with carboxy-terminal CaaX sequences typically undergo two additional post-translational modifications in which the aaX is cleaved by the endoprotease Ras Converting Enzyme 1 (Rce-1) followed with subsequent methylation of cysteine by isoprenyl–cysteine carboxyl methyltransferase (ICMT) [144] (Figure 1.5).

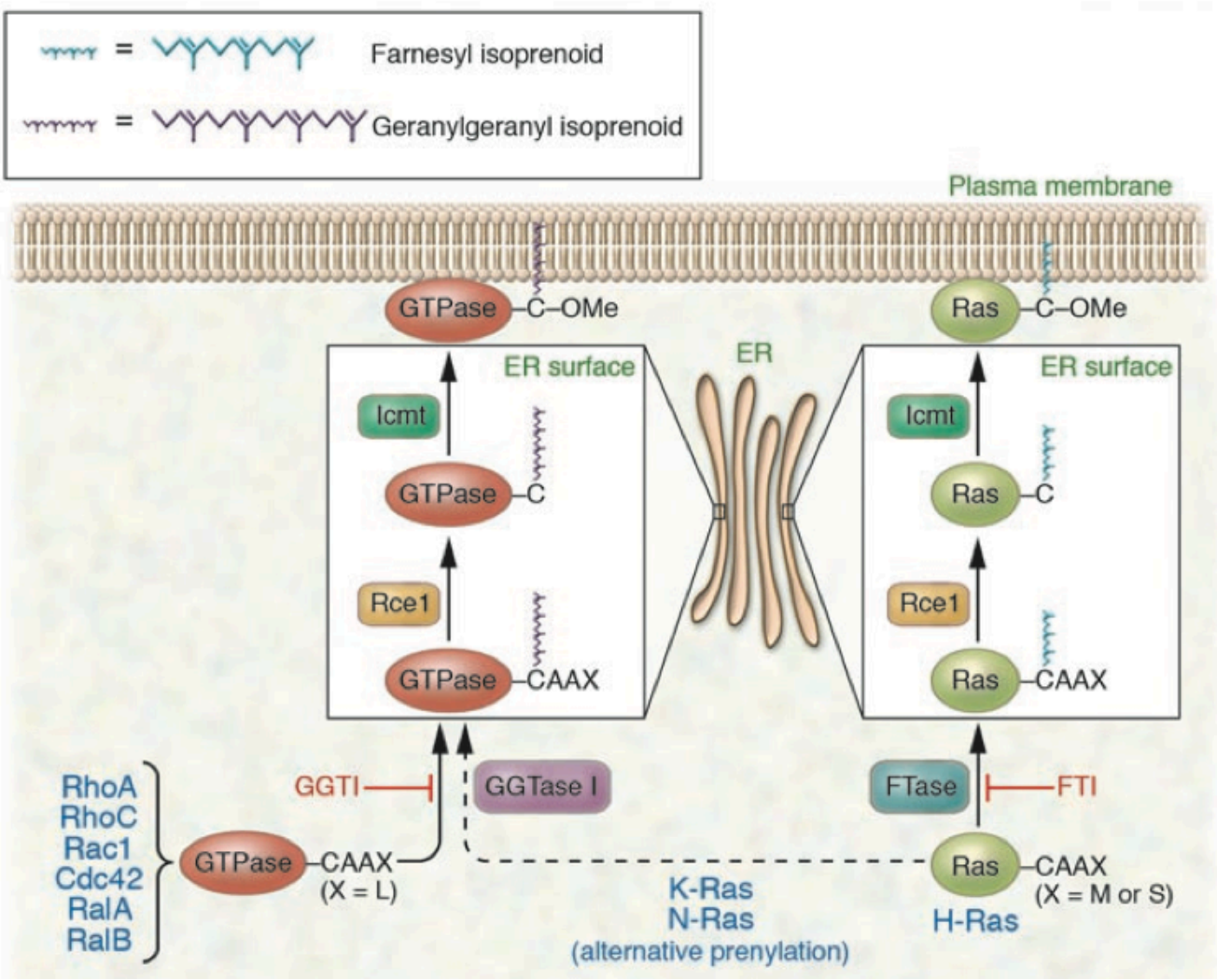


Figure 1.5 The three-step prenylation process (I) Prenyltransferase adds either a farnesyl or geranylgeranyl group to the cysteine of the CAAX motif. (II) RCE1 removes the AAX residues with subsequent methylation of cysteine by ICMT. (III) Methylation of cysteine by ICMT. Adapted from Philips, M.R., 2001 [145].

1.7.2 Prenylated Ras-like GTPases

The number of possible prenylated proteins is estimated to be 2% of total cellular proteins and include the small G proteins [146]. The small G proteins are monomeric G proteins with molecular masses of 20–40-kDa [147] and are often referred to as the Ras superfamily GTPases. These small GTPases function as GDP/GTP-regulated molecular switches [148], and are divided into five major subfamilies: Ras, Rho, Rab, Ran and Arf. The Rho, Ras, and Rab GTPases are discussed below:

The Ras Family The Ras sarcoma (Ras) GTPases have been under intensive research, partly due to their critical roles in human oncogenesis [149]. Ras proteins serve as signaling nodes activated in response to diverse extracellular stimuli. Activated Ras interacts with multiple downstream effectors, which regulate cytoplasmic signaling networks that control gene expression and regulation of cell proliferation, differentiation, and survival [149].

The Rho Family Like Ras, Ras homologous (Rho) proteins also serve as key regulators of extracellular-stimulus-mediated signaling networks that regulate actin organization, cell cycle progression and gene expression [150]. Rho GTPases have been implicated in the regulation of cell polarity, cell movement, cell shape, and cell-cell and cell-matrix interactions, as well as in regulation of endocytosis and exocytosis [151].

The Rab Family First described as Ras-like proteins in brain (Rab), Rab GTPases are regulators of intracellular vesicular transport and the trafficking of proteins between different organelles of the endocytic and secretory pathways [152]. Rab proteins localize to specific intracellular compartments consistent with their function in distinct vesicular transport

processes [152].

1.7.3 Inhibitors of prenylated GTPases

Interest in developing inhibitors of farnesylation as anti-cancer drugs was prompted by the realization more than 20 years ago that a significant percentage of human cancers (8% - 93% depending on the tumor type) harbor activating oncogenic mutations in the RAS genes [153], and that RAS GTPases require this lipid post-translational modification for their malignant transforming activity [154]. Many signal transduction pathways activated by RAS involve proteins that require farnesylation or geranylgeranylation in order to mediate tumor cell survival, growth, proliferation, migration and metastasis. These discoveries led to the development of farnesyltransferase inhibitors (FTIs) and geranylgeranyltransferase 1 inhibitors (GGTIs) as potential anticancer drugs.

Various preclinical studies in the 1990s showed that FTIs are highly successful in killing cancer cells *in vitro* and in animals with very little toxicity, which generated much excitement and raised hope that a novel anticancer drug has been discovered [155]. Unfortunately, the efficacy of these agents against tumors has been disappointing. Although some single-agent activity toward hematopoietic malignancies has been demonstrated [155], no such activity has been found for solid tumors. However, combinations of FTIs with cytotoxic agents improve the responses of patients with locally advanced breast cancer or other advanced solid tumors [156-159]. Ongoing trials are designed to test FTIs in combination with other chemotherapeutic agents [155].

FTI research resulted in two major discoveries: First the efficacy of these agents in preclinical testing did not correlate with the presence or absence of activating mutations of

Ras [160], suggesting that FTIs limit cell proliferation and survival through at least one other target. Second, although model tumors driven by oncogenic H-Ras were highly susceptible to FTIs, tumors driven by oncogenic N-Ras and K-Ras were not [155]. The vast majority of human solid tumors driven by oncogenic Ras involve K-Ras and hematopoietic malignancies are driven by N-Ras. The resistance of K-Ras and N-Ras suggest that there's an alternative prenylation, where in the presence of FTIs they are geranylgeranylated and retain full biological activity [161]. This discovery helped propel interest in GGTase-I as an additional therapeutic target.

Numerous geranylgeranylated CAAX proteins are critical for oncogenesis downstream of Ras [162-166]. GGTIs have now been developed to target these substrates. Similar to FTIs, GGTIs have shown promising results *in vitro* and in animal models, and one GGTI (GGTI-2418) has recently entered Phase I clinical trials [167]. As monotherapies, GGTase-I–selective inhibitors have shown the ability to impair transformation *in vitro* and tumor growth *in vivo* [168-171]. Unlike FTIs, GGTIs cause both cell cycle arrest in G1 and apoptosis [168-170]. Our previous studies determined that protein isoprenylation was critical to the cytotoxic effects of lovastatin. According to these results, GGPP reversed the effects on tumor cell viability induced by lovastatin in HNSCC. Interestingly FPP had only partial protective effects [172]. Additionally, our microarray analysis identified rhoA as a gene that was regulated by lovastatin [172]. RhoA is a member of the Rho family of small GTPases that regulate actin cytoskeletal reorganization, thereby regulating cell shape and motility [173, 174]. Rho proteins have been implicated in cancer cell invasion, growth, and survival [175, 176] and require the isoprenoid moiety GGPP for their cellular localization and function. GGPP facilitates their membrane localization and transduction of upstream signals

[176]. Furthermore, our studies have also shown that lovastatin induces actin disorganization through inhibition of geranylgeranylation of the rho family of proteins, resulting in the inhibition of EGFR dimerization, activation and downstream signaling [132].

Given these results, further investigation of GGTIs as antitumor agents in HNSCC is an important avenue. These agents have biological activities that are desirable for novel anticancer drugs as they inhibit human tumor growth *in vitro* and *in vivo* with a mechanism that is consistent with a cell cycle arrest at the G1 phase. Further evidence for targeting GGTase I for the development of novel anticancer drugs includes not only the fact that many of its substrates, such as RhoA, Rac1, cdc42, and R-Ras mediate oncogenesis and/or metastasis [177] but also that K-Ras becomes a substrate for GGTase I when human cancer cells are treated with FTIs [156, 161, 178].

1.8 Rationale and Hypothesis

Treatment of advanced HNSCC has mainly consisted of a combination of surgery and radiation, or combined radiation and chemotherapy. Unfortunately, these standard therapies are not adequate in this patient population, due to the high incidence of locoregional failure and poor HNSCC patient survival [5]. Combinations of currently available treatment modalities have been moderately successful; however, these combination therapies often result in an unacceptable high degree of toxicity and damage to the surrounding normal tissues without any survival advantage [5, 15]. This is mostly due to the lack of specificity for the cancer cells and the high degree of toxicity of these standard therapies. Current research has led to a better understanding of tumor physiology and the differences between tumor and normal cells. As a result, a variety of novel therapeutic approaches have been developed to exploit these differences in order to increase specificity

for the tumor cells and avoid damage to normal tissues [22]. Over the past decade, significant progress has been made in the development of targeted therapies, selectively targeting malignancies while sparing normal tissues. One such approach revolves around targeting the EGFR that is overexpressed in HNSCC and plays a role in its pathogenesis and not highly expressed in adult normal tissues. This has provided the rationale for the development and implementation of EGFR- targeted therapies, with particular interest to receptor tyrosine kinases inhibitors (RTKs). Unfortunately, EGFR TKIs as a monotherapy have had limited results in patients with HNSCC. The mevalonate pathway metabolites are a critical requirement for the function or the appropriate expression/localization of RTKs. Previous studies done in our laboratory has demonstrated that the combination of lovastatin with EGFR tyrosine kinase inhibitors induced robust synergistic cytotoxicity [132]. However, the use of statins was associated with significant toxicities including higher than anticipated rate of muscle pathologies [140]. The objective of this study is to evaluate the efficacy of combining the downstream mevalonate pathway inhibitor, GGTI-298, with an EGFR TKI on cell viability and downstream function of EGFR in HNSCC.

Hypothesis The combination of GGTI-298 with tarceva inhibits proliferation and induces enhanced cytotoxicity of HNSCC cells.

1.9 Approach

1.9.1 Objective 1: To validate the effects of GGTI-298 on protein prenylation focusing on Rho GTPases

The effects of GGTI-298 on protein prenylation were determined by western immunoblot assay and compared to the effects of lovastatin in SCC9 and SCC25 cells. The effect of protein geranylgeranylation inhibition on actin cytoskeleton was observed by phalloidin staining of actin in SCC25 treated with increasing concentrations of GGTI-298. The role of Rho GTPases on cell migration was determined by a scratch wound assay in SCC9 and SCC25.

1.9.2 Objective 2: To assess the effects on cell viability of GGTI-298 in combination with tarceva

The effects of GGTI-298 in combination with tarceva on cell viability were assessed in both SCC9 and SCC25 HNSCC cell lines. The role of induction of apoptosis as a mechanism, by which GTTI-298 and tarceva induce cytotoxicity, was assessed by flow cytometry and validated by PARP cleavage.

1.9.3 Objective 3: To evaluate GGTI-298 as a novel inhibitor of EGFR function and its downstream signaling pathways

The effects of GGTI-298 treatment on ligand binding induced dimerization of the EGFR were determined by cross-linking experiments. The effects of GGTI-298 on EGFR internalization were evaluated by immunohistochemistry in SCC9 cells and validated by fluorescence microscopy of internalized EGF ligand in SCC25 cells treated with GGTI-298. The combination effects of GGTI-298 and tarceva on EGFR activation and downstream function were assessed by western immunoblot assay: pEGFR, pAKT, and pERK.

Chapter 2: MATERIALS AND METHODS

2.1 Cell Culture

SCC25 and SCC9 HNSCC cells (American Type Culture Collection [ATCC], Manassas, VA) were grown as monolayers in Dulbecco's Modified Eagle medium (DMEM, Cellgro, Manassas, VA) supplemented with 10% HyClone fetal bovine serum (FBS, Fischer Scientific Co., Toronto, ON); penicillin, 100 U/mL; streptomycin sulfate, 100 µg/mL (Sigma, St. Louis, MI). All cells were grown at 37ºC in 5% CO_2 in a HERA cell incubator (Kendro Laboratory Products, Newton, CT). Cells were regularly passaged after reaching 80-90% confluency by a wash with warm PBS (Dulbecco's Phosphate Buffered Saline, Cellgro, Manassas, VA) and released using Trypsin EDTA 1X (Cellgro, Manassas, VA) for 4-10 minutes at 37ºC depending on the level of adherence of each cell type. Cells were then resuspended in 10% DMEM and a cell count was performed using the Vi-CELL® XR cell viability analyzer (Beckman Coulter, Mississauga, ON) performing the trypan blue dye exclusion method in order to determine cell concentration and viability. Cells were maintained in 75cm^2 or 175cm^2 CORNING® flasks (Corning Inc., Corning, NY). EGFR was induced with human recombinant EGF (Sigma, St. Louis, MI) diluted from a 50µg/ml stock in 10mM acetic acid and 0.1% bovine serum albumin (Sigma).

2.2 Rhodamine-Conjugated Phalloidin Treatment

In a six-well tissue culture dish, SCC25 were seeded at 2x10^5 cells in each well containing a 1 cm x 1 cm microscope cover slip. The following day, control, 0.5µM, 1µM, and 5µM GTTI-298 treated cells were incubated for 24h at 37ºC. Following treatments, cells

were fixed with 4% PBS buffered paraformaldehyde for 15 minutes at 37ºC followed by three washes with PBS. The cells were then permeabilized using 0.1% Triton X-100 (Sigma) in PBS for 15 minutes and followed by three washes with PBS. In order to label the actin cytoskeleton, cells were incubated with 100uL/well of reconstituted rhodamine-conjugated phalloidin (Cytoskeleton) in PBS for 15 minutes at room temperature. After two washes with PBS, the cover slips were mounted on microscope slides with DAPI (diluted in 50% glycerol) containing immunofluorescent mounting medium. Cells were then visualized with fluorescent microscopy (AxioCam HR, Carl Zeiss).

2.3 Scratch Wound Assay

SCC25 and SCC9 cells were seeded into 6-well tissue culture plates and grown in DMEM supplemented with 10% FCS and 1% Pen/Strep at a density that ensures a confluent state (~90%) the next day. The following day, the cell monolayers were scratched using a P20 pipette tip. Media was removed and cells were washed twice with warm PBS. Cells were then treated as control or 5μM GGTI-298. Five images per well were taken using a digital camera attached to an Eclipse TE2000-U microscope (Nikon, Missisauga, ON) for time 0 hours. Additional images were taken at 24 hours. Wound diameters for the five images taken for each condition, were measured and averaged and percentage wound closure was calculated using the following formula: [(average wound diameter at 24 hours – average wound diameter at 0 hours) / wound diameter at 0 hours] x 100.

2.4 MTT Assays

In 96-well tissue culture plates (Costar®, Corning Inc., Corning, NY) SCC9 and SCC25 were seeded at approximately 5000 cells/75µL in each well. The cells were incubated overnight to allow for cell attachment and recovery. Cells were either pre-treated with 1µM or 10µM Lovastatin (Apotex, Mississauga, ON), diluted from a 10mM stock in ethanol, for 24 hours following a combination treatment with increasing concentrations of tarceva (Biovision Inc., Milpitas, CA), diluted from a 10mM stock in DMSO, for a total of 72 hours or a combination treatment with 1µM or 10µM Tarceva with increasing concentrations of GGTI-298 (*N*-[4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-(1-naphthalenyl)benzoyl]-L-leucine methyl estertrifluoroacetate salt) (Tocris Bioscience, Bristol, UK), diluted from a 10mM stock in DMSO, for a total of 48 hours. Each condition contained 8 wells for a total of 8 replicates. Following treatments, 42µL/well of a 5mg/ml solution in phosphate buffered saline of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium substrate (Sigma) was added to each and incubated for 4 hours at 37ºC. The resulting violet formazan precipitate was solubilized by the addition of 84µL/well lysis solution composed of 0.01N HCl in 10% SDS (Fischer Scientific Co., Toronto, ON) overnight at 37ºC. The following day, absorbance readings at 570nm were done on the plates in a Multiskan Ascent plate reader (MTX Lab systems Inc., Vienna, VA).

2.5 Cell Viability Assays

For each cell line, duplicates of this experiment were performed in 6-well tissue culture plates (Corning Incorporated, Corning NY). SCC9 and SCC25 cells were seeded at $1x10^5$ cells/well. Cells were treated at 90% confluency with medium, 10µM Tarceva, 5µM

GGTI-298, 10μM GGTI-298 or a combination treatment of 10μM GGTI-298 with 10μM Tarceva and incubated for 24hrs and 48hrs at 37ºC (5% CO_2). Following treatment cells were washed with PBS and trypsinized for 4-10 minutes at 37ºC depending on the level of adherence of each cell type. Cells were then resuspended in 10% DMEM and a cell count by trypan blue assay was performed as described in 2.1 at 24 hours and 48 hours. The average and standard deviation for each cell line and treatment were calculated and plotted on a graph.

2.6 Propidium Iodide Flow Cytometry

In 10-cm BD Falcon™ plates (Becton Dickinson, Mississauga, ON), SCC9 and SCC25 cells were seeded at $2x10^6$ cells in each plate and incubated overnight at 37ºC (5% CO_2) to allow for cell attachment and recovery. The following day, one set of cells were treated as single treatments with 10μM Lovastatin for 72 hours or as pre-treatment with 10μM Lovastatin for 24 hours followed by combination with 10μM Tarceva for a total of 72 hours. Another set of cells was treated either with 10μM GGTI-298 alone or in combination with 10μM tarceva for 48 hours. Each treatment was performed in triplicates in 10% DMEM. Following treatments, the media for each sample was collected in 50mL Falcon tubes (Becton Dickinson, Mississauga, ON). Cells were washed with 10mL PBS and the wash was added to the same 50mL tube. Plates were trypsinized and resuspended in 10mL PBS and collected into the same conical tube. Cells were pelleted by centrifugation at 2-8ºC for 5 minutes at 2500 RPM. Supernatant was washed and cells were resuspended in 10-20mL of cold PBS and pellet was collected once again by centrifugation at 2-8ºC. Supernatant was aspirated and cells were fixed with 80% ethanol in 15mL Falcon tubes and incubated at -

20ºC for a minimum of 1 hour. Fixed cells were washed and pelleted by centrifugation at 2-8ºC for 5 minutes at 2500 RPM. Cells were resuspended in 3mL of staining buffer made of 0.2% Triton X-100 and 1mM EDTA (Sigma) at pH 7.4 and pelleted by centrifugation. Each sample was resuspended in staining buffer containing 50μg/mL propidium iodide (Sigma) and 100μg/ml RNaseA (BioShop Canada Inc., Burlington, ON) and transferred to a 5mL round bottom tube (Becton Dickinson, Mississauga, ON). Tubes were incubated for a minimum of 1 hour, in the dark, at 18-25ºC. Following incubation, cells were evaluated using the Epics XL Flow Cytometer (Beckman Coulter, Mississauga, ON) on the FL2 channel and the percentage of cells in pre-G_1 phase was determined using the ModFit LT program (Verity Software House, Topsham, ME).

2.7 Surface EGFR Dimer Crosslinking

SCC25 and SCC9 cells were seeded in 10-cm BD Falcon™ plates and grown in DMEM supplemented with 10% FCS and 1% Pen/Strep at a density that ensures a confluent state (~90%) the next day. The following day cells were treated with medium, 10μM Lovastatin, and 10μM GGTI-298. Plates were treated in duplicates for each drug treatment, as one half is treated as control and the other with EGF ligand. After 24 hours of drug treatment, cells were stimulated with 50ng/mL EGF ligand for 15 minutes at 37ºC. Cells were then washed three times with ice-cold calcium and magnesium free (CMF) PBS. Following the washes, cells were incubated for 20 minutes at 4ºC with 1mL/10-cm plate of the membrane impermeable crosslinker BS^3 (bis[sulfosuccinimidyl] substrate) (Thermo Scientific, Rockford, IL), diluted in PBS for a final concentration of 3mM. The crosslinking reaction was quenched by adding 5mL/10-cm plate of 250mM glycine (Fischer Scientific

Co., Toronto, ON) and incubated for 5 minutes at 4ºC. Cells were then washed three times with ice-cold CMF-PBS. Cells were then lysed with 150µL/10-cm plate of RIPA lysis containing 1X protease inhibitor cocktail and 200mM glycine. Samples were homogenized and supernatants were collected followed by protein quantification as described in 2.10.

2.8 EGFR immunofluorescence

SCC25 cells were seeded at $2x10^5$ cells/well in six-well tissue culture plates containing a 1 cm x 1 cm microscope cover slip in each well and grown in DMEM supplemented with 10% FBS and 1% Pen/Strep. The following day cells were treated with medium, 10µM Lovastatin, and 10µM GGTI-298, and 10uM FTI-277 (Tocris Bioscience, Bristol, UK) for 24 hours and then stimulated with 50ng/ml EGF for 15 minutes. Following treatments, cells were fixed for 15 minutes in 4% PBS buffered paraformaldehyde at 37^0C. The cells were then washed two times with PBS and permeabilized using 0.1% Triton X-100 in PBS for 15 minutes at room temperature. Cells were then blocked with 3% FBS in PBS for 30 minutes at room temperature. The primary antibody EGFR (Cell Signaling Technology, Inc., Danvers, MA) was added at a 1:50 dilution in 3% FBS in PBS for 1 hour followed by three 5-minute washes with PBS. The cells were then blocked with 5% goat serum (Sigma) in PBS for 30 minutes at room temperature. Following the second blocking, the cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Carlsbad, CA) at a working dilution of 10µg/ml in the dark for an hour followed by three 5-minute washes with PBS (protected from light with aluminum foil). The cover slips were then mounted on microscope slides with DAPI (diluted in 50% glycerol) containing

immunofluorescent mounting medium. Cells were then visualized with fluorescent microscope (AxioCam HR, Carl Zeiss).

2.9 EGF-Alexa 488 Treatment

In a six-well tissue culture dish, SCC25 were seeded at $2x10^5$ cells in each well containing a 1 cm x 1 cm microscope cover slip. The following day, cells were treated as control and 0.5μM, 1μM, and 5μM GTTI-298 in serum free media and incubated for 24 hours at 37ºC. Following drug treatments, cells were treated with EGF-Alexa 488 from Molecular Probes® **(**Life Technologies Inc., Burlington, ON) at 50ng/mL for 30 minutes on ice. For internalization of the fluorescent EGF, a three times wash with ice cold PBS followed by the addition of warm PBS and an incubation at 37ºC for 15 minutes was performed. This was followed by a three times wash with ice cold PBS in order to stop internalization. Cells were then incubated on ice in an acid wash (0.2M acetic acid and 0.5M NaCl) for 5 minutes in order to wash off surface EGF-Alexa 488. Following three washes with cold PBS, cells were fixed with 4% PBS buffered paraformaldehyde for 15 minutes at 37ºC and then washed two times with PBS. The cover slips were then mounted on microscope slides with DAPI (diluted in 50% glycerol) containing immunofluorescent mounting medium. Cells were then visualized with fluorescent microscope (AxioCam HR, Carl Zeiss).

2.10 Cell Lysis and Protein Extraction and Quantification

On a cold surface, media was aspirated and cells were washed with cold PBS. Cells were then lysed with radio immuno precipitation assay (RIPA) lysis buffer containing 50mM

Tris-Cl (Fischer Scientific Co., Toronto, ON), 150mM NaCl, 1mM EDTA (Sigma), 1% Triton X-100 (Sigma), 1% Sodium deoxycholate (Sigma), and 0.1% SDS (Fischer Scientific Co., Toronto, ON) pH to 7.5 with 1X Protease inhibitor cocktail (PIC, Sigma) diluted from a 10X stock. When probing with phospho-antibodies additional protease inhibitors were added to RIPA: 5mM Sodium fluoride (NaF, Sigma) diluted from a 1M stock, 0.2M Sodium orthovanadate (Na_2VO_4, Sigma) diluted from a 1mM stock, and 17mM β-glycerophosphate (β-GP, Sigma) diluted from a 1.75M stock. Plates were scrapped using rubber policemen and the lysis solution was transferred to a 1.5mL tube. Cells were then sonicated at 1.5 power for 10 seconds using a Sonicator 3000 (Misonix, Newton, CT). The homogenized samples were placed on ice for 20-30 minutes and then centrifuged for 15 minutes at 14000 RPM at 4ºC. Supernatants were collected into 1.5mL tubes and stored at -70ºC. Total protein concentration was assessed using Pierce BCA protein assay protocol (Thermo Scientific) and protein absorbance was measured at 595nm.

2.11 Western Immunoblotting and Antibodies

Total protein concentration was assessed using a commercially available protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL, USA) and absorbance was measured in a Multiskan Ascent plate reader (MTX Lab systems Inc., Vienna, VA). For western blot analysis, equal amounts of total protein per sample (20-60 μg), were separated on a 6% (for EGFR dimer crosslinking) or a 10% SDS page gel (per ~20 mL 10%-separating gel: 6.67 mL of 30% acrylamide/Bis solution, 5 mL of 4X Tris-Cl/SDS (pH8.8), 8.33 mL ddH_2O, 0.133 mL 10% ammonium persulfate, and 0.016 mL TEMED. Per ~10 mL 10%-stacking gel: 1.3 mL of 30% acrylamide/Bis solution, 2.50 mL mL of 4X Tris-Cl/SDS (pH8.8), 6.10 mL

ddH_2O, 0.0.050 mL 10% ammonium persulfate, and 0.010 mL TEMED. For the electrophoresis, gels were placed in a freshly made 1X Running Buffer (for a 10X solution: 29g Tris base, 144g glycine, 10g SDS, filled to 1L with ddH_2O) and run for 20 minutes at 100 volts then from 1 hour to 3 hours (depending on the size of protein of interest) at 120 volts. Proteins were then transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA) in 1x transfer buffer [100mL of 10X TB, 10-20% methanol, filled to 1L with ddH_2O (for a 10X TB solution: 30.3g Tris base, 144g glycine, filled to 1L with ddH_2O)]. After transfer, membranes were washed with 1x Tris-buffered saline (TBS) (for a 20x solution (4L): 193.6g Tris base, 640g NaCl, bring volume to 3.2L with ddH_2O; adjust pH to 7.6 with HCl; bing volume to 4L with ddH_2O) then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. Membranes were then probed with a primary antibody dilution in 5%BSA/TBST and incubated with rocking overnight at 4^oC.

Monoclonal mouse anti-β-actin from Sigma-Aldrich (St. Louis, MO) was used at a 1:10,000 dilution. Monoclonal mouse anti-rhoA from Santa Cruz (CA, USA) was used at a 1:500 dilution. Monoclonal rabbit anti-cdc42 from from Santa Cruz (CA, USA) was used at a 1:500 dilution. Monoclonal rabbit anti-rab5 from Santa Cruz (CA, USA) was used at a 1:500 dilution. Monoclonal mouse anti-rac1 from Transduction Laboratories, BD Bioscences (Mississauga, ON) was used at a 1:1000 dilution. Monoclonal rabbit anti-PARP from Cell Signaling Technology (Lake Placid, NY, USA) was used at a 1:1000 dilution. Monoclonal rabbit anti-EGFR from Cell Signaling Technology (Lake Placid, NY, USA) was used at a 1:1000 dilution. Monoclonal mouse anti-pEGFR from Transduction Laboratories, BD Bioscences (Mississauga, ON) was used at a 1:1000 dilution. Monoclonal rabbit anti-ERK from Cell Signaling Technology (Lake Placid, NY, USA) was used at a 1:1000 dilution.

Monoclonal mouse anti-ERK from Cell Signaling Technology ((Lake Placid, NY, USA) was used at a 1:1000 dilution. Monoclonal rabbit anti-AKT from Cell Signaling Technology (Lake Placid, NY, USA) was used at a 1:1000 dilution. Monoclonal rabbit anti-pAKT from Cell Signaling Technology (Lake Placid, NY, USA) was used at a 1:1000 dilution. All antibodies were diluted to the appropriate concentration in 5% BSA/TBST and incubated with blots overnight at 4^{o}C.

The following day, the membranes were washed from the primary antibody 3 times with TBST for 5 minutes each, and an anti-mouse (1:3000) or anti-rabbit (1:2500) conjugated secondary antibodies diluted in TBST were added to the membranes and incubated at room temperature for 1 hour. Membranes were washed from the secondary antibody 2 times with TBST and 1 time with TBS for 5 minutes. The chemiluminescent substrate used was Supersignal West Pico (Pierce) and the visualization of the protein bands was performed using the GeneSnap image acquisition system followed by densitometry analysis with the GeneTools software (Syngene, Frederick, MD, USA).

2.11 Graphical and Statistical Analysis

The presented data was plotted using GraphPad Prism software (version 3.02). Statistical differences for single comparison were determined using two-tailed unpaired t-test with a 95% confidence interval. Statistical differences for multiple comparisons were determined using a non-parametric one-way ANOVA test with a 95% confidence interval using the Bonferroni correction. Results with $p<0.05$ were deemed significant.

Chapter 3: RESULTS

3.1 GGTI-298 Inhibits Protein Prenylation of Rho-GTPases

RhoA, rac1 and cdc42 have significant roles in actin cytoskeleton reorganization and require geranylgeranylation for their proper localization and function [141]. The effects of inhibiting protein geranylgeranylation with GGTI-298 were evaluated in SCC9 and SCC25 cells by western immunoblotting. The induction of rhoA and cdc42 expression in these cell lines treated with either 10μM lovastatin or 5μM GGTI-298 for 24 hours was reversed by the co-administration of 10μM GGPP with lovastatin but not with GGTI-298. RhoA, rac1, and cdc42 showed a slight shift in the mobility on the gel due to the lack of prenylation in either 10μM lovastatin or 5μM GGTI-298 treated cells. This effect was reversed with the co-administration 10μM GGPP with lovastatin but not with GGTI-298. Rab5 is doubly geranylgeranylated and was used as a control. The shift in mobility on the gel for rab5 is observed in the lovastatin treated cells but not the GGTI-298 treated cells. Hence, GGTI-298 targets the prenylation of geranylgeranylated proteins, such as the Rho GTPases (Figure 3.1).

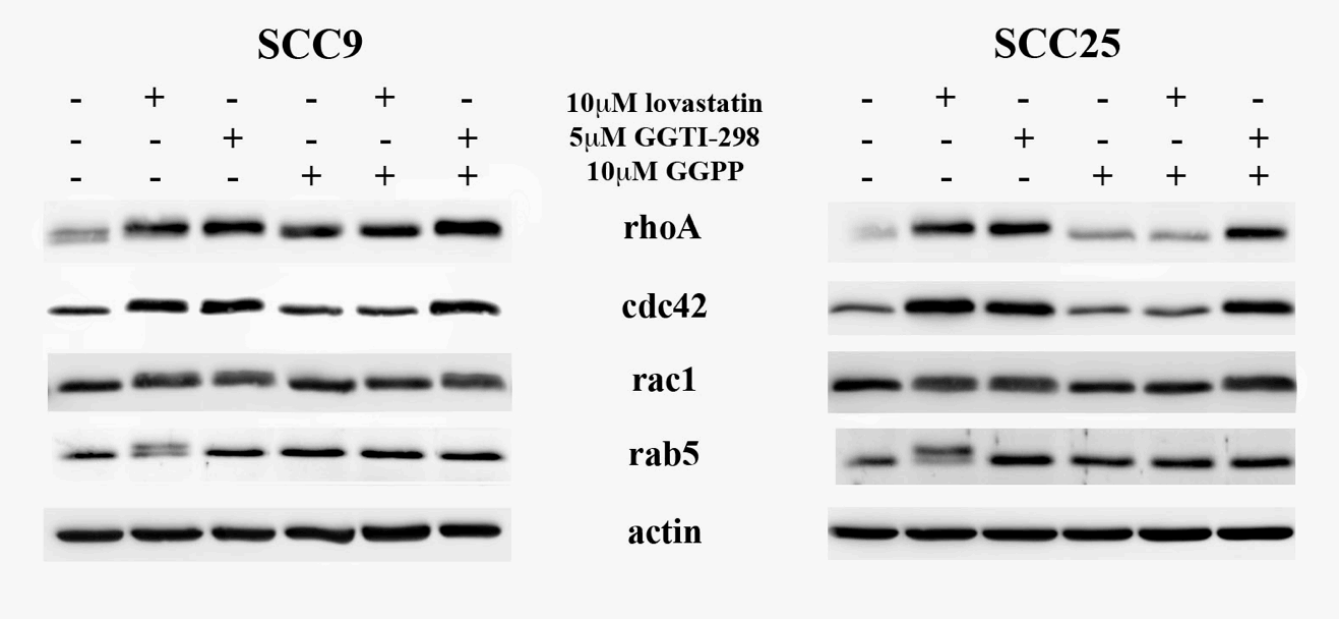


Figure 3.1 GGTI-298 induces enhanced expression of non-geranylgeranylated rho family of proteins Western blot analysis of rhoA, cdc42, rac1, and rab5 protein expression levels in GGTI-298 and lovastatin treated SCC9 and SCC25 cell lines for 24 hours. Both cell lines were treated with 10μM lovastatin or 5μM GGTI-298. Treatments with 10μM GGPP alone or in combination with 10μM lovastatin or 5μM GGTI-298 were also evaluated. Expression levels of actin were assayed as the loading control.

3.2 GGTI-298 Induces Actin Cytoskeletal Disorganization

The Rho GTPases have been associated in a wide variety of cellular processes including cytoskeletal organization, cell adhesion to the extracellular matrix, cell polarity, and transcriptional activation. They have also been implicated in motility, invasion, and some aspects of metastasis under aberrant conditions [179]. In an attempt to determine the effects of protein geranylgeranylation on cytoskeletal organization, actin architecture was visualized with rhodamine-conjugated phalloidin, which has a high affinity for F-actin following 0.5 μM, 1 μM, and 5 μM GGTI-298 treatment for 24 hours in SCC25 cells. Increasing concentration of GGTI-298 caused enhanced inhibition of stress fiber formation as visualized by fluorescence microscopy (Figure 3.2).

3.3 GGTI-298 Reduces Cell Migration

We next evaluated the effects of GGTI-298 on cell migration of SCC9 and SCC25 cells in a scratch wound assay. Confluent cell monolayers were scratched to create a wound opening and 5 different pictures were taken along the wound opening from which the average diameter was calculated. Percentage wound closure at 24 hours was calculated in comparison to the wound diameter at time 0 hour (Figure 3.3A and 3.3B). GGTI-298 significantly reduced the migration of SCC9 and SCC25 cells as compared to the control treated cells (Figure 3.3C and 3.3D). In order to confirm that GGTI-298 did in fact inhibit migration, we performed a trypan blue exclusion cell viability test to remove the variability of altered cell migration due to the cytotoxic effects of GGTI-298 on cell proliferation. A cell count of SCC9 and SCC25 treated with 5μM GGTI-298 showed no significant change in cell number compared to the control treated cells (Figure 3.3E and 3.3F).

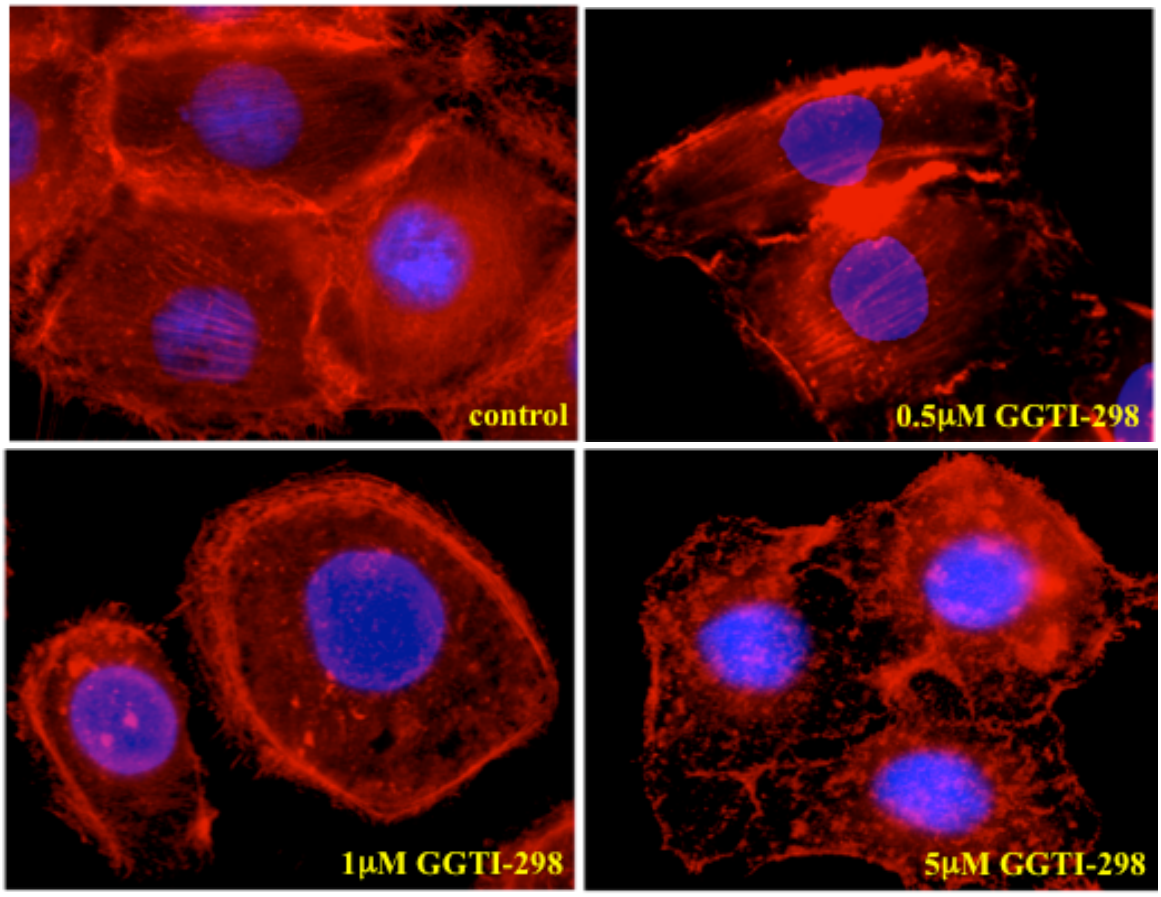
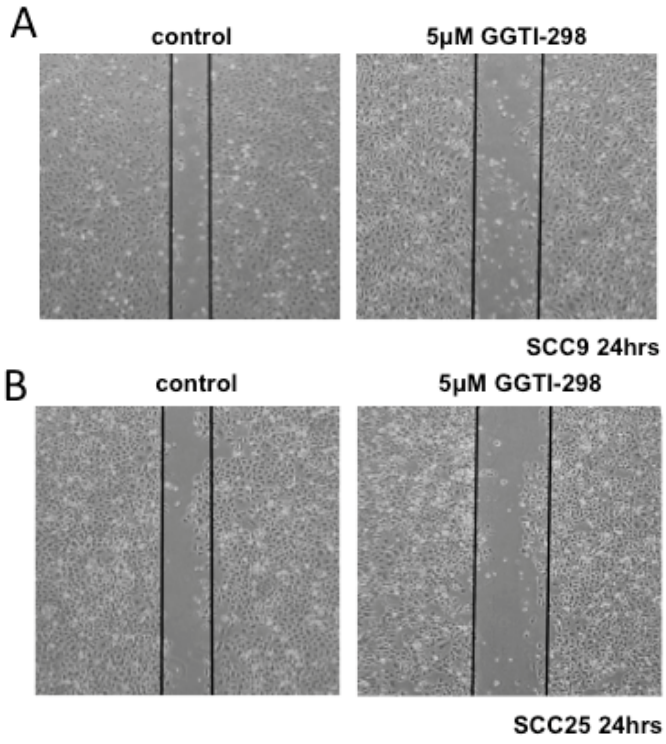
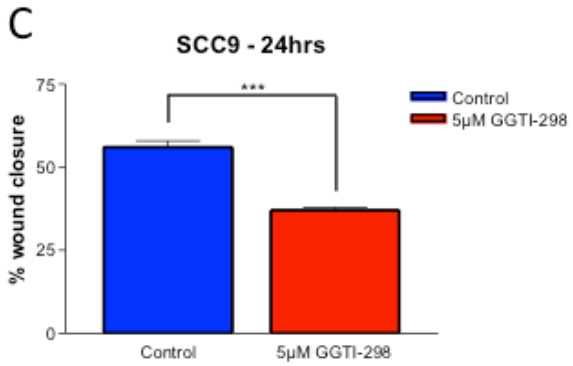
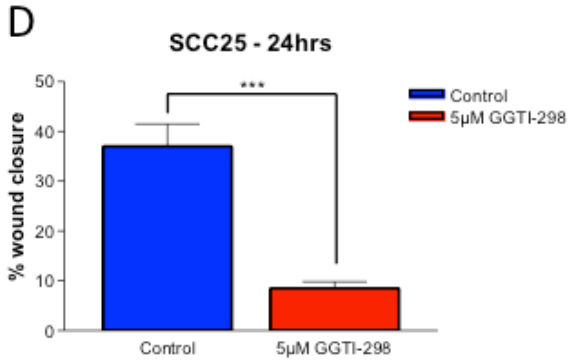
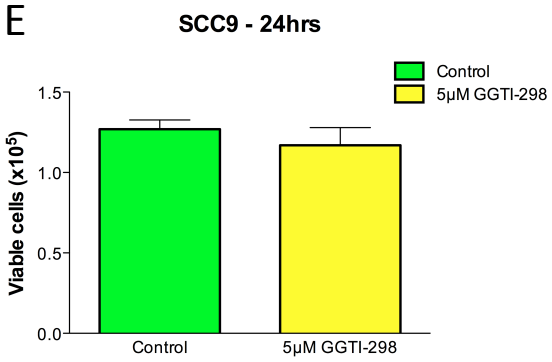


Figure 3.2 GGTI-298 induces cytoskeletal disorganization Fluorescence microscopy examining the effect of increasing concentrations of GGTI-298 on actin cytoskeletal organization in SCC25 cells. Rhodamine phalloidin staining of solvent control, 0.5μM, 1μM, and 5μM GGTI-298 for 24 hours. Nuclei were counterstained with DAPI. Original magnification 630x.


A
control
5μM GGTI-298
SCC9 24hrs
B
control
5μM GGTI-298
SCC25 24hrs


C
SCC9 - 24hrs
% wound closure
75
50
25
0
***
Control
5μM GGTI-298
Control
5μM GGTI-298


D
SCC25 - 24hrs
% wound closure
50
40
30
20
10
0
***
Control
5μM GGTI-298
Control
5μM GGTI-298


E
SCC9 - 24hrs
Viable cells (x10⁵)
1.5
1.0
0.5
0.0
Control
5μM GGTI-298
Control
5μM GGTI-298

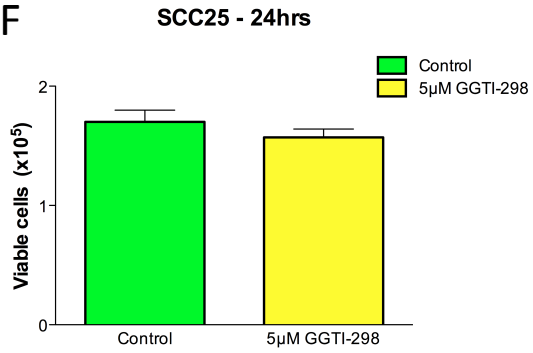

F
SCC25 - 24hrs
Viable cells (x10⁵)
2
1
0
Control
5μM GGTI-298
Control
5μM GGTI-298

Figure 3.3 GGTI-298 inhibits cell migration (A, B) Representative photos of scratch wound assay at 24 hours post wounding in control, and 5μM GGTI-298 treated SCC9 and SCC25 cells. Photos were taken at x10 magnification. (C, D) SCC9 and SCC25 migration was measured as percentage wound closure as described in section 2.3. Average wound closure measurement at five different points along wound edge was quantified at time points 0 hour and 24 hours. Bars represent pooled mean with standard error. GGTI-298 significantly reduced cell migration in SCC9 ($p<0.001$) and SCC25 ($p<0.001$) cells. (E, F) Treatment of 5μM GGTI-298 in SCC9 and SCC25 had no significant effect of cell viability evaluated by trypan blue exclusion cell viability assay.

3.4 GGTI-298 in Combination with Tarceva Enhances Cytotoxicity in HNSCC

The sensitivity of the head and neck cell lines to the combination of GGTI-298 with tarceva was determined by MTT assay, a colorimetric assay used to measure cell viability [180]. Previous studies have demonstrated that lovastatin in combination with gefitinib enhances cytotoxicity in HNSCC cells [132]. We reassessed the combination of lovastatin with the receptor tyrosine kinase inhibitor, tarceva (Figure 3.4A,B) and compared these results to the effects of combining varying concentrations of GGTI-298 (0.001-10μM) with 1μM or 10μM tarceva for 48 hours in SCC9 and SCC25 cell lines (Figure 3.4C,D). We demonstrated that GGTI-298 single treatment did not significantly affect cell viability of SCC9 cells. However, treatment of SCC25 cells with 10μM GGTI-298 for 48 hours did increase the cytotoxicity, where approximately 25% survival was observed. The combination of GGTI-298 with tarceva showed significant cytotoxicity, almost completely abolishing cell viability in both head and neck cancer cells.

We confirmed the sensitivity of the HNSCC cell lines to the combination of GGTI-298 with tarceva by trypan blue cell viability assay. In this assay, a cell suspension is mixed with the trypan blue, where viable cells with intact cell membranes will exclude the dye, whereas dead cells won't [181]. Cells were treated with control, 10μM tarceva, 10μM GGTI-298 single treatments or the combination of 10μM tarceva with 10μM GGTI-298. Cell viability was measured at 24 hours (Figure 3.5A,B) and 48 hours (Figure 3.5C,D) and the average of the viable cells was calculated from 2 independent studies. The combination of tarceva with GGTI-298, enhanced cytotoxicity on SCC9 and SCC25 cells compared to each agent alone.

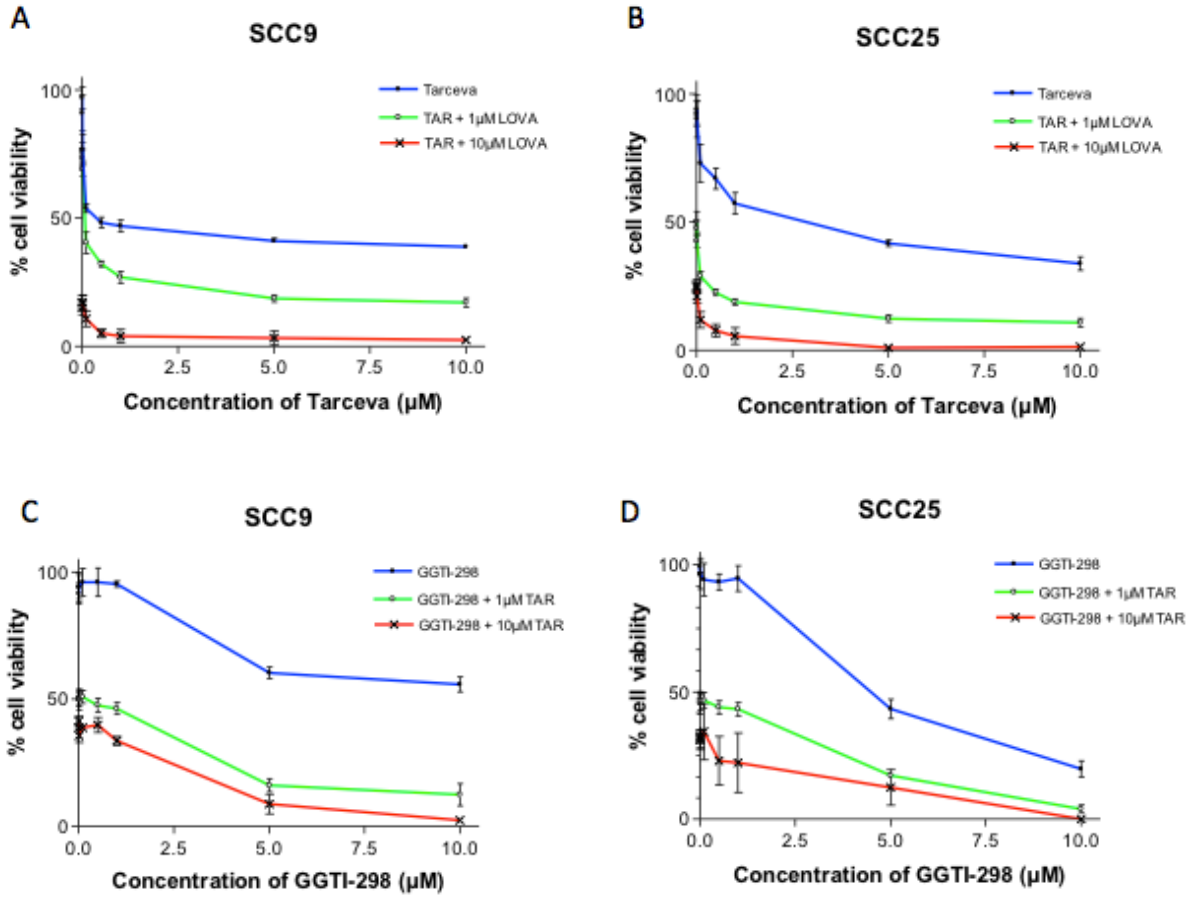


Figure 3.4 MTT assay showing enhanced cytotoxicity with GGTI-298 and tarceva combination treatment MTT cell viability assay of SCC9 and SCC25 cell lines comparing response of the combinations of lovastatin and tarceva to the combinations of GGTI-298 and tarceva. (A, B) The cell lines were treated with 0-10μM tarceva for 48 hours as monotherapy or in combination with 24-hour pre-treatment of 1μM or 10μM lovastatin for a total of for 72 hours. (C, D) The cell lines were treated with 0-10μM GGTI-298 for 48 hours as monotherapy or in combination with 1μM or 10μM tarceva for 48 hours. Cell viability was assessed with the activity of untreated cells taken to be 100%. In all experiments, error bars indicate standard deviation of 8 measurements.

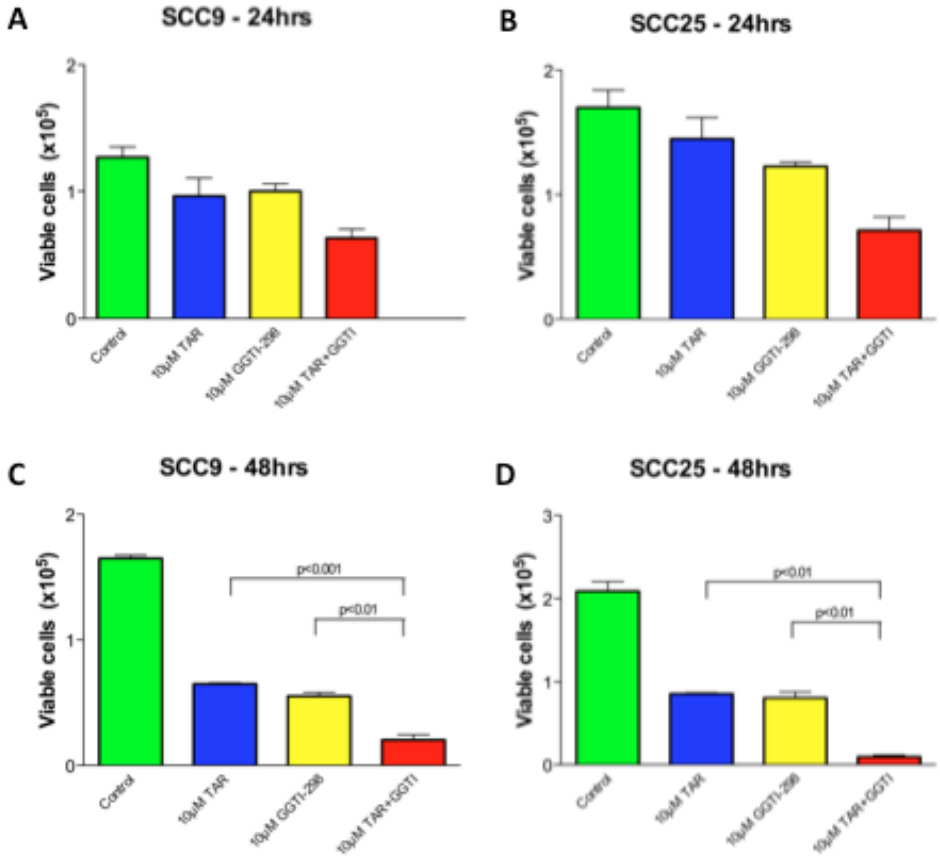
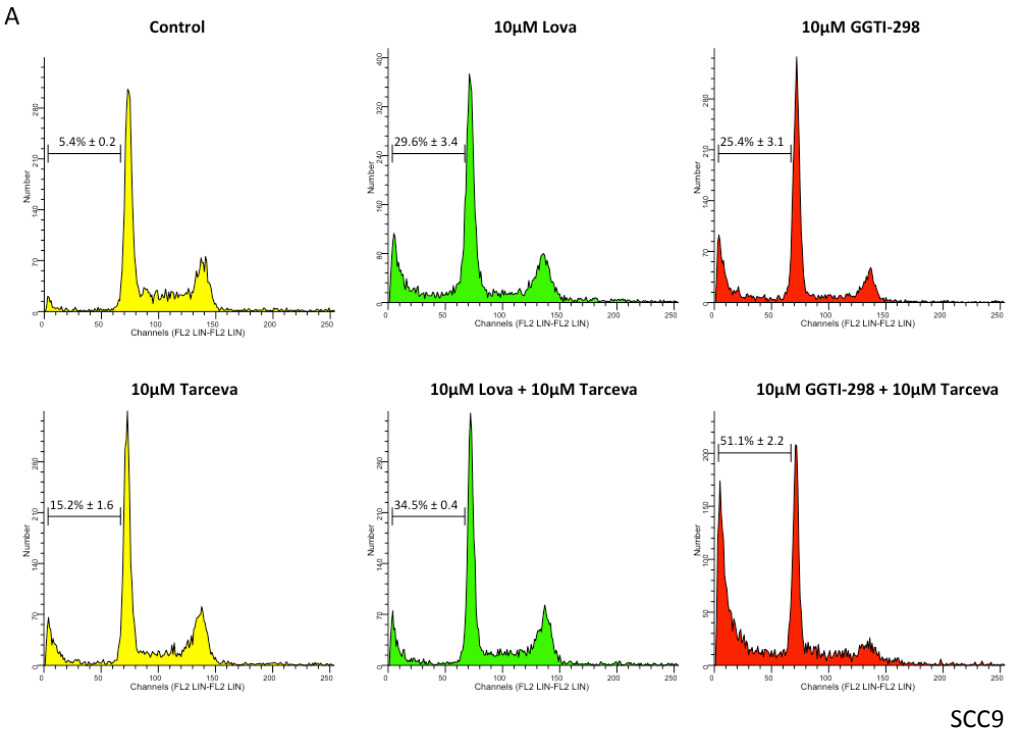


Figure 3.5 Trypan blue exclusion cell viability assay showing enhanced cytotoxicity with GGTI-298 and tarceva combination treatment Trypan blue exclusion cell viability assay of SCC9 and SCC25 cell lines comparing response of monotherapy treatment and combination treatment with GGTI-298 and tarceva at 24 hours and 48 hours. (A, B) The cell lines were treated for 24 hours with control, 10μM tarceva, 10μM GGTI-298, or the combination of 10μM tarceva and 10μM GGTI-298. (C, D) The cell lines were treated with the same conditions for 48 hours. In all experiments, error bars indicate standard deviation of 2 independent studies.

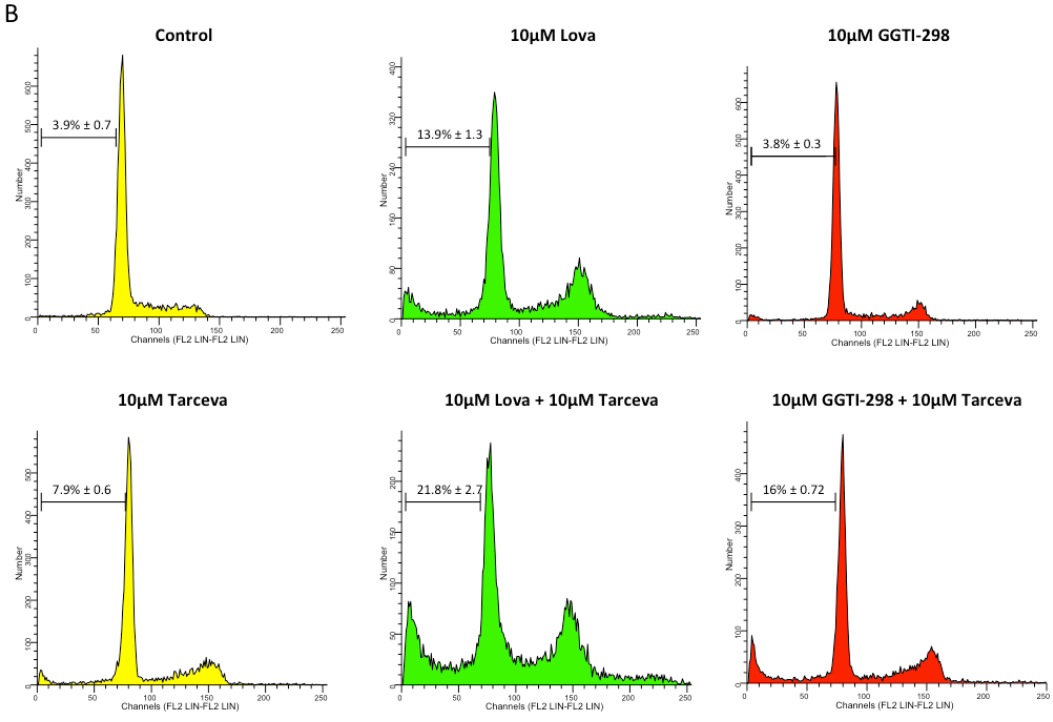
3.5 Enhanced Cytotoxicity of GGTI-298 in Combination with Tarceva through the Induction of Apoptosis

Using flow cytometric analysis of propidium iodide stained SCC9 and SCC25 cell lines, we evaluated the role of the induction of apoptosis as the mechanism by which the combination of GGTI-298 and tarceva induces enhanced 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 [182]. Treating SCC9 (Figure 3.6A) and SCC25 (Figure 3.6B) cell lines with 10μM GGTI-298 and 10μM tarceva showed a significant induction of apoptosis with this combination compared to either agent alone.

To confirm that the effects and the combined enhanced cytotoxicity with tarceva of GGTI-298 were due to induction of apoptosis, we evaluated PARP cleavage by Western blot. SCC9 (Figure 3.7A) and SCC25 (Figure 3.7B) cells were treated with 1μM, 5μM, and 10μM GGTI-298 alone or in combination with 10μM tarceva for 48 hours. In this assay, increased levels of cleaved PARP (lower band) compared to uncleaved PARP (upper band) represents an increase in apoptosis. In SCC9 cells we observed no cleavage of PARP with GGTI-298 treatment alone but increased cleavage with the addition of tarceva. The combination of 10μM GGTI-298 with 10μM of tarceva showed a significant increase in the levels of cleaved PARP when compared with tarceva as a single agent or in combination with 1μM and 5μM GGTI-298. In SCC25 cells, there was no PARP cleavage after 48-hour treatment with GGTI-298 alone. The addition of 10μM tarceva showed an increase in the levels of cleaved PARP with increasing concentrations of GGTI-298. Therefore, the combination of GGTI-298 and tarceva displayed enhanced cytotoxicity in head and neck cancer cells by inducing a potent apoptotic response through targeting of protein geranylgeranylation.


A
Control
5.4% ± 0.2
10μM Lova
29.6% ± 3.4
10μM GGTI-298
25.4% ± 3.1
10μM Tarceva
15.2% ± 1.6
10μM Lova + 10μM Tarceva
34.5% ± 0.4
10μM GGTI-298 + 10μM Tarceva
51.1% ± 2.2
Number
Channels (FL2 LIN-FL2 LIN)

SCC9


B
Control
3.9% ± 0.7
10μM Lova
13.9% ± 1.3
10μM GGTI-298
3.8% ± 0.3
10μM Tarceva
7.9% ± 0.6
10μM Lova + 10μM Tarceva
21.8% ± 2.7
10μM GGTI-298 + 10μM Tarceva
16% ± 0.72
Number
Channels (FL2 LIN-FL2 LIN)

SCC25

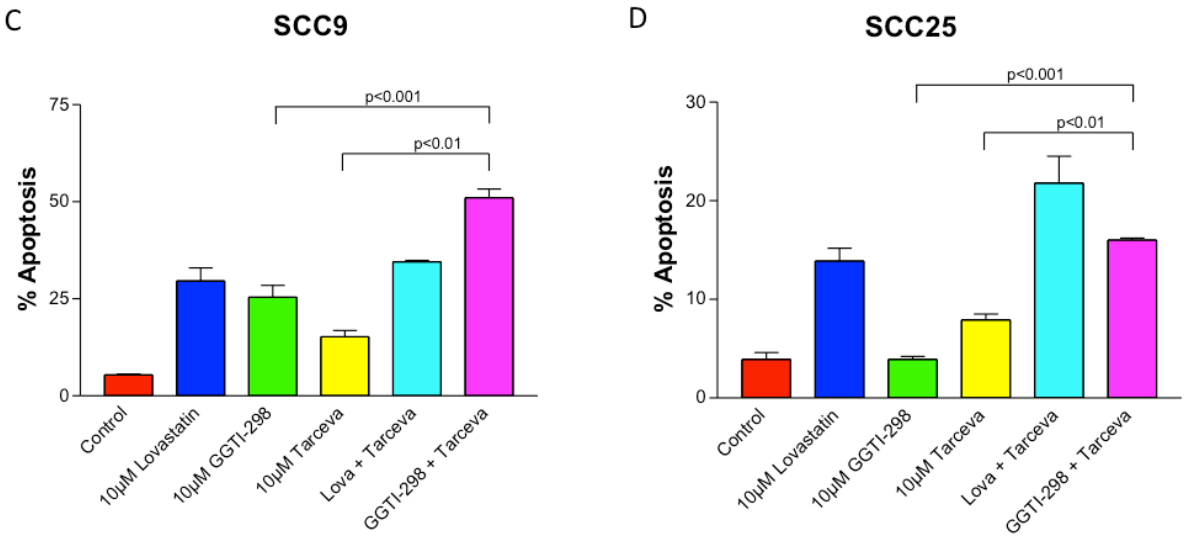


Figure 3.6 Flow cytometric analysis showing enhanced apoptotic response to combined treatment of GGTI-298 and tarceva (A, B) Flow cytometric analysis of subG1 apoptotic fraction as determined by propidium iodide staining of cellular DNA content comparing the response of SCC9 and SCC25 cell lines to lovastatin, GGTI-298, tarceva and combination treatments. Both cell lines were treated with 10μM lovastatin, 10μM GGTI-298, and 10μM tarceva for 48 hours as monotherapies or 24 hours pre-treatment with 10μM lovastatin followed by the combination with 10μM tarceva for 48 hours or the combination of 10μM GGTI-298 together with 10μM tarceva for 48 hours. The average percentage of apoptotic cells with standard deviation of 3 independent experiments is shown in the upper left quadrant of each histogram. (C, D) Bar graph representation with error bars for the standard deviation from 3 independent studies of the flow cytometry results for SCC9 and SCC25 cell lines.

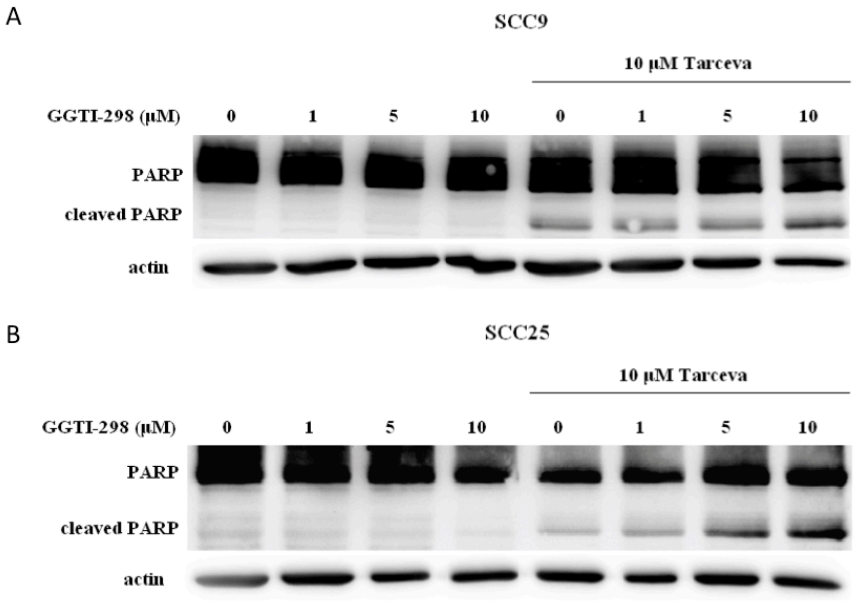


Figure 3.7 GGTI-298 in combination with tarceva induces PARP cleavage (A, B) Western blot analysis of PARP cleavage upon 48-hour treatment with increasing concentrations of 1μM, 5μM, and 10μM GGTI-298 alone or in combination with 10μM tarceva in SCC9 and SCC25 cells. Actin is used as a loading control.

3.6 GGTI-298 Inhibits EGFR Dimerization

Firstly, we determined the effects of GGTI-298 treatment on ligand binding induced dimerization of the EGFR by performing cross-linking experiments (Figure 3.8). SCC25 cells were treated with control, 10μM lovastatin and 10μM GGTI-298 in serum-free media for 24 hours and then stimulated with 50ng/ml EGF for 15 minutes. Using Western blot analysis, dimers were visualized as >300kD bands and the monomers served as loading control. GGTI-298 treatment inhibited ligand stimulated EGFR dimerization in SCC25 cells and showed similar results as previously published data with lovastatin.

3.7 GGTI-298 Inhibits the Proper Trafficking of EGFR

We then examined whether GGTI-298 treatment can regulate EGFR trafficking by performing an immunnofluroscence assay for EGFR (Figure 3.9). SCC9 cells were treated with control, 10μM FTI-277, 10μM lovastatin, and 10μM GGTI-298 in serum-free media for 24 hours. Following treatments, cells were incubated for 15min with 50ng/mL EGF, washed, probed with a primary EGFR antibody followed by a secondary Alexa Fluor 488 conjugated antibody. In control and FTI-277 treated SCC9 cells, there was a distinct punctate intracellular staining pattern indicating efficient intracellular trafficking of the EGFR. However, in lovastatin and GGTI-298 treated SCC9 cells, there was a significant reduction in the intracellular staining pattern, suggesting that EGFR remained sequestered close to the cell surface. These results indicate inhibition of proper trafficking of EGFR in GGTI-298 treated SCC9 cells.

To further examine whether GGTI-298 is regulating the trafficking of EGFR, we examined the EGF ligand by fluorescence microscopy (Figure 3.10). SCC25 cells were treated with control, 0.5μM, 1μM, and 5μM GGTI-298 in serum-free media for 24 hours then stimulated for 30 minutes with 50ng/mL Alexa Fluor 488-EGF on ice. Following treatments, cells were washed with an acid solution to remove all the EGF that has not been internalized into the cell. Cells were then fixed and visualized by fluorescence microscopy. In control and 0.5μM GGTI-298 treated SCC25 cells, there was efficient intracellular trafficking of the EGF ligand. However, in the 1μM and 5μM GGTI-298 treated SCC25 cells, EGF while internalized was sequestered close to the cell membrane indicating inhibition of proper trafficking of the ligand in the cell.

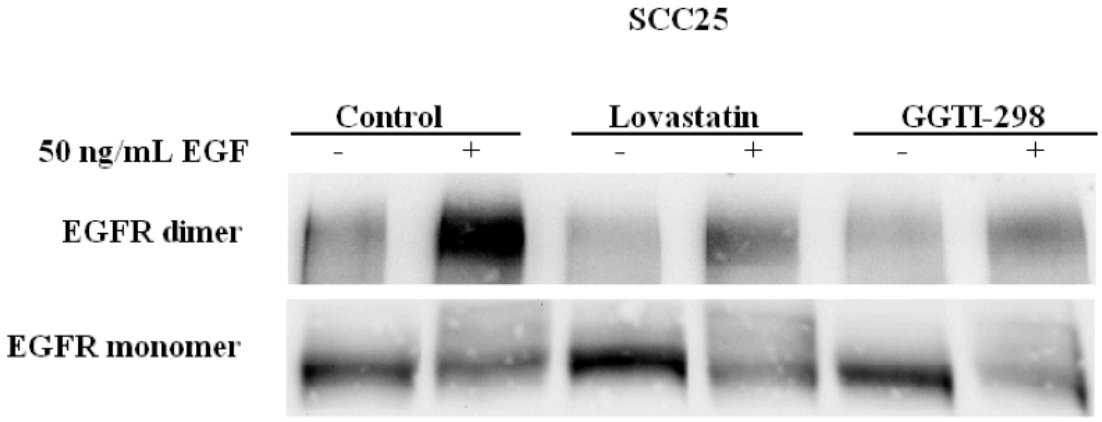


Figure 3.8 GGTI-298 inhibits EGFR dimerization Control, 10μM lovastatin, and 10μM GGTI-298 (serum-free) treated cells for 24 hours were stimulated with 50ng/ml of EGF for 15 minutes. Cells were washed with ice-cold PBS and incubated on ice for 30 minutes with the cross-linking reagent bis (sulfosuccinimidyl) substrate (3 mM in PBS). Dimers were visualized as >300kD bands. GGTI-298 treatment significantly decreased the amount of EGFR dimers formed as previously shown with lovastatin treatment.

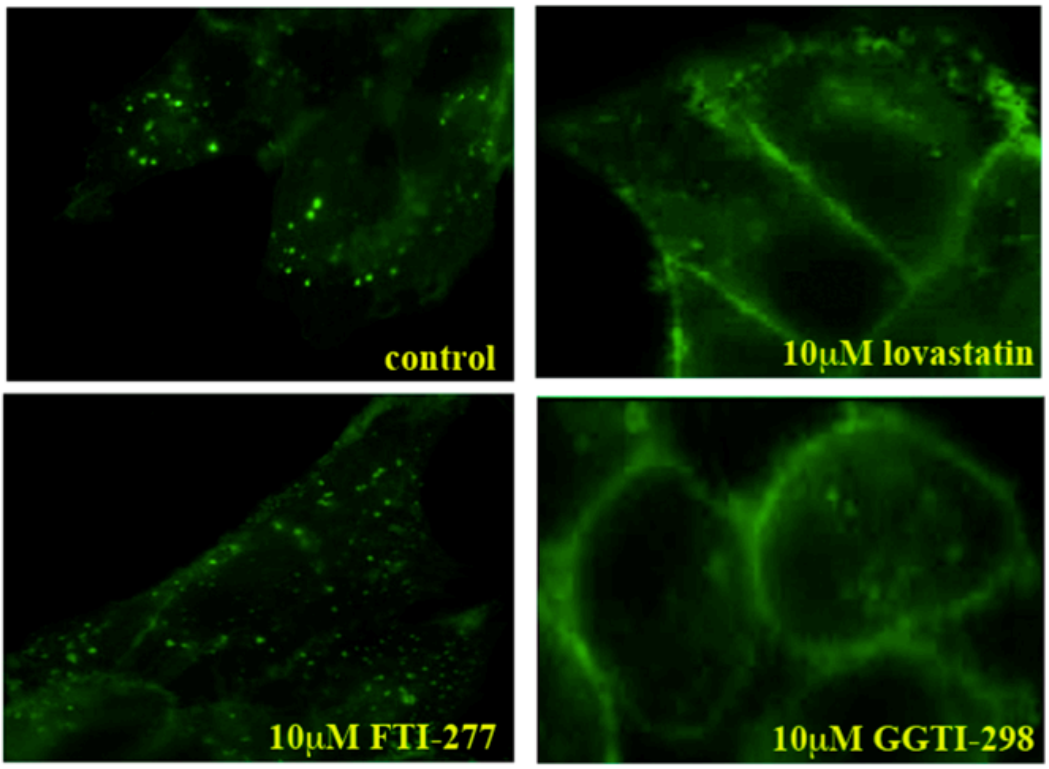


Figure 3.9 GGTI-298 treatment inhibits the proper trafficking of EGFR EGFR trafficking in SCC9 was evaluated by immunofluorescence. SCC9 were treated with solvent control, 10μM lovastatin, 10μM FTI-277, or 10μM GGTI-298 for 24 hours in serum-free media followed by 15 minutes of stimulation with EGF. Immunofluorescence staining of SCC9 revealed a punctate intracellular staining pattern upon EGF ligand binding in the control and FTI-277 treated cells but not in cells treated with 10μM lovastatin and 10μM GGTI-298. Original magnification 630x.

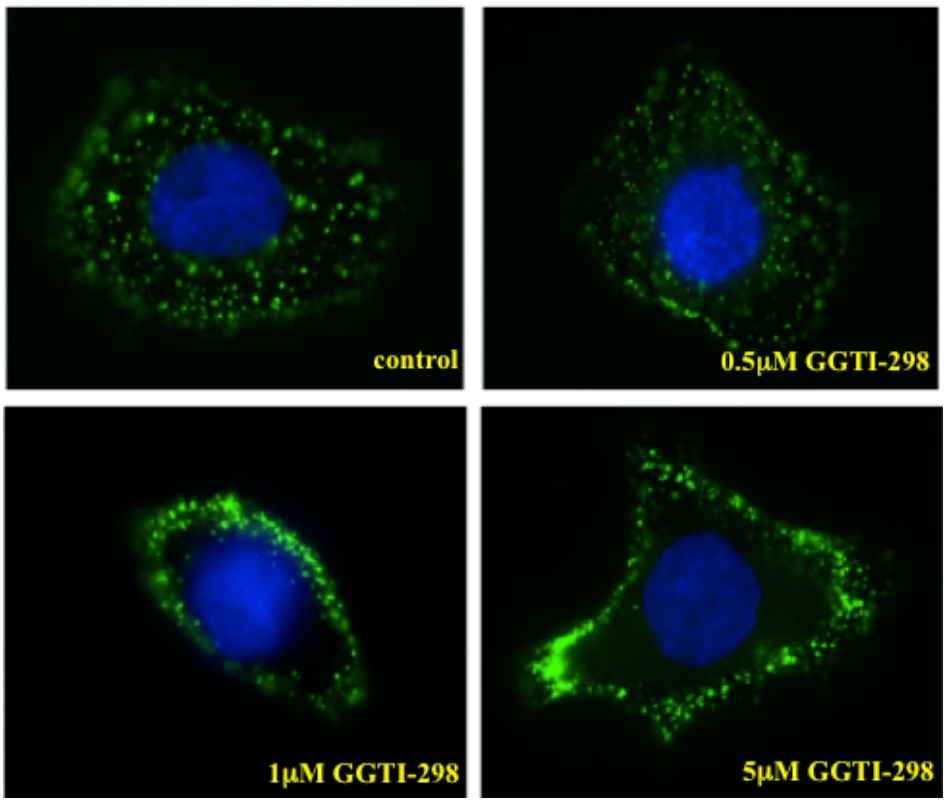


Figure 3.10 GGTI-298 treatment inhibits the proper trafficking of the EGF ligand Fluorescence microscopy examining the effect of increasing concentrations of GGTI-298 on EGF internalization in SCC25 cells. The cells were treated with solvent control, 0.5μM, 1μM, or 5μM GGTI-298 followed by stimulation with 50ng/mL EGF-Alexa 488 for 30 minutes on ice. Following treatments, cells were washed with an acid solution to remove all the EGF that has not been internalized into the cell. Nuclei were counterstained with DAPI. Original magnification 630x.

3.8 GGTI-298 in Combination with Tarceva Shows Enhanced Inhibition of EGFR Autophosphorylation and Activation of Its Downstream Pathway

Based on the ability of GGTI-298 to inhibit EGFR dimerization and its proper trafficking inside the cell, we further evaluated the effect of GGTI-298 treatment on EGFR autophosphorylation and the signaling cascades triggered by EGFR activation. The Ras/Mek/ERK pathway has diverse effects, which can regulate cell cycle progression, apoptosis and differentiation [36] and the PI3K/AKT pathway is involved in cell growth, apoptosis resistance, invasion, and migration [44]. Serum starved SCC9 and SCC25 cells were treated with 0.001μM, 0.01μM, 0.1μM, or 1μM tarceva for 2 hours alone or following a 22 hours pre-treatment with 10μM GGTI-298 followed with 50ng/ml EGF stimulation for 15mins. The autophosphorylation of EGFR and functional activation of the AKT and ERK pathways were evaluated by Western blot analysis, employing phospho-specific antibodies recognizing the active form and control antibodies for total EGFR, AKT, and ERK. GGTI-298 treatment enhanced the effects of tarceva on the inhibition of EGFR autophosphorylation in SCC9 (Figure 3.11 A, C) and SCC25 (Figure 3.11 B, D) cells. The combination of GGTI-298 and tarceva also enhanced the inhibition of the activation of AKT (Figure 3.12 A-D) and little effect on the ERK (Figure 3.12 E-H) pathway.

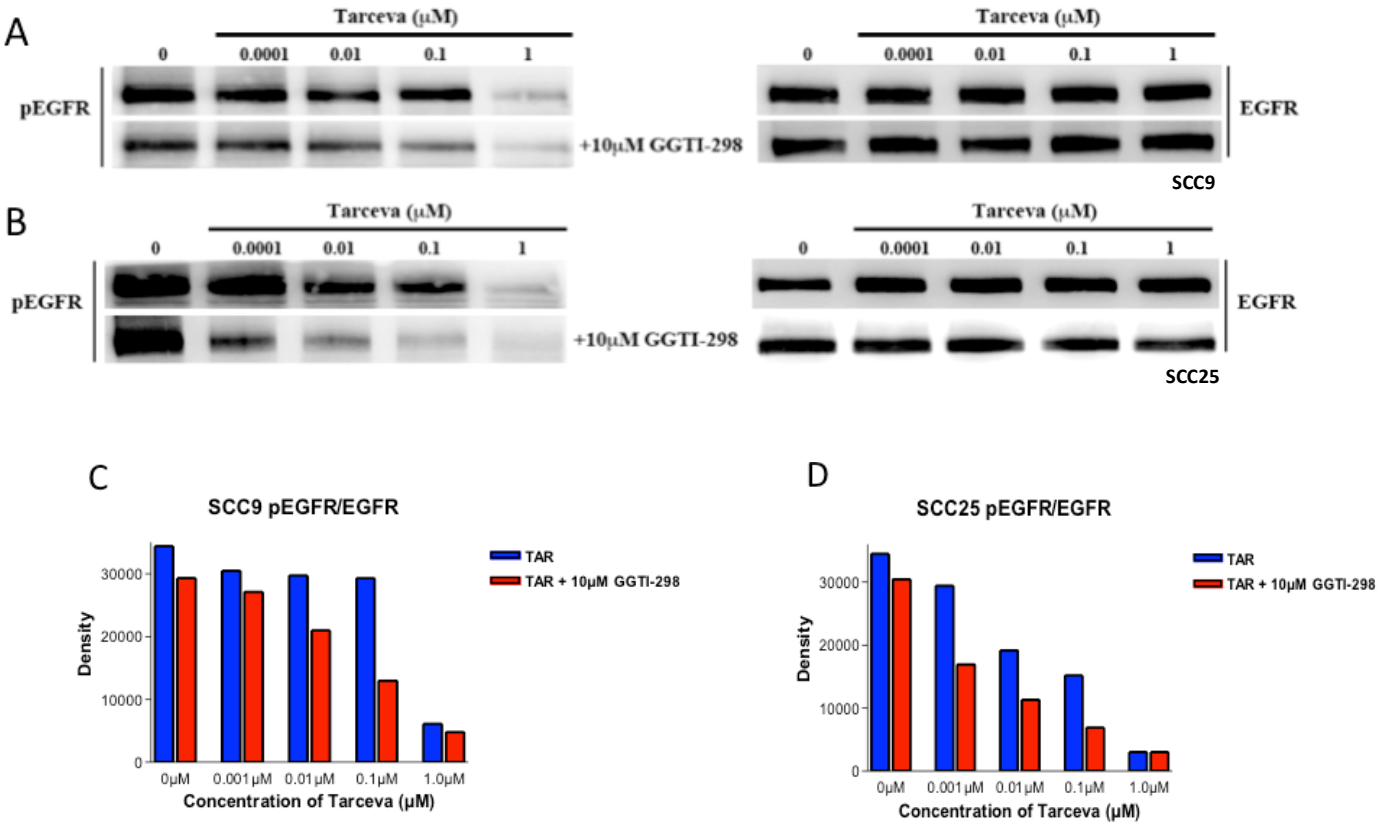
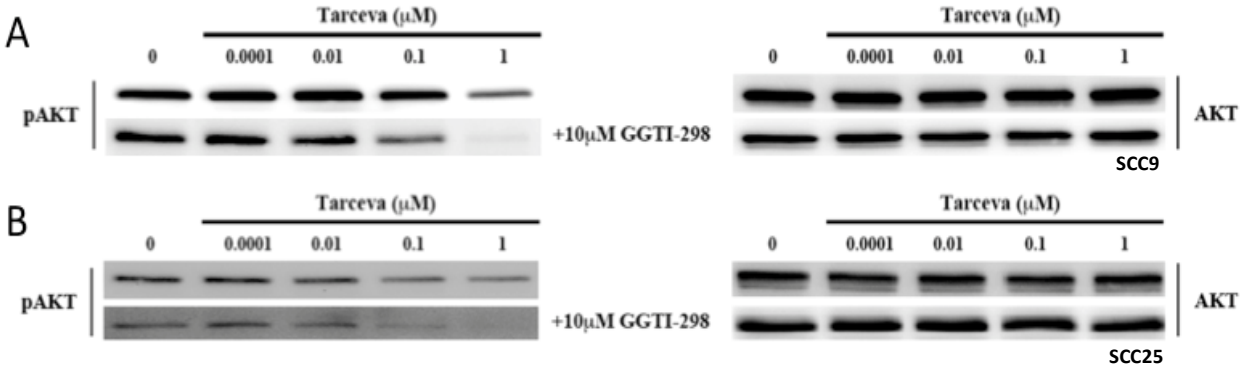
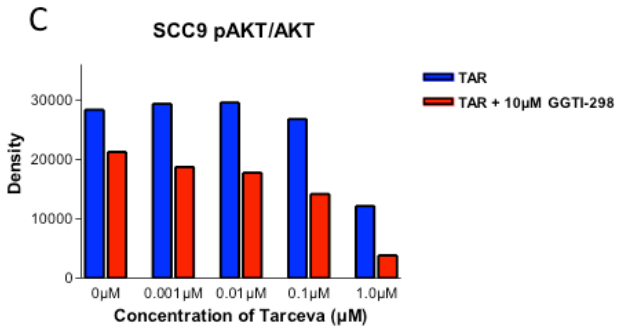
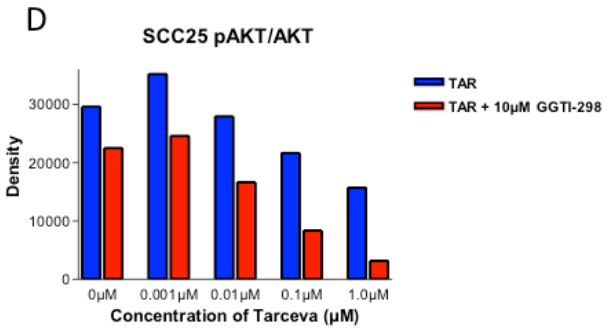


Figure 3.11 GGTI-298 in combination with tarceva enhances inhibition of EGFR autophosphorylation (A, B) Western blot analysis of EGFR autophosphorylation upon 2-hour treatment with increasing concentrations of tarceva (0.001μM, 0.01μM, 0.1μM, or 1μM) alone or in combination with a 22-hour pre-treatment of 10μM GGTI-298 for a total of 24 hours (in serum free media) in SCC9 and SCC25 cells. Following treatment cells were stimulated with 50ng/ml of EGF for 15 minutes. Total EGFR is used as a loading control. (C, D) Bar graph representation of protein expression.


A
Tarceva (μM)
0
0.0001
0.01
0.1
1
pAKT
+10μM GGTI-298
AKT
SCC9
B
Tarceva (μM)
0
0.0001
0.01
0.1
1
pAKT
+10μM GGTI-298
AKT
SCC25


C
SCC9 pAKT/AKT
TAR
TAR + 10μM GGTI-298
Density
30000
20000
10000
0
0μM
0.001μM
0.01μM
0.1μM
1.0μM
Concentration of Tarceva (μM)


D
SCC25 pAKT/AKT
TAR
TAR + 10μM GGTI-298
Density
30000
20000
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0μM
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1.0μM
Concentration of Tarceva (μM)

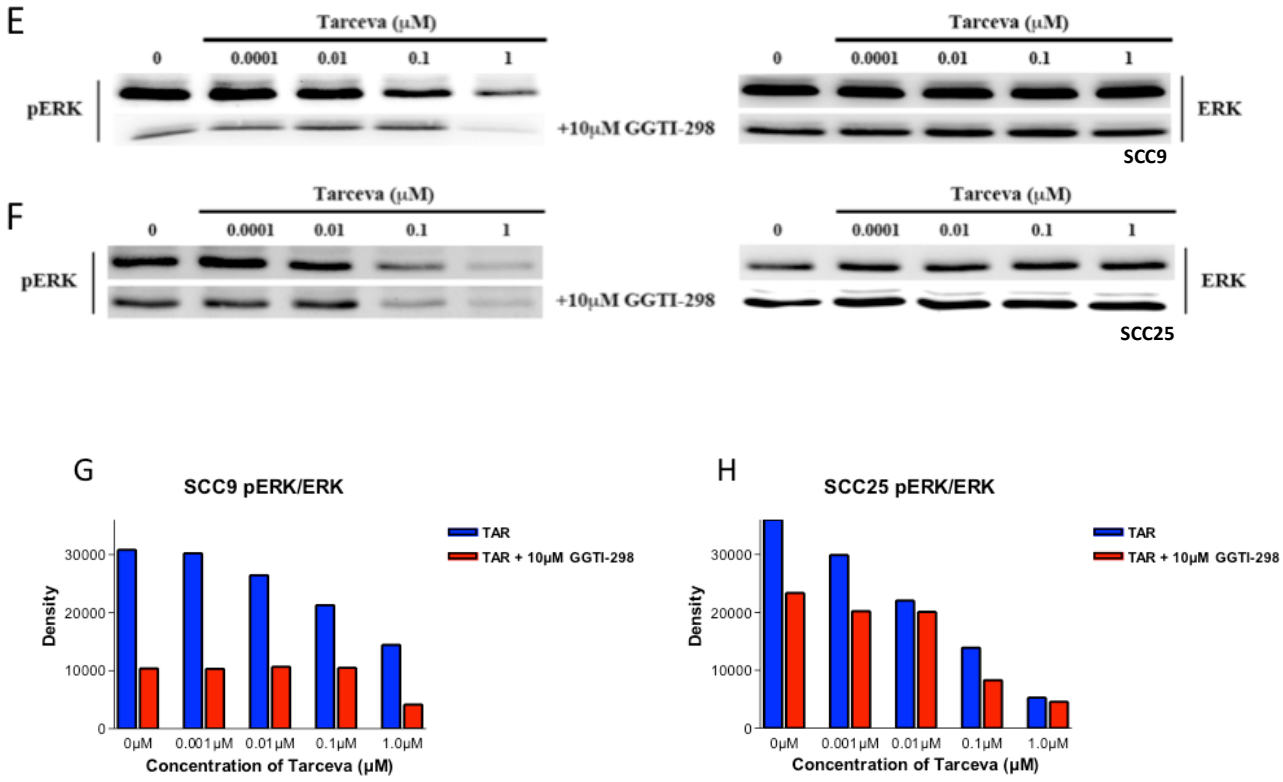

Figure 3.12 GGTI-298 in combination with tarceva enhances inhibition of EGFR downstream signalling (A, B, E, F) Western blot analysis of AKT and ERK activation upon 2-hour treatment with increasing concentrations of tarceva (0.001μM, 0.01μM, 0.1μM, or 1μM) alone or in combination with a 22-hour pre-treatment of 10μM GGTI-298 for a total of 24 hours (in serum free media) in SCC9 and SCC25 cells. Following treatment cells were stimulated with 50ng/ml of EGF for 15 minutes. Total AKT and ERK were used as loading controls. (C, D, G, H) Bar graph representation of protein expression.

Chapter 4: DISCUSSION

4.1 GGTI-298 Enhances the Effects of Tarceva in the Treatment of HNSCC

Interest in targeting protein geranylgeranylation was prompted from the realization in our previous studies that the administration of GGPP, through add back approaches, was able to reverse the cytototoxic and apoptotic effects of lovastatin in HNSCC. From our overall data we observed that GGTI-298, through its ability to inhibit protein geranylgeranylation results in the dis-organization of actin cytoskeleton that is likely the mechanism regulating its inhibitory effects on ligand-induced dimerization of the EGFR and its subsequent trafficking in the cell. GGTI-298 combined with tarceva enhanced the cytotoxic effects on HNSCC. This combination also demonstrated enhanced inhibitory effects on EGFR activation and its downstream signaling pathways involved in cell cycle progression, proliferation and cell survival. These results provide new insights in a potential novel treatment for advanced HNSCC.

4.1.1 GGTI-298 causes actin cytoskeleton disorganization and suppression of migration through inhibition of protein prenylation

Several studies in the last 3 decades have shown the important and fundamental implication of Ras proteins in cancer and in physiological processes controlling cellular proliferation, differentiation, and survival [183]. Many signal transduction pathways that are activated by RAS involve proteins that require prenylation in order to mediate tumor cell survival, growth, proliferation, migration and metastasis. These discoveries led to the development of farnesyltransferase inhibitors (FTIs) and geranylgeranyltransferase 1 inhibitors (GGTIs) as potential anticancer drugs.

Our previous studies determined that protein prenylation was critical to the cytotoxic effects of lovastatin. This was based on the observation that GGPP reversed the effects on tumor cell viability induced by lovastatin in HNSCC; however, FPP had only partial protective effects [172]. Furthermore, the administration of GGPP reversed the effects of lovastatin on EGFR dimerization and activation [132]. These results led us to evaluate the role of specifically targeting protein geranylgeranylation in HNSCC as a means to recapitulate the effects shown by lovastatin on EGFR activation and enhanced cytotoxicity with tarceva employing an inhibitor of GGTase-I, GGTI-298.

Cell migration is required for many biological processes, and its aberrant regulation drives progression of many diseases, including cancer invasion and metastasis [184]. The rearrangement of the actin cytoskeleton plays an important role in cell motility [185]. The geranylgeranylated rho proteins regulate actin cytoskeleton and agents that affect actin polymerization have been shown to inhibit EGFR function as well [133]. The Rho family of small GTP binding proteins, rhoA, rac1 and cdc42, have significant roles in the rearrangement of the actin cytoskeleton [186].

We examined the effects of GGTI-298 on rhoA, rac1, and cdc42 in the HNSCC cell lines, SCC9 and SCC25. Using western blot analysis, we demonstrated a shift in gel mobility in all 3 Rho GTPases of GGTI-298 treated HNSCC cells indicative of the absence of their prenylation. Rho GTPases contain a CAAX sequence at their carboxy-terminal. Following their prenylation (C20), the AAX is cleaved by the endoprotease Ras Converting Enzyme 1 (Rce-1) followed with subsequent methylation of cysteine by isoprenyl–cysteine carboxyl methyltransferase (ICMT) [144]. Unprenylated proteins retain the 3 amino acids (AAX) and display reduced mobility in SDS-PAGE compared to their prenylated forms; a hallmark for lack of geranylgeranylation. Protein prenylation is important for the proper functioning and

localization of small G proteins [141]. GGTI-298 induced significant expression of rhoA and cdc42 but not that of rac1. Although there is an increase in expression of these proteins, they remain nonfunctional due to their lack of GGPP, as our previous studies have shown with lovastatin-induced rhoA inactivation [132]. The co-administration of GGPP reversed the effects of lovastatin but not of GGTI-298 further confirming that rhoA, cdc42, and rac1 are geranylgeranylated.

GGTI-298 treatment inhibits the geranylgeranylation and proper membrane localization of rho family of proteins, which resulted in actin cytoskeletal disorganization observed in the rhodamine-conjugated phalloidin stained SCC25 cells. Normal fibroblast cells, such as N1H 3T3, can be used to further confirm the effects of GGTI-298 on the proper organization of the actin cytoskeleton particularly its effects on stress fiber formation.

The acquisition of migratory and invasive properties are key events in the oncogenic progression of cells [179]. In order to illuminate the importance of Rho GTPases on cell migration in HNSCC, we performed a wound scratch assay and demonstrated a decrease in cell migration; though, this difference could have been affected by the inhibition of cell proliferation or induced cytotoxicity of GGTI-298. However, the concentration of 5μM GGTI-298 we used to test effects on cell migration did not have significant effect on cell viability or number. Hence, treatment with GGTI-298 in HNSCC cells inhibited their cell migration and motility.

4.1.2 GGTI-298 enhances the cytotoxic effects of tarceva in HNSCC

As stated, mevalonate metabolites play an essential role in transducing EGFR-mediated signaling. Due to the potential of statins to affect EGFR signaling through its

ability to target HMG-CoA reductase, our previous studies have demonstrated enhanced cytotoxicity of receptor tyrosine kinase inhibitors in combination with lovastatin through the induction of apoptosis in various malignancies, including HNSCC [131, 132, 187, 188]. These results have led to a Phase I study (ClinicalTrials.gov Identifier: NCT00966472) in which rosuvastatin demonstrated therapeutic benefit when combined with tarceva in a subset of these patients. However, this enhanced cytotoxicity was associated with significant muscle toxicities at a significantly higher rate than at cholesterol lowering doses of statins [140], which limits the potential therapeutic benefit of this approach.

To determine which end products downstream of the mevalonate pathway are essential for statin-induced cytotoxicity, we demonstrated that GGPP co-administration reverses the apoptotic effects of lovastatin, its inhibitory effects on EGFR function, and its synergistic cytotoxicity in combination with tarceva [132]. Hence, our goal in this study was to demonstrate that a selective GGTase-I inhibitor can affect EGFR function and show enhanced cytotoxicity in combination with tarceva in HNSCC cells mimicking the effects lovastatin.

GGTI-298 demonstrated enhanced cytotoxicity with the combination of tarceva in HNSCC. However, the limitations of the MTT assay must be delineated. The assay is based on cell metabolism, as tetrazolium salts such as MTT are taken up by viable cells and metabolically reduced into highly colored and measurable end products called formazans [189]. The decrease in cell viability observed in our experiment could be due to a decrease in cell proliferation or an increase in cell apoptosis, because in both cases fewer cells are present in order to metabolize the MTT. We first confirmed the enhanced cytotoxicity of the combination treatment of GGTI-298 and tarceva in HNSCC using a non-enzymatic dye

exclusion viability assay. Similar results were demonstrated with the combination of GGTI-298 and tarceva. However, this test does not differentiate between necrotic and apoptotic cells. To address this issue, we employed flow cytometric analysis of propidium iodide stained SCC9 and SCC25 cell lines that can identify subG1 apoptotic cells. Apoptotic bodies are cell fragments that also contain nuclear fragments that are visualized as a pre-G1 peak in the DNA histogram that is characteristic of this programmed cell death modality [182]. In both cell lines, there was a measurable increase in apoptosis levels in GGTI-298 and tarceva combination treatment when compared to the monotherapy of each drug. Additionally, we confirmed our results by evaluating the induction of apoptosis at a molecular level by assessing the cleavage of PARP by western blot analysis. PARP is a nuclear DNA repair protein that during apoptosis is cleaved by activated caspases resulting in the emergence of a signature 89 kDa fragment [190]. SCC9 and SCC25 showed an increase in the levels of cleaved PARP when treated with a combination of GGTI-298 and tarceva. This provides evidence that this combination treatment induced apoptosis in our HNSCC models.

4.1.3 GGTI-298 inhibits EGFR function and activation of its downstream signaling pathways

Activation of the EGFR is stimulated by ligand binding that results in receptor dimerization and its subsequent autophosphorylation required to activate its downstream signaling targets that mediate its effects on growth, differentiation, migration and cell survival [191]. In this study, we demonstrated that GGTI-298 inhibited EGF induced EGFR activation and receptor dimerization. The inhibitory effects of GGTI-298 on EGFR dimerization were likely mediated through targeting of the protein geranylgeranylation. As we have demonstrated, GGTI-298 has significant effects on actin architecture in HNSCC

cells and actin organization has been shown to play a role in EGFR localization and activation [133].

Furthermore, we evaluated the effects of GGTI-298 on trafficking of EGFR and EGF ligand in HNSCC. In this study, we have shown that GGTI-298 does indeed affect trafficking of ligand-induced EGFR in SCC9 cells and also inhibited the trafficking of fluorescent EGF treated SCC25 cells. Subsequently, GGTI-298 enhanced the effects of tarceva on EGFR activation visualized as a reduction in EGFR autophosphorylation compared to tarceva treatment alone in both SCC9 and SCC25 cells.

EGFR dimerization and autophosphorylation leads to subsequent activation of downstream signaling cascades, including the Raf/MEK/ERK and the PI3K/AKT/mTOR pathways. The activation of these signaling events is responsible for regulating key tumorigenic processes such as proliferation, inhibition of apoptosis, cell adhesion/ motility, growth, and survival [192]. In this study, we have shown that GGTI-298 does indeed enhance the inhibitory effects of tarceva on the activation of the AKT and ERK pathways as assessed by the levels of pAKT and pERK expression.

4.2 Novel Mechanism of GGTI-298 Inhibition of EGFR Activation and its Downstream Signaling Pathways

4.2.1 Linking effects of GGTI-298 on actin cytoskeleton disorganization to EGFR's inactivation

Previous reports have demonstrated direct evidence for the involvement of Rho proteins in cytoskeleton organization using activated mutant forms of the small GTPase. For example injection of RhoV14, the active mutant for Rho, in fibroblasts resulted in the induction of stress fibers and the appearance of focal adhesions [193]. On the other hand, injection of RacV12, the active form of Rac1, induced lamellopodia and membrane ruffles

formation [194]. Injection of yet a third member of the family, Cdc42, led to the formation of filopodial protrusions at the cell periphery [195]. Our proposed model based on the experimental results contained herein suggests that GGTI-298 treatment inhibits the geranylgeranylation and proper membrane localization of the rho family of proteins. This results in cytoskeletal disorganization and subsequent effects on ligand induced receptor dimerization, activation and downstream signaling (Figure 4.1). There are multiple mechanisms by which the cytoskeleton may influence EGFR activation:

The cytoskeleton may stabilize the receptor by maintaining receptor dimer formation. *In vitro* studies have shown the colocalization of F-actin and the EGFR using immunofluorescence and immunoelectron microscopy [196, 197]. Additionally, using a co-immunoprecipitation method, a previous study in our laboratory demonstrated that F-actin is readily pulled down with EGFR in control-treated cells, but not in lovastatin-treated cells suggesting that EGFR can associate directly with the actin cytoskeleton (Zhao et al, manuscript in preparation).

The cytoskeleton may form a matrix that facilitates the interaction of downstream signaling proteins by sequestering substrates and keeping them in close contact with the receptor. Upon ligand binding, EGFR undergoes a conformational change that results in its dimerization and its subsequent activation as well as the activation of its downstream signaling targets. The signal transduction mediated by EGFR activation involves a series of downstream signaling molecules, which interact with proteins associated with the cytoskeleton [198].

The actin cytoskeleton may also play a role in EGFR internalization and proper trafficking within the cell. Previous reports have shown that the actin cytoskeleton is critical

to the endocytosis of the receptor [199]. EGFR complexes and downstream signaling molecules that associate with the actin cytoskeleton are involved in receptor endocytosis [200-203].

In this study, we demonstrated a novel mechanism in which GGTI-298 affects EGFR function through inhibition of its dimerization and proper trafficking. This effect was regulated by GGTI-298's ability to prevent proper actin cytoskeleton organization and confirms previous reports that actin plays a role in EGFR activity [204].

4.2.2 Effects of geranylgeranylated proteins on downstream targets of EGFR

Elevated expression of EGFR in HNSCC correlates with poor prognosis and the downstream effectors of EGFR such, ERK and AKT, are activated in HNSCC. A study showed that expression of activated ERK correlated with EGFR overexpression in HNSCC. Importantly, activated ERK was associated with a higher proliferative index and advanced HNSCC tumor stage, and was present in the nucleus of tumor cells [205]. Another survival signal downstream of EGFR, AKT, is overexpressed in 57% to 81% of HNSCC tumors [206]. Thus, EGFR promotes growth and survival through several oncogenic signaling pathways.

On the basis of the data presented herein, we demonstrated that GGTI-298 inhibited ligand-induced receptor dimerization, trafficking and combined with tarceva, enhanced the effects on EGFR autophosphorylation and subsequent inactivation of its downstream targets, AKT and ERK, in HNSCC. At present, little is known about the precise mechanism of GGTI-298 inactivation of EGFR downstream targets. However, a study showed that GGTI-298 monotherapy was able to inhibit the activation of pERK and pAKT, but had no effect on

Ras activation in human mesangial (HM) cells [207]. This suggests that the effects of GGTI-298 on ERK and AKT activation are directly owing to geranylgeranylation inhibition and not simply a secondary consequence of the effects of GGTI-298 on cell proliferation and apoptosis. Future experiments could extrapolate these findings in cancer cells, particularly in HNSCC, in order to further elucidate GGTI-298's mechanism of inhibition of EGFR downstream signaling.

Our data shows that GGTI-298 addition enhances tarceva's cytotoxicity on SCC9 and SCC25 HNSCC cells. This enhanced cytotoxicity is due to the induction of apoptosis as demonstrated by PARP cleavage and flow cytometry analysis. Although the mechanism by which GGTIs induce apoptosis has yet to be uncovered, these results suggest that inhibiting protein geranylgeranylation could inhibit tumor cell survival. Recent work in human cancer epithelial cells demonstrated that GGTIs might promote apoptosis by inhibiting the activation of AKT [208]. Although it is not clear which geranylgeranylated protein activates AKT, both Rac and Cdc42 have been shown to interact specifically with PI3K and activate AKT [209]. Furthermore, Rac appears to play a key role in transducing integrin-mediated survival signals [210]. RhoA may also protect against apoptosis by up-regulating the expression of the survival protein Bcl-2 [211] or down regulating the expression of the tumor suppressor protein, p53 [212].

Based on these results, the treatment of HNSCC with GGTI-298 in combination with tarceva could overcome resistance to TKIs and would constitute a strong rational approach to cancer treatment.

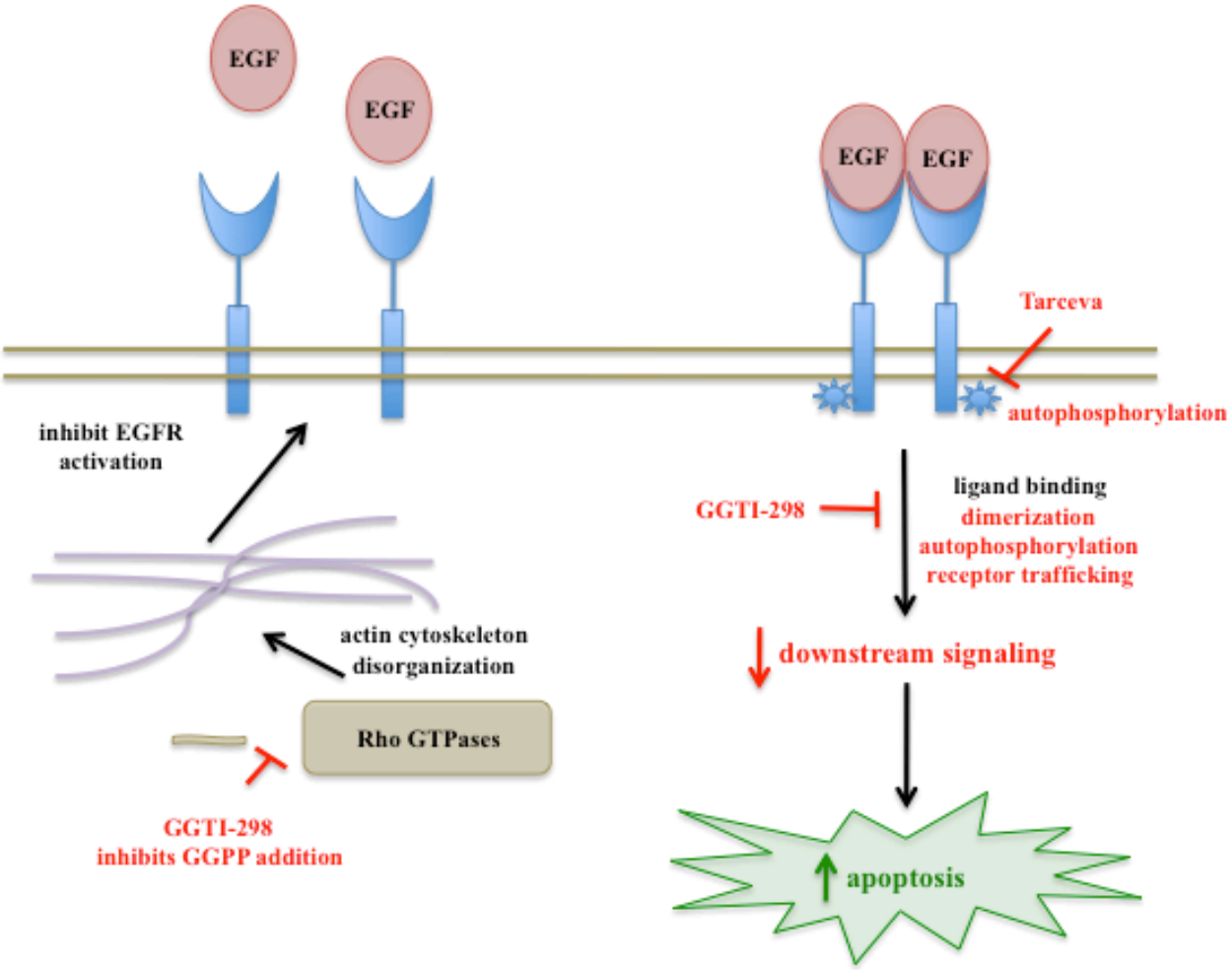

Figure 4.1 Proposed model of the mechanism of GGTI-298 inhibition on EGFR dimerization, activation and trafficking GGTI-298 exerts its inhibitory effects possibly through the inhibiton of protein geranylgeranylation of the Rho GTPases.

4.3 Future Prospects and Perspectives

Over the past 30 years, patients with advanced HNSCC presented with a poor prognosis [5, 213]. More than 50% of newly diagnosed patients do not achieve complete remission, and approximately 10% relapse with metastasis to distant organs [85]. Therefore, research focused on gaining a better understanding of this disease and on the development of novel treatment strategies. High expression of EGFR is a common feature of the majority of epithelial malignancies including HNSCC [214]. Because of its prevalence and its crucial role in the pathogenesis, targeting EGFR has become a rational approach for the treatment of HNSCC. Despite the high levels of EGFR expression within the tumor, clinical data indicates that many patients fail to respond to EGFR inhibitor treatment, and of particular interest, to receptor tyrosine kinase inhibitors (TKIs). A number of laboratory studies including our own have strongly linked protein geranylgeranylation inhibition to statin-induced apoptosis in several models. In endothelial cells, statins potentiate TNF-α–mediated apoptosis by blocking rhoA geranylgeranylation [215]. RhoA geranylgeranylation has also been linked to statin-induced apoptosis in malignancies, including thyroid cancer [216] and multiple myeloma [217]. Statins were also shown to prevent migration through the alteration of rho signaling [218] in breast and thyroid cancers as well as in normal T cells.

In this study, we have successfully shown that targeting protein geranylgeranylation with GGTI-298 affects EGFR function through inhibition of its dimerization and proper cellular trafficking. This effect was regulated by GGTI-298's ability to disrupt actin cytoskeleton organization. This results in enhanced inhibition of EGFR-induced AKT and ERK activation compared to tarceva monotherapy. We also identified the ability of GGTI-298 to enhance cytotoxicity through the induction of a potent apoptotic response in

combination with tarceva in HNSCC. Combining these two approaches, with each targeting the EGFR through distinct mechanisms, represents a novel combinational therapeutic approach in HNSCC, as well as in other cancers in which EGFR is involved in their pathogenesis.

Recently, the GGTI-2418 inhibitor underwent a Phase I clinical trial evaluation and was shown to be well tolerated with minimal side effects, with nausea as the main adverse event. Plasma concentrations of GGTI-2418 achieved in this trial exceeded concentrations required for the in-vitro inhibition of geranylgeranyltransferase I, with the C_{max} at 1050 $mg/m^2/day$. Durable stable disease was achieved in two colorectal cancer patients and one hepatocellular patient [219]. Furthermore, the application of GGTI-298 in combination with tarceva in *ex vivo* HNSCC human biopsies and mice injected with SCC9 or SCC25, will allow the validation of the efficacy and toxicity of this combination treatment. These experiments will allow translation of this combination-based approach into a Phase I clinical study in order to determine the maximum tolerable dose (MTD) of this novel combination therapy for HNSCC.

4.4 Summary

Overall our findings suggest that targeting protein geranylgeranylation can inhibit the function of EGFR. Furthermore, combining GGTI-298 with tarceva induced apoptotic and cytotoxic effects in HNSCC. This was demonstrated by the enhanced effects of GGTI-298 on tarceva's inhibition of the AKT and ERK pathways. Expanding these studies together with further pre-clinical studies will provide invaluable data that will play a role in developing this novel therapeutic strategy in the treatment of HNSCC.

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CURRICULUM VITAE

EDUCATION

University of Ottawa
January 2011-Present *Ottawa, Ontario*
Master of Science in biochemistry with specialization in human and molecular genetics

- Average of A over two seminar courses
- Degree to be completed winter 2013

University of Ottawa
September 2005 -April 2010 *Ottawa, Ontario*
Honours Bachelor of Sciences with Specialization in Biochemistry

- Graduated with an average of 70%

Collège Catholique Samuel-Genest
September 2002-July 2005 *Ottawa, Ontario*

- Graduated with honours
- Received numerous academic and extracurricular awards

AWARDS

- Received the *Admission Scholarship* from the University of Ottawa - 2005
- Obtained the *Aiming for the Top Tuition Scholarship* - 2005

LANGUAGES

Fluent and able to write in English, French, and Arabic

RESEARCH EXPERIENCE

Ottawa Hospital Research Institute – Cancer Center
January 2011-Present *Ottawa, Ontario*
Master of Science

- Laboratory research in cancer therapeutics and drug development
- Discovered novel drug combination in the treatment of head and neck cancers
- Contributed work for a publication currently in press
- Presented multiple posters and seminars at BMI and OHRI events

PUBLICATIONS

Nima Niknejad, Ivan Gorn-Hondermann, Laurie Ma, Stephanie Zahr, Stephanie Johnson-Obeseki Matthew Tang and Jim Dimitroulakos
Lovastatin-Induced Apoptosis is Mediated by Activating Transcription Factor 3 and Enhanced in Combination with Salubrinal
International Journal of Cancer - 2013

PROFFESIONAL EXPERIENCE

University of Ottawa
September 2008-Present *Ottawa, Ontario*
Teaching Assistant

- Laboratory demonstrator

- Explained theories related to protocol for each session
- Proctor and corrector

Cyrville Community Centre
October 2009-2010 *Ottawa, Ontario*
After-School Program Counselor
- Supervised and participated in educational activities with the children while maintaining an orderly and safe environment.
- Assisted a diverse range of children in homework of all subjects in English and French.
- Performed general administrative duties.

VOLUNTEER

Roger's House
May 2012-Present *Ottawa, Ontario*
- Assist with the care of children/youth diagnosed with life limiting illnesses.
- Work with a multidisciplinary team, supervising, playing and entertaining children/youth.

Let's Talk Science
January 2011-Present *Ottawa, Ontario*
- Sharing knowledge and passion with kids.
- Inspiring and engaging them in Sciences.
- Helping them discover the relevance of science to their lives.

Delta Delta Delta Sorority
September 2009-Present *Ottawa, Ontario*
- Raised money by organizing events such as student events and bake sales with all proceeds go to charities such as Relay for Life, CHEO, Harmony House, St. Joseph's Parish Women's Centre, and St. Jude Children's Hospital.
- Volunteered at a variety of local organizations such as Harmony House and CHEO.

Children Hosptial of Eastern Ontario
September 2008-February 2010 *Ottawa, Ontario*
Emergency Department Volunteer
- Provided administrative support for the Emergency Department's functioning.

Playrooms for in-patient and out-patient oncology
- Engaged in therapeutic play program by preparing and planning recreational activities for age groups.

Rideau Dental Clinic
May 2009-May 2010 *Ottawa, Ontario*
Volunteer
- Shadow dentists on various operations to gain knowledge on procedures.
- Organizing patient information for future appointments while creating daily charts for the dentist.
- General administrative duties

Marochel Manor Retirement Home
May 2007-August 2007 *Ottawa, Ontario*
Volunteer
- Coordinated planned activities such as teaching a class, make presentations on topics of

interest, and spending quality time with residents.
- Provided companionship, guidance, and connection to the outside community.

Saint Stephano Maronite Catholic Church
May 2006-July 2006 *Batroun, Lebanon*

Sunday-School Teacher/Tutor
- Tutored 10-15 elementary children to read, write, and speak the English language, critiqued weekly homework tasks.
- Evaluated students from prior classes having tested their improvements in English reading, writing, and oral abilities.
- Consulted with children's parents to stimulate lesson planning encouraged for students with special attention outside class.