Targeting the Mevalonate Pathway Inhibits the Function of the Epidermal Growth Factor Receptor

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ABSTRACT

Purpose: The epidermal growth factor receptor (EGFR) is a key regulator of growth, differentiation, and survival of epithelial cancers. In a small subset of tumors, the presence of activating mutations within the ATP binding site confers increased susceptibility to gefitinib, a potent tyrosine kinase inhibitor of EGFR. Agents that can inhibit EGFR function through different mechanisms may enhance gefitinib activity in patients lacking these mutations. Mevalonate metabolites play significant roles in the function of the EGFR; therefore, mevalonate pathway inhibitors may potentiate EGFR-targeted therapies.

Experimental Design: In this study, we evaluated the effect of lovastatin on EGFR function and on gefitinib activity. Effects on EGFR function were analyzed by Western blot analysis using phosphospecific antibodies to EGFR, AKT, and extracellular signal-regulated kinase. Cytotoxic effects of lovastatin and/or gefitinib were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and flow cytometry.

Results: Lovastatin treatment inhibited EGF-induced EGFR autophosphorylation by 24 hours that was reversed by the coadministration of mevalonate. Combining lovastatin and gefitinib treatments showed enhanced inhibition of AKT activation by EGF in SCC9 cells. The combination of 10 μmol/L lovastatin and 10 μmol/L gefitinib treatments showed cooperative cytotoxicity in all 8 squamous cell carcinomas, 4 of 4 non–small cell lung carcinoma and 4 of 4 colon carcinoma cell lines tested. Isobologram and flow cytometric analyses of three representative cell lines with wild-type EGFR ATP binding sites confirmed that this combination was synergistic inducing a potent apoptotic response.

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Conclusions: Taken together, these results show that targeting the mevalonate pathway can inhibit EGFR function. They also suggest the potential utility of combining these clinically relevant therapeutic approaches.

INTRODUCTION

Communication between individual cells is essential for the regulation and coordination of complex cellular processes such as growth, differentiation, migration, and apoptosis. Signal transduction networks mediating these biological processes are regulated in part by polypeptide growth factors that activate cell surface receptors either in a paracrine or autocrine manner (1). The primary upstream mediators of these cell responses are receptor tyrosine kinases that couple ligand binding to downstream signaling cascades and gene transcription (2). An integral member of this family is the epidermal growth factor receptor (EGFR), a transmembrane glycoprotein that is activated by binding to its ligands (mainly EGF and tumor growth factor-α; refs. 3, 4). Ligand binding triggers receptor dimerization and autophosphorylation at specific tyrosine residues of the EGFR, followed by the activation of a signal transduction cascade that results in enhanced proliferation and cell survival by suppressing apoptosis (3, 4).

The EGFR is a key regulator of growth, differentiation, and survival of epithelial cells. It is also involved in the development and progression of cancers derived from these tissues including squamous cell carcinomas of the head and neck (HNSCC) and of the cervix, non–small cell lung carcinomas (NSCLC), and colon cancer (4). In these prevalent human cancers, chemotherapy has provided only marginal survival benefits for patients with metastatic disease (5, 6). Clearly, novel therapeutic approaches are urgently needed. As such, targeting EGFR function has been an intensive focus of anticancer therapeutic approaches in these cancers (4, 7). Many of the proliferative and antiapoptotic effects elicited by this pathway are regulated through the ability of EGFRs to activate RAS proteins (3, 4). The ras/raf/extracellular signal-regulated kinase (ERK) pathway can regulate the effects of EGFR on growth whereas antiapoptotic as well as mitogenic responses can be mediated by the ras/phosphoinositide-3 kinase/AKT pathway (3, 4).

EGFR signaling is triggered by binding to its ligands that results either in homodimerization or heterodimerization with closely related receptors such as Erb2 (3, 4). Dimerization leads to the autophosphorylation of their tyrosine kinase domains with subsequent activation of downstream effectors (3, 4). Gefitinib (Iressa, ZD1839) is the most clinically advanced EGFR-targeted therapy (8). Gefitinib efficiently binds to the ATP binding domain of this receptor and inhibits the autophosphorylation of tyrosine residues in its kinase domain on ligand activation (8). Clinical and preclinical evaluations have identified significant variability in patients response to gefitinib that were not correlated with the expression levels of EGFR or related family members in tumors (9). This variability has been recently
attributed to the presence versus absence of mutations affecting residues contributing to the ATP binding site of the EGFR (10, 11). These studies provided evidence that NSCLC patients responding to this agent almost exclusively developed tumors that had acquired these specific EGFR mutations. Such mutated EGFRs show enhanced sensitivity to stimulation by its ligands and to gefitinib inhibition potentially through the stabilization of ATP and gefitinib binding, respectively (10, 11). Cell lines expressing these mutated EGFRs require lower concentrations of gefitinib to induce cytotoxicity and to inhibit ligand-induced EGFR autophosphorylation and downstream signaling (11).

These landmark studies imply that in tumors not carrying these activating mutations either gefitinib does not sufficiently inhibit EGFR to evoke a clinical response or that over-expression of EGFR alone, when it occurs, does not play a critical role in the majority of patients (10, 11). In gliomas, where frequent amplification and rearrangements of the EGFR gene result in deletion of the extracellular receptor binding domain producing a constitutively active receptor (12), gefitinib failed to induce clinical responses (13). Furthermore, over-expression of EGFR is a well-established prognostic indicator of response to therapy and of patient survival in a range of epithelial cancers, which includes HNSCC, NSCLC, and colon carcinomas (14). Clearly, the EGFR plays a significant role in the pathogenesis of these tumor types. Therefore, combining gefitinib with other agents that target EGFR by alternative mechanisms showing cooperative inhibition of EGFR and enhanced cytotoxicity may provide more wide-ranging clinical benefits.

Mevalonate metabolites play an essential role in transducing EGFR-mediated signaling (15, 16). The diverse array of critical biosynthetic products of the mevalonate pathway include sterols, especially cholesterol, involved in membrane structure and steroid production; ubiquinone, involved in electron transport; farnesyl and geranylgeranyl isoprenoids of which covalent binding to proteins such as ras facilitates their membrane localization; and dolichol, required for glycoprotein synthesis (17). Several of these metabolites have been shown to play significant roles in the function of the EGFR and the components of its signaling cascades. Therefore, the depletion of mevalonate metabolites may have a significant effect on EGFR function (15, 16). Lovastatin is a specific and potent inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), the rate-limiting enzyme of the mevalonate pathway (18, 19). Targeting HMG-CoA reductase using lovastatin induces a potent tumor specific apoptotic response in a variety of tumor types including HNSCC-derived cell lines at therapeutically achievable levels of this drug (20, 21). We recently showed that inhibiting EGFR function potentiated the apoptotic effects of lovastatin in HNSCC cells (22). The additive effects shown in HNSCC-derived cell lines suggested the possibility that these agents, although acting through distinct mechanisms, may share a common target. In this study, we evaluated the effects of lovastatin on the function of the EGFR and its signaling cascades as well as the cytotoxic effects elicited when combining lovastatin and gefitinib on various squamous cell carcinomas, NSCLC, and colon cancer-derived cell lines.

**MATERIALS AND METHODS**

**Tissue Culture.** The HNSCC cell lines SCC9, SCC25, FADU, and CAL27 and the breast adenocarcinoma cell line MCF-7 were obtained from the American Type Culture Collection (Rockville, MD). The cervical carcinoma cell lines CASKI, SIHA, HeLa, and ME180 were provided by Dr. D. Hedley (University Health Network, Toronto, Canada). The NSCLC cell lines A549, H226, H23, EKVVX, and HOP92, the colon carcinoma cell lines HCT116, HT29, HCT15, and SW620, and the epidermoid carcinoma cell line A431 were provided by Dr. J. Bell (Ottawa Regional Cancer Centre, Ottawa, Canada). The cell lines were maintained in DMEM (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal, Canada). Cells were exposed to solvent control or to 0 to 100 μmol/L lovastatin (generously provided by Apsophex, Mississauga, Canada, diluted from a 10 mmol/L stock in ethanol prepared as previously described; ref. 23). Pravastatin (generously provided by Bristol-Myers Squib, Outside Investigator Program) was diluted from a 100 mmol/L stock in DMSO and human recombinant EGF (Sigma, St. Louis, MO) was diluted from a 50 μg/mL stock in 10 mmol/L acetic acid/0.1% bovine serum albumin (Sigma).

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay.** In a 96-well flat-bottomed plate (Nunc, Naperville, IL) ~5,000 cells/150 μL of cell suspension were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Following treatment, 50 μL of a 5 mg/mL solution in PBS of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium substrate (Sigma) were added and incubated for up to 6 hours at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 100 μL of a 0.01 mol/L HCl/10% SDS (Sigma) solution, shaking overnight at 37°C. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies (West Sussex, United Kingdom) at 570 nm to determine the absorbance of the samples. Isobologram combination index values were determined as described by Chou et al. (24).

**Colony Growth Assay and Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Staining.** SCC9 cells were plated at a density of 500 cells in 60-mm tissue culture plates (Nunc) and allowed to attach overnight. After treatments were completed, the cells were incubated for 10 days in control media that was replenished every 3 days. The plates were washed twice in PBS and the colonies that formed were stained with 2 mg/mL methylene blue (Sigma) in methanol for 1 hour. The plates were washed with liberal volumes of water, dried, and colonies were visually counted.

SCC9 cells were plated in 35-mm tissue culture plates (Nunc) containing a sterilized 20 × 20 mm glass coverslip (Fisher, Ottawa, Canada) at a density of 10^4 cells and allowed to attach overnight. After treatments were completed, the cells were rinsed twice with PBS and fixed with 4% paraformaldehyde (Sigma) in PBS for 30 minutes at room temperature. After two washes in PBS, the cells were permeabilized using PBS + 0.2% Triton X-100 for 2 minutes and subsequently washed twice with...
PBS. DNA strand breaks were detected using the TUNEL In situ Cell Death Kit (fluorescein) from Roche Applied Science (Laval, QC, Canada) following the protocol of the manufacturer. Cover slips were mounted onto slides and cell nuclei counterstained with 4′,6-diamidino-2-phenylindole (Vectashield, Vector, Burlington, CA). Thirty random fields per coverslip at 4,000× magnification were counted and the percentages of fluorescein-positive nuclei versus 4′,6-diamidino-2-phenylindole stained nuclei were determined using the Zeiss Axioskop2 microscope. The mean and SD of the mean were calculated for each treatment.

Flow Cytometry. Cell cycle variables were determined by flow cytometry using propidium iodide labeling of single cells as described previously (21). Single cell suspensions were labeled with 50 μg/mL propidium iodide (Sigma) and ~10⁶ cells in 1 mL were analyzed by flow cytometry. Ten thousand cells were evaluated and the percentage of cells in sub-G₁ phase was determined using the Modfit LT program (Verity Software House, Topsham, ME).

Western Blot Analysis. Total cellular protein was extracted using a buffer that consisted of 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 0.2 mM sodium orthovanadate (Sigma) and 0.2 mM phenyl methyl sulfonyl fluoride (Sigma) in 2× PBS. Approximately 200 μL of extraction buffer were used to treat 10⁶ cells. Total protein was quantified with the Bio-Rad Protein Assay using bovine serum albumin (Sigma) as standard. Protein extracts representing 50 μg total protein were separated on a 10% SDS-PAGE gel and electrophoretically transferred onto polyvinylidene difluoride membranes (Amersham, Toronto, Canada). Membranes were blocked in 5% skim milk powder in PBS overnight at 4°C. Primary antibody, diluted in 5% skim milk powder in PBS, was incubated with the membrane for 1 hour at room temperature. The monoclonal antibodies used were specific for phosphotyrosine (clone PY20) and ERK1 (Santa Cruz Biotechnology, Santa Cruz, CA) and the polyclonal antibodies were specific for EGFR and phospho-EGFR at sites 1,068, 1,045, and 845, phospho-ERK at site threonine 202/tyrosine 204, and actin (clone PY20) as shown above but the coadministration of 100 μM mevalonate reversed this effect (Fig. 1B). In this experiment, the phosphorylation status of EGFR was determined by Western blot analysis using antiphosphotyrosine (clone PY20) and antiphosphotyrosine 1,068 antibodies. These results showed the ability of lovastatin to inhibit the activation of the EGFR through the targeting of the mevalonate pathway.

We expanded these results and evaluated the potential of lovastatin to inhibit EGFR phosphorylation in various tumor-derived cell lines. In a panel of HNSCC (SCC9 and SCC25), cervical carcinomas (SiHa and ME180), NSCLC (EKVX and A549), colon carcinomas (HCT15 and HT29), and a breast adenocarcinoma (MCF-7), the expression levels of EGFR varied significantly (Fig. 2A). The effects of lovastatin were evaluated on cell lines expressing relatively high (SCC25 and ME180), low (SCC9 and EKVX), and undetectable (MCF-7) levels of EGFR. Following a 24-hour exposure to 10 μM lovastatin treatment, EGFR-induced autophosphorylation was inhibited in both the high expressing cell lines SCC25 and ME180 as well as the low expressing cell lines EKVX (Fig. 2B) and SCC9 (see Fig. 1). Neither EGFR nor phosphorylated EGFR (1,068) was detectable in MCF-7 under these conditions (data not shown). Therefore, lovastatin can inhibit EGFR autophosphorylation in a number of cell lines expressing variable levels of this receptor.

Lovastatin Effects on Downstream Epidermal Growth Factor Receptor Signaling Pathways. Based on its ability to inhibit EGFR-induced activation of the EGFR, we evaluated the effect of lovastatin treatment on the signaling cascades triggered by this receptor as well. The signaling pathways induced by activated EGFR include the ras/mitogen-activated protein kinase/ERK and the ras/phosphoinositide-3 kinase/AKT pathways, both of which play a significant role in the mitogenic and cell survival responses mediated by this receptor (3, 4). Serum-starved SCC9 cells were treated with 10 μM lovastatin for 24 hours, conditions that evoke significant inhibition of EGFR-induced activation of the EGFR. The functional activation
of these pathways was evaluated by Western blot analysis, employing phosphospecific antibodies recognizing their active forms and control antibodies for EGFR, ERK, and AKT. Lovastatin did not affect EGF-induced phosphorylation of ERK, whereas both the basal and EGF-induced levels of phosphorylated AKT were reduced (Fig. 3A). However, this reduction was not apparent when SCC9 cells were treated in complete medium with 10% serum (Fig. 3B). These results imply that lovastatin interferes with the transduction of EGFR signals to the ras/phosphoinositide-3 kinase/AKT pathway.

Similar to the effects of lovastatin in SCC9 cells, gefitinib treatment at 10 μmol/L for 2 hours also displayed potent inhibition of both EGF-induced EGFR autophosphorylation and ERK activation but had minimal effect on AKT (Fig. 3B). However, both the basal and EGF-induced activation of AKT were considerably reduced when lovastatin and gefitinib were combined (Fig. 3B). This suggests that these agents act cooperatively to target at least one arm of the EGFR signaling pathway.

Inhibition of Epidermal Growth Factor Receptor Potentiates Lovastatin-Induced Cytotoxicity. To examine the more downstream consequences of lovastatin and gefitinib cotreatment, we evaluated the cytotoxic response of this combination in SCC9 cells, using an MTT assay. Gefitinib alone (1-20 μmol/L treatment for 48 hours) induced pronounced cytotoxicity in the HNSCC cell line SCC9 (Fig. 4A). The further addition of 10 μmol/L lovastatin did not enhance the cytotoxic effects of gefitinib when the agents were added simultaneously. However, lovastatin significantly potentiated the cytotoxic effects of gefitinib when added 24 hours before the combination treatment for 48 hours (Fig. 4A). This schedule allowed for EGFR to be functionally inactivated before the addition of the combination of lovastatin and gefitinib in SCC9 cells. This pretreatment regimen was implemented in all subsequent assays where cell cultures were treated with either solvent control or 10 μmol/L lovastatin for 24 hours. This time point and concentration of lovastatin showed significant inhibitory effects on EGF-induced EGFR activation in the SCC9 cell line.

The MTT assay is a measure of cell viability and does not distinguish between the cytotoxicity and the growth inhibition responses of cells to drugs. We therefore evaluated the combined effects of lovastatin and gefitinib in a clonogenic assay described in MATERIALS AND METHODS. A 50% reduction in colony formation was observed when SCC9 cells were treated with lovastatin alone (10 μmol/L, 72 hours; Fig. 4B). Gefitinib treatment (10 μmol/L, 48 hours) produced a 20% reduction in clonogenicity. The combined lovastatin and gefitinib treatment induced a more than 90% reduction (Fig. 4B). To determine if the combined effects of these agents were the result of an enhanced apoptotic response in SCC9 cells, we evaluated these treatments using a terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay. In this assay, DNA strand breaks that are characteristic of apoptosis (26) are detected and can be visualized by fluorescence microscopy. SCC9 cells were pretreated with either solvent control (control, 10 μmol/L gefitinib treatment) or 10 μmol/L lovastatin (10 μmol/L...
lovastatin, combination treatment) for 24 hours. Treatment with either agent alone or their combination was continued for 24 and 48 hours. The percentage of terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling positive cells was determined for each treatment (Fig. 4C). The combination treatment for 48 hours resulted in significant cell loss and was not evaluable in this study. The combination treatment for 24 hours, however, displayed an enhanced apoptotic response compared with 10 μmol/L lovastatin or 10 μmol/L gefitinib treatment alone (Fig. 4C). These results show an enhanced cytotoxic and apoptotic response with this combination in SCC9 cells.

**Gefitinib and Lovastatin Induce a Cooperative Cytotoxic Response in a Variety of Tumor-Derived Cell Lines.** Based on the cytotoxic effects exhibited by gefitinib and lovastatin in combination in SCC9 cells, we expanded this study and evaluated a variety of tumor-derived cell lines. We evaluated four HNSCC, four cervical carcinoma, five NSCLC, four colon carcinomas, a breast adenocarcinoma, and an epidermoid carcinoma-derived cell lines. All were treated with either lovastatin alone (10 μmol/L, 72 hours), gefitinib alone (10 μmol/L, 48 hours), or pretreated with lovastatin (10 μmol/L, 24 hours), followed by the combination of agents (10 μmol/L each, 48 hours; Fig. 5A). These cell lines showed significant variability in their response to each of these agents with only the HNSCC cell lines being consistently sensitive to both. Gefitinib induced relatively weak responses in the NSCLC and colon carcinoma–derived cell lines examined, in line with previously published results (27).

All four HNSCC cell lines tested showed a significant cooperative effect with the combination of lovastatin and gefitinib (Fig. 5). Significant differences in response between each agent added alone and their combination were observed (Fig. 5; *, P < 0.01, paired t test). Similar significant cooperative effects were shown in all 4 cervical carcinomas, all 4 of 4 evaluable NSCLC and in all 4 of the colon carcinoma cell lines examined. The combination was not evaluable in HOP92 cells, the fifth NSCLC cell line analyzed, due to their hypersensitivity to lovastatin treatment alone. The A431 epidermoid carcinoma cell line that highly expresses EGFR and is sensitive to gefitinib (27) also showed cooperative effects with this combination of agents. In contrast, the MCF-7 breast adenocarcinoma cell line, which does not express detectable levels of EGFR, was unresponsive to both of these agents and did not respond to the combination lovastatin and gefitinib (Fig. 5A). In this study, we have shown that a wide range of tumor-derived cell lines show cooperative cytotoxicity with this combination of agents irrespective of their expression levels of EGFR and response to these agents individually.

To show that the cytotoxic effects of lovastatin and gefitinib in combination were a result of the ability of lovastatin to target the mevalonate pathway, we evaluated another statin inhibitor as well. Statins comprise a large family of related inhibitors with varying physical properties (28). Lovastatin is a lipophilic inactive lactone prodrug that requires chemical or enzymatic activation whereas pravastatin is a water-soluble active inhibitor. These two agents represent the spectrum of physical properties associated with this class of drugs (28). We evaluated the effects of the combination of pravastatin and gefitinib in the MCF-7 and the SCC9 cell lines, unresponsive and responsive cell lines with respect to the cooperative effects of lovastatin and gefitinib treatments, respectively. A 10-fold higher concentration of pravastatin was necessary to mimic the effects ofLovastatin due to solubility differences. The treatments with pravastatin in combination with gefitinib recapitulated the responses with lovastatin in these cell lines (Fig. 5C), indicating that the cooperative effects observed with gefitinib were a result of targeting mevalonate synthesis.

**Absence of Specific ATP Binding Site Mutations in Epidermal Growth Factor Receptor.** Because recent studies have shown that the anticancer effects of gefitinib were more pronounced in tumor cells that harbored mutations in the ATP binding pocket of the EGFR tyrosine kinase domain (10, 11), we determined the mutational status of this region in 10 representative cell lines used in this study. In this study, we sequenced exons 18 to 21 of the EGFR that encompass its ATP binding site (10, 11) in various HNSCC (SCC9 and SCC25), cervical carcinoma (SIHA, HeLa, and ME180), NSCLC (A549 and EKVX), colon carcinomas (HT29 and HCT116), and the breast adenocarcinoma (MCF-7) cell lines. No point mutations or deletions were detected in the ATP binding sites of their EGFRs (data not shown). The only nucleotide alterations that were detected were neutral polymorphisms that did not result in alterations of amino acid sequence.

**Combinations of Gefitinib and Lovastatin Induce a Synergistic Cytotoxic Response.** To further characterize the cooperative effects of the combination of lovastatin and gefitinib, we analyzed various representative cell lines in greater detail. The HNSCC cell lines SCC9 and SCC25 showed significant cytotoxicity with both agents; the cervical carcinoma cell line SIHA was sensitive to lovastatin only; and the
Colon carcinoma cell line HCT116 did not display significant cytotoxicity with either agent. All four cell lines showed significant cooperative effects with the combination of lovastatin and gefitinib. Using MTT analysis, we showed that the addition of 10 μmol/L lovastatin induced cooperative effects within a range of gefitinib concentrations (1-20 μmol/L) in all of the cell lines examined (Fig. 6, data for SCC9 in Fig. 4A).

We further employed flow cytometric analyses to determine the potential of the induction of apoptosis to the cellular response to these agents in combination. Apoptosis was visualized as a sub-G1 peak that identifies apoptotic bodies that result from cellular fragmentation (29). Lovastatin (10 μmol/L) treatment was for 72 hours; gefitinib (10 μmol/L) treatment was for 48 hours; and the combination of treatments was a 24-hour pretreatment with lovastatin followed by 48-hour treatment with the combination of agents. In the SCC25 cell line, both agents individually induced significant apoptosis with a more dramatic response in combination with 88.5% of cells in the sub-G1 fraction. In the SIHA cell line, only lovastatin induced a significant response in 42.9% of sub-G1 cells with the combination inducing a more pronounced apoptotic response in 68.9%. In the HCT116 cell line, lovastatin and gefitinib induced very weak apoptosis, however, a significant apoptotic response was observed with their combination (Fig. 6). Our flow

![Flow Cytometry](image_url)

**A.** Western blot analysis of 24-hour 10 μmol/L lovastatin–treated serum-starved SCC9 cells with anti-EGFR and phospho-EGFR (tyrosine-1,068), ERK and phospho-ERK (threonine 202/tyrosine 204), and AKT and phospho-AKT (serine 473) antibodies. Following addition of 50 ng/mL of EGF for 15 minutes, the activation of EGFR and AKT was inhibited but not ERK. **B.** Western blot analysis of SCC9 cells treated with 10 μmol/L lovastatin for 24 hours, 10 μmol/L gefitinib for 2 hours, or their combination (24 hours of lovastatin followed by 2 hours of gefitinib treatments). Activation of EGFR, ERK, and AKT following EGF addition was determined as above.

![MTT Analysis](image_url)

**A.** MTT analysis of 48-hour gefitinib treatment in SCC9 cells alone or in combination with lovastatin concurrently or with a 24-hour pretreatment followed by the combination for 48 hours. In the 24-hour pretreatment with lovastatin, significant differences in response between either agent alone and the combination were observed (*, P < 0.005, paired t test). **B.** Colony growth assays of SCC9 cells using the lovastatin pretreatment conditions where control cells (solvent alone), lovastatin-treated cells (24-hour 10 μmol/L lovastatin pretreatment followed by 48-hour 10 μmol/L lovastatin treatment), gefitinib-treated cells (24-hour solvent control pretreatment followed by 48-hour 10 μmol/L gefitinib treatment), and their combination (24-hour 10 μmol/L lovastatin pretreatment followed by 48-hour combination treatment) were evaluated. In the SCC9 cell line, the combination of lovastatin and gefitinib treatments showed significant additive cytotoxic effects compared with each agent alone (P < 0.0001, paired t test). **C.** Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay of treated SCC9 cells detecting DNA strand breaks characteristic of apoptosis. Treatment conditions were as outlined in B; however, due to extensive cell death at the lovastatin pretreatment with the 48-hour combination treatment, drug treatments were evaluated at 24 hours. In the SCC9 cell line, the combination of lovastatin and gefitinib treatments showed enhanced apoptosis compared with each agent alone.
cytometric analyses showed the potential of these agents to induce a synergistic apoptotic response in the cell lines examined.

To determine if lovastatin and gefitinib are synergistic in combination, we evaluated their response using the classic isobologram approach (24) in cell lines lacking activating mutations in the ATP binding site of the EGFR (Fig. 7). In the SCC9, SiHa, and HCT116 cell lines, we determined the MTT50 values (concentration required to reduce MTT activity by 50%) for 72-hour lovastatin treatment and 48-hour gefitinib treatment. These values are represented on the axis of our isobolograms. Combinations of these drugs that induce MTT50 can be plotted on this graph; values on the line represent additive effects of that combination, values above the line are antagonistic, and values below the line represent synergistic activity (24). In this study, we determined the concentration of gefitinib that was required to obtain an MTT50 with 10 μmol/L lovastatin (24-hour pretreatment followed by 48-hour treatment with the combination). Under these conditions in the three cell lines examined, the combination of lovastatin and gefitinib was synergistic. The combination index (24) determined by the individual responses and the response to the combinations clearly showed synergy as values ranged from 0.38 to 0.79 (combination index: >1, antagonism; =1, additive; <1, synergy).

DISCUSSION

In this study, we have shown that the targeting of HMG-CoA reductase, which results in mevalonate depletion, can inhibit the function of the EGFR. EGFR inhibition is substantially amplified when gefitinib and lovastatin are combined. Furthermore, their apoptotic and cytotoxic effects in combination were found to be synergistic, to be manifested in several types of tumor cell lines, to potentially involve the phosphoinositide-3 kinase/AKT pathway, and to be independent of the presence of activating ATP binding site mutations within the EGFR. These findings suggest that mevalonate pathway and receptor tyrosine kinase inhibitors may represent a novel combinational therapeutic approach in a variety of human cancers.

Inhibiting the function of the EGFR has been the focus of a number of therapeutic approaches (4). The limited clinical
responses associated with gefitinib treatment in a relatively small subgroup of NSCLC patients have been recently associated with the presence of activating mutations within the ATP binding site of the EGFR (10, 11). Mutations in this region confer enhanced sensitivity of EGFR to stimulation by ligand binding, and tumor cells show marked enhanced sensitivity to gefitinib-induced cytotoxicity and inhibition of ligand activation of EGFR and its downstream signaling pathways. This is thought to result from enhanced binding of ATP and gefitinib to the mutated ATP binding domains (10, 11). Agents that can target this pathway through alternative mechanisms, like lovastatin, may augment the anticancer effects of gefitinib, increasing their therapeutic activity in the majority of patients that lack these activating mutations. A representative sampling of 10 of the cell lines used in this study, which showed cooperative cytotoxic responses with lovastatin and gefitinib treatments in combination, did not carry mutations within the ATP binding site of this receptor.

Inhibition of HMG-CoA reductase has been evaluated as an anticancer therapeutic approach owing to its ability to inhibit tumor cell proliferation, induce tumor specific apoptosis, and inhibit cell motility and metastasis in several tumor models (28). A number of phase I clinical trials evaluating toxicity, pharmacokinetic variables, and potential efficacy of lovastatin have shown that high doses of lovastatin are relatively safe and target HMG-CoA reductase function (30). Although lovastatin was well tolerated, it failed to show significant antitumor activity (28, 30). The tumor types evaluated in these studies did not include those that we identified as being highly sensitive to lovastatin-induced apoptosis, including HNSCC and cervical carcinomas (21). As a result, a phase I clinical evaluation of lovastatin in recurrent HNSCC and cervical carcinoma patients was undertaken by our group. Although no tumor regressions were observed, a number of patients with HNSCC (5 of 11

Fig. 6 MTT analysis of gefitinib treatments (0-25 μmol/L, 48 hours) in the SCC25 (HNSCC), SIHA (cervical carcinoma), and HCT116 (colon carcinoma) with or without 10 μmol/L lovastatin treatment (24 hours of pretreatment, 48 hours in combination). Flow cytometric analysis of 10 μmol/L lovastatin treatment for 72 hours, 10 μmol/L gefitinib for 48 hours, and pretreatment with lovastatin for 24 hours followed by the combination of agents for 48 hours. The sub-G1 (apoptotic) percent of cells is displayed in the upper left region of the individual histograms.

Mutational Status

Fig. 7 Isobologram analysis of the combination of lovastatin and gefitinib. MTT50 (concentration of agent that reduces MTT activity by 50%) values were determined for the SCC9 (HNSCC), SIHA (cervical carcinomas), and HCT116 (colon carcinoma) cell lines for 72-hour lovastatin and 48-hour gefitinib treatments. These MTT50 values are represented on the axis of each respective isobologram. The concentration of gefitinib that produced an MTT50 value in combination with 10 mmol/L lovastatin (24 hours of pretreatment, 48 hours in combination) was identified and plotted. A combination index (CI) was determined based on the method of Chou (24) where combination index = 1, additive; combination index >1, antagonistic; combination index <1, synergistic. We also sequenced the EGFR ATP binding domains in each of these three cell lines and no mutations were found.
treated) exhibited stable disease. Another phase I trial using pravastatin in combination with 5-fluorouracil showed enhanced overall survival compared with 5-fluorouracil treatment alone in hepatoma patients. Taken together, these results suggested that the most effective use of lovastatin would be as part of a combined modality.

Due to the potential for mevalonate metabolite depletion to functionally alter the EGFR signaling pathway, HMG-CoA reductase and EGFR-targeted therapies may be associated. This study has shown that the combination of lovastatin and gefitinib induced significant cooperative cytotoxicity in all 16 squamous cell carcinomas, NSCLC, and colon carcinomas that were tested irrespective of their EGFR expression levels. More detailed analysis in three representative cell lines showed that the enhanced cytotoxic response with this combination was synergistic. These results suggest the potential of combining these two therapeutic approaches. Because mevalonate metabolites can play a role in modulating EGFR function, we evaluated the effect of lovastatin on ligand activation of EGFR and its downstream signaling cascades. In this study, we have shown that lovastatin treatment in vitro of relatively high and low EGFR expressing cells inhibited ligand-induced activation of EGFR in a time-dependent manner. Importantly, this inhibitory effect was independent of both basal EGFR expression levels as well as EGF-induced phospho-EGFR levels. In the SCC9 cell line, the inhibitory effects of lovastatin on EGF-induced EGFR autophosphorylation showed a range of inhibition that was greater than 50% by 24 hours. Inhibitory effects were greater under serum-starved conditions, indicating that this variability is likely the result of experimental conditions. The inhibition of mevalonate synthesis and depletion of one or more mevalonate metabolites is the mechanism regulating this phenomenon. In the HNSCC cell line SCC9, lovastatin did not affect EGF-induced ERK activation. In contrast, AKT activation, which mediates cell survival, was significantly inhibited in serum-starved SCC9 cells. Furthermore, combining lovastatin with gefitinib induced a more significant inhibition of AKT activation in SCC9 cells than either agent alone. Therefore, the combination of lovastatin and gefitinib showed synergistic cytotoxic effects and enhanced EGFR inhibition.

The combination of statins and EGFR tyrosine kinase inhibitors represents an attractive therapeutic approach as clinical trials have shown a different spectrum of toxicities with these agents. Our results indicate that inhibitors of HMG-CoA reductase and of EGFR may act cooperatively to target this receptor and that this approach may have implications for a variety of tumor types. The ability of lovastatin to inhibit EGFR function is intriguing and requires further study to elucidate its underlying mechanism. Evaluating therapeutic approaches based on combinations of mevalonate pathway and receptor tyrosine kinase inhibitors is also of great interest. In that regard, a number of pediatric tumor-derived cell lines and leukemias are also sensitive to lovastatin-induced apoptosis where other receptor tyrosine kinases play a role in their survival. This suggests the potential for HMG-CoA reductase inhibition to affect the activity of a number of receptor tyrosine kinases potentially through a similar, novel, and as yet uncharacterized mechanism.

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Targeting the Mevalonate Pathway Inhibits the Function of the Epidermal Growth Factor Receptor

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