Monensin Inhibits Epidermal Growth Factor Receptor Trafficking and Activation: Synergistic Cytotoxicity in Combination with EGFR Inhibitors

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Abstract

Targeting the EGFR, with inhibitors such as erlotinib, represents a promising therapeutic option in advanced head and neck squamous cell carcinomas (HNSCC). However, they lack significant efficacy as single agents. Recently, we identified the ability of statins to induce synergistic cytotoxicity in HNSCC cells through targeting the activation and trafficking of the EGFR. However, in a phase I trial of rosuvastatin and erlotinib, statin-induced muscle pathology limited the usefulness of this approach. To overcome these toxicity limitations, we sought to uncover other potential combinations using a 1,200 compound screen of FDA-approved drugs. We identified monensin, a coccidial antibiotic, as synergistically enhancing the cytotoxicity of erlotinib in two cell line models of HNSCC, SCC9 and SCC25. Monensin treatment mimicked the inhibitory effects of statins on EGFR activation and downstream signaling. RNA-seq analysis of monensin-treated SCC25 cells demonstrated a wide array of cholesterol and lipid synthesis genes upregulated by this treatment similar to statin treatment. However, this pattern was not recapitulated in SCC9 cells as monensin specifically induced the expression of activation of transcription factor (ATF) 3, a key regulator of statin-induced apoptosis. This differential response was also demonstrated in monensin-treated ex vivo surgical tissues in which HMG-CoA reductase expression and ATF3 were either not induced, induced singly, or both induced together in a cohort of 10 patient samples, including four HNSCC. These results suggest the potential clinical utility of combining monensin with erlotinib in patients with HNSCC. Mol Cancer Ther; 13(11); 2559–71. ©2014 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and accounts for approximately 90% of the cancers occurring in the mucosa of the head and neck (1). Presently, early-stage HNSCC tumors are treated with radiation or surgery (2) and in advanced cases, a multimodal approach is used often incorporating chemotherapy (3). Unfortunately, long-term survival remains relatively unchanged (4) and new therapeutic options are required to enhance overall survival (OS) of patients with HNSCC. Expression of the EGFR is associated with HNSCC pathogenesis and is upregulated in 90% of these tumors (5, 6). EGFR expression is associated with poor prognosis (7–9). Preclinical studies that target the EGFR in HNSCC models have shown promising results (10, 11); however, ineffective receptor inhibition and deregulated downstream signaling pathways can lead to resistance to these agents (12). Thus, targeting the EGFR in combination with other strategies that can overcome these resistant mechanisms is critical to enhance the efficacy of this approach.

Tyrosine kinase inhibitors (TKI) are a class of therapeutic agents that consist of low molecular weight ATP mimics that bind the intracellular kinase domain of receptors like the EGFR (13). TKIs compete with and reversibly bind to the ATP-binding site preventing the activation of the receptor and its downstream signal transduction cascades (13). Gefitinib (ZD 1839, Iressa) and erlotinib (OSI 774, Tarceva) are the most clinically advanced EGFR–TKIs (13, 14). Interestingly, recent studies have revealed that a subset of patients with non–small cell lung cancer (NSCLC) respond more robustly to treatment with these drugs due to the presence of mutations in the kinase domain of the EGFR (15, 16). These mutations stabilize ATP binding conferring enhanced activity to this mutated
expression of receptor; however, TKIs can also demonstrate enhanced binding to the ATP-binding pocket rendering these NSCLC cells more sensitive to these agents (15, 16).

Monensin is an antibiotic secreted by the bacteria Streptomyces cinnamonensis and classified as a polyether antibiotic or ionophore possessing a cyclic conformation with protruding alkyl groups making the molecule highly lipid-soluble (17). This allows monensin to freely pass across the lipid bilayer of the cytoplasmic membrane or cellular organelles transporting ions along by passive diffusion (17). During ion transport, monensin crosses the lipid membrane, loses the proton on the carboxylic group, and chelates a cation and then it crosses back to the opposite side of the membrane losing the ion in exchange for a proton and the cycle repeats (18). During the past decade, monensin has been the focus of many cancer studies in which its effectiveness in targeting cell lines derived from many cancer types, including renal cell carcinoma, colon cancer, myeloma, lymphoma, and prostate has been demonstrated (19–22). Furthermore, Ketola and colleagues (23) compared the cytotoxic effect of monensin between malignant versus nonmalignant cell lines and concluded that nonmalignant cell lines are more than 20-fold more resistant than their malignant counterparts. Furthermore, it has been shown to have a positive safety profile in veterinary medicine as it has been used in cattle and poultry feed for more than 40 years (17, 18). Our laboratory has previously identified high-dose statins, a widely prescribed family of agents used to treat hypercholesterolemia (24), as potent inducers of tumor-specific apoptosis particularly in various pediatric malignancies, acute myelogenous leukemias and HNSCC cells (25–27). This led to a phase I study in squamous cell carcinoma evaluating lovastatin as a single agent that demonstrated potential utility likely as part of a combination-based regimen (28). We then demonstrated the potential of lovastatin to induce synergistic cytotoxicity in combination with EGFR-TKIs (29). The mechanism of action revolves around the ability ofLovastatin to target the activity ofEGFR and other RTKs enhancing the cytotoxicity ofEGFR-TKIs in HNSCC cells (30, 31). These studies eventually led to a phase I clinical trial of rosuvastatin and erlotinib at our Institute that showed disease stabilization, although the high dose of statins used had undesirable muscle pathologies in a significant number of patients (ClinicalTrials.gov Identifier: NCT00966472), suggesting an alternative approach to improve cancer treatment while minimizing the side effects of the treatment regimen. In this study, we performed a high-throughput analysis of 1,200 FDA-approved compounds in combination with lovastatin or erlotinib and identified monensin as a potent enhancer of their cytotoxicity in two HNSCC (SCC9 and SCC25)-derived cell lines. Importantly, monensin has been shown to similarly inhibit the trafficking of the EGFR as well as other receptors and may represent an alternative to statins with a similar mechanism of action.

**Materials and Methods**

**Tissue culture**

The HNSCC SCC9 and SCC25 and the normal lung fibroblasts GM-38 cell lines were purchased from the ATCC. The SCC9 and SCC25 cells lines were authenticated using short tandem repeat genetic profiling (Centre for Applied Genomics). PCR analysis confirmed that all cell lines showed undetectable mycoplasma contamination using routine methodology as outlined (32). All experiments were performed within 10 passages of SCC9 and SCC25 cells and within three passages of the GM-38 cells. Cells were grown and maintained in DMEM (Cellgro) supplemented with 1% (v/v) penicillin/streptomycin (Sigma-Aldrich) and 10% (v/v) fetal calf serum (Hyclone). Incubation settings were maintained at 37°C and 5% CO2.

Erlotinib (Tarceva) hydrochloride was purchased from BioVision and resuspended and diluted to 10 mmol/L stock in DMSO. Lovastatin was obtained from Apotex and suspended in ethanol to 10 mmol/L. Sodium salt of monensin was purchased from Sigma-Aldrich and suspended in ethanol to 10 mmol/L solution.

**High-throughput chemical library screen**

The SCC9 and SCC25 cell lines were treated with a chemical library of 1,200 FDA-approved compounds (Preswick Chemical; ref. 33). All compounds were supplied in a 10 mmol/L stock diluted in DMSO and were used at a final concentration of 1 μmol/L. Base-line cytotoxicity was evaluated for the drug library alone for both the 48- and 72-hour time points to be compared with the combination treatments. Two combination screens were performed (1), SCC9 and SCC25 pretreated with 10 μmol/L lovastatin for 24 hours then exposed to the drug library at 1 μmol/L for a further 48 hours, and (2) SCC9 and SCC25 pretreated with the drug library for 24 hours at 1 μmol/L followed by 10 μmol/L erlotinib treatment for 48 hours. The MTT assay was used to determine cell viability as described below.

**2.4 (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

SCC9, SCC25, and GM-38 cell lines were seeded on 96-well flat-bottomed plates (Corning Costar #3595) at a density of 5,000 cells per well. The next day, cells were pretreated with lovastatin for 24 hours followed by monensin (0–5 μmol/L) for a further 48 hours or pretreated with monensin (0–5 μmol/L) for 24 hours followed by erlotinib (0, 1, 5, or 10 μmol/L) for 48 hours. This sequence of treatments recapitulates the chemical library screen used in this study. After 48 hours, 10 μg/mL MTT reagent (Sigma-Aldrich) dissolved in PBS was added for 2 hours followed by cell lysis and MTT solubilization with 0.005N HCl (in 10% SDS) for 24 hours. MTT activity was measured using a BioTek Synergy MX plate reader at 570 nm and analyzed using Gen5 software (BioTek). The combination effects of lovastatin or erlotinib in combination with monensin were determined by the Chou–Talalay method (34) using CalcuSyn computer software (Biosoft). The dose-effect curves of each drug alone, and in combination, were...
produced by MTT assay. These data were entered into the CalcuSyn software and combination index (CI) values were graphed on fraction-affected CI (Fa-CI) plots. A CI < 1 is a synergistic interaction, CI = 1 is additive, and CI > 1 is antagonistic.

**Propidium iodide flow cytometry**

One million SCC25 cells were seeded in 10-cm plates and incubated overnight to allow for attachment and recovery. The following day, cells were pretreated with 0, 1, or 5 μmol/L monensin for 24 hours then treated with 10 μmol/L erlotinib alone or in combination with monensin for a further 24 hours. Adherent and cells in suspension were collected by centrifugation and fixed in 3 mL of cold 80% ethanol overnight at −20°C. Before analysis, cell pellets were washed with PBS resuspended in staining buffer containing 25 μg/mL propidium iodide (Sigma-Aldrich) and 40 μg/mL RNase A (BioShop) and incubated for a minimum of 1 hour in the dark at room temperature. Data (10,000 events) were acquired on an EPICS XL flow cytometer (Beckman Coulter) and analyzed with Modfit software (Verity Software House).

**Western blotting**

SCC9 and SCC25 cells were pretreated with 0, 0.1, or 1 μmol/L monensin (Sigma-Aldrich), for 22 hours followed by 2 hours of treatment with control, 10, 1, 0.01, and 0.001 μmol/L/erlotinib under serum-free conditions. Cells were then stimulated for 15 minutes with 50 ng/mL EGF before lysis with RIPA buffer (50 mmol/L Tris-Cl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% (v/v) Triton X-100, 0.25% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS and pH 7.5) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 17.5 mmol/L beta-glycerophosphate, and 0.2 mmol/L Na3VO4 (Sigma-Aldrich). The samples’ protein contents were quantified using the Pierce BCA protein assay protocol (Pierce). Protein samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P; Millipore). Blocking the membrane was performed with 5% BSA (Sigma-Aldrich) in Tris-buffered Saline with 0.1% Tween-20 (TBS-T) and Western blotted with the following primary antibodies: EGFR, Akt, pAkt, ERK, pERK (Cell Signaling Technology) overnight at 4°C and diluted 1/1,000, pY20 (BD Biosciences) overnight at 4°C and diluted 1/2,000, and actin antibody (Sigma-Aldrich) for 1 hour at room temperature and diluted 1/10,000. Dilution of antibodies was made in 5% BSA 0.1% TBS-T. Blots were washed twice with 0.1% TBS-T and once with TBS for 5 minutes each wash, then incubated for 1 hour at room temperature with the appropriate horseradish peroxidase–tagged secondary antibody (anti-rabbit 1/2,500 and anti-mouse 1/300 Jackson ImmunoResearch). Supersignal west pico chemiluminescence substrate (Pierce) was applied to the blot and developed using Syngene bioimaging system (Syngene Bio-imaging).

Densitometric analysis was performed using the Gene-Tools software from Syngene (Syngene Bio-imaging). The density of the nonphosphorylated EGFR antibody was normalized with that of the actin antibody, then the density of the pY20 antibody of the 170 kDa band was divided by the normalized density of the nonphosphorylated EGFR and taken as percentages. This was done in triplicate to obtain the SD of the mean.

**Fluorescence microscopy**

SCC9 and SCC25 cells were seeded in 6-well plates containing a cover slip at a density of 2.5 × 10^5 cells per well in complete media and incubated overnight and replaced by serum-free media containing 0, 0.1, 1, 10 μmol/L erlotinib, 10 μmol/L lovastatin, or 1 μmol/L monensin for 24 hours. The following day the cells were treated with 50 ng/mL EGF that is conjugated to the Alexa-488 fluorophore (Molecular probes) for 30 minutes at 4°C to allow the ligand to bind to the receptor but not internalize. After that, the cells were washed three times with 2 mL ice-cold PBS followed by 15 to 120 minutes incubation at 37°C to allow for internalization. Then, cells were incubated on ice to stop internalization, rinsed three times with ice-cold PBS and then acid washed for 5 minutes with 2 mL of acetic acid solution (0.2 mol/L acetic acid and 0.5 mol/L NaCl, pH 2.8) to remove ligand bound receptor on the cell surface. Cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich; 4% w/v paraformaldehyde, 2 mmol/L MgCl2, 1.25 mmol/L EDTA in PBS, pH 7.3) for 15 minutes at 37°C and mounted on a microscope slide with VectaShield mounting media with DAPI (Vector Laboratories). The slides were then examined under a Zeiss inverted microscope (Zeiss) using oil immersion microscopy. A similar procedure was followed for the low density lipoprotein (LDL) internalization using 1 μg/mL LDL-Alexa 488 (Molecular Probes).

**Transcriptome analysis (RNASeq)**

Total RNA was isolated using the RNeasy Isolation Kit (Qiagen). mRNA expression profiling was determined using NuGen reagents (www.nugeninc.com). After amplification, libraries compatible with Illumina NGS methods were prepared using the Ovation Ultra Low Library Prep Kit (NuGen). The quality of each library was assessed using a Bioanalyzer 2100 (Agilent Technologies). Kappa Library Quant Kits (KappaBiosystems; www.kapabiosystems.com) were used for library quantitation. Cluster generation and 2 × 36 bp paired-end sequencing were performed over a single lane using the Illumina GAIIx or HiSeq Genome Analyzer workstation. We obtained approximately 42 million reads per sample with 89% to 90% of reads mapped back to the genome. The Illumina CASAVA pipeline was used to generate fastq sequence files. TopHat/Cufflinks pipeline was used for mapping reads to the genome and quantification of gene expression. Computational assessments were conducted by the Ottawa Bioinformatics Core Facility.
RNA isolation and RT-PCR from cell lines

SCC25 and SCC9 cell lines were treated with either 1 μmol/L monensin or control for 24 hours and mRNA extracted using the RNeasy Kit (Qiagen) following the manufacturer's protocol. The concentration of RNA was quantified using Take3 Micro-Volume plate and BioTek Synergy MX plate reader and analyzed using Gen5 software (BioTek). Of note, 1 μg of total RNA was used for reverse transcription to cDNA using a High Capacity cDNA reverse Transcription Kit (Applied Biosystems) in an ABI Thermal Cycler (Applied Biosystems). The synthesized cDNA was used to carry out real-time PCR (RT-PCR). The total reaction volume of 20 μL contained 2 μL of cDNA, 1 μL TaqMan Gene-Expression Assay Primer/Probe (20×), 10 μL of TaqMan Universal PCR Master Mix (2×; Applied Biosystems; 4304437), and 7 μL of RNase-free water. The endogenous control for the assay was the housekeeping gene, human GAPDH (20×; Applied Biosystems; HS4333764-F). The reaction was performed in a 7500 Real-Time PCR system (Applied Biosystems). TaqMan Primers/Probes (ATF3, HS00231069; DDIT4: HS01111686_G1; HMGCS1: HS00168352_M1; INSIG1: HS01680977_G1; HMGCR: HS00168352_M1; BNIP3: HS0969291_M1)

Ex vivo tumor analysis

Surgical tissue from 4 HNSCC, a mucosal hyperplasia and an oropharynx lymphoma all from the head and neck region (HN), 2 lung cancer (LU), and 2 ovarian cancer (OV) patients undergoing routine surgical procedures were obtained and analyzed as approved by the Ottawa Hospital Research Ethics Board (Protocol# 20120559-01H). Areas containing tumor were identified by gross pathologic examinations. Approximately 2-mm cores were obtained using a sterile biopsy punch that was further sliced with a scalpel to obtain approximately 2 × 1-mm tumor slices. The slices were randomized and three slices were placed into each well of 24-well plate and cultured in DMEM (HyClone) supplemented with 10% heat-inactivated FBS (Medicorp) and 100-U/mL antibiotic/antimycotic solution (Sigma). After 48 hours drug treatments, the tumor slices were processed for RNA extraction and RT-PCR analysis of HMG-CoA reductase and activation of transcription factor (ATF) 3 mRNA levels as described above in triplicate. Initial tumor cell viability was assessed by addition of a 10% Alamar Blue (Invitrogen) solution and measured in Synergy Mx Monochromator-Based Multi-Mode Microplate Reader using Gen5 software, both from Biotek Instruments at 560-nm excitation wavelength and 590-nm emission wavelength.

Results

Monensin synergistically enhances the cytotoxicity of lovastatin and erlotinib

Our goal is to identify more specific agents that enhance erlotinib activity without the toxicities associated with high-dose statin use in our clinical trials (28). Thus, a high-throughput drug screen to identify potential enhancers of both lovastatin and erlotinib cytotoxicity was performed. Two HNSCC cell line models, SCC9 and SCC25, were treated with a chemical library of 1,200 FDA-approved compounds all at a dose of 1 μmol/L, alone or in combination with either 10 μmol/L lovastatin or 10 μmol/L erlotinib and assayed for changes in cell viability using the MTT assay. Monensin, used as an antibiotic in animal feed (18), enhanced the cytotoxic action of both lovastatin and erlotinib treatments in both cell lines examined (Supplementary Table S1). Interestingly, the EGFR TKIs gefitinib and erlotinib were also enhancers of lovastatin treatment, whereas the mevalonate pathway inhibitors fluvastatin and alendronate enhanced erlotinib cytotoxicity highlighting our previous identification of the synergistic cytotoxicity induced by this combination of agents (29). As expected, MTT assay results showed increased lovastatin cytotoxicity (Fig. 1A) and erlotinib cytotoxicity (Fig. 1B) when combined with monensin in both SCC9 and SCC25 cell lines. To understand the nature of the combination effect, the Chou–Talalay method was used to distinguish between additive and synergistic interactions (34). Fraction-affected (on cell viability) plots were generated to show the combination effect (CI). CI values of 1 are additive, less than 1 are synergistic, and more than 1 are antagonistic. In HNSCC cell lines, SCC9 and SCC25, monensin in combination with lovastatin or erlotinib resulted in a marked decrease in viability in a dose-dependent manner, which was also found to be a synergistic (Fig. 1C).

Monensin induces a potent apoptotic response in combination with erlotinib

To further confirm the ability of monensin to potentiate the cytotoxicity of erlotinib, we performed propidium iodide flow cytometric analysis to assess apoptosis induction (26, 29). SCC25 and SCC9 cells were pretreated with 1 or 5 μmol/L monensin for 24 hours, followed treatment with 10 μmol/L erlotinib alone or in combination with monensin for 24 hours; each condition performed in triplicate. The histograms of the various treatment conditions with the percentage of cells in the subG1 apoptotic peak are depicted in the upper left of each treatment for SCC25 cells (Fig. 2A). Untreated cells displayed 2.5% apoptosis; 48 hours treatment with 1 μmol/L monensin showed 4.5% whereas 5 μmol/L monensin for 48 hours induced a greater apoptotic response (16.4%). The erlotinib treatment response for 24 hours at a 10 μmol/L dose was similar to control (3.4%); however, pretreatment with either 1 or 5 μmol/L monensin for 24 hours followed by 10 μmol/L erlotinib treatment for another 24 hours resulted in a marked increases in apoptotic events (14.6%).
arrest as well as inducing apoptosis in SCC25 cells. The results of these flow cytometric analyses are tabulated in Fig. 2B showing a statistically significant induction of apoptosis in the monensin and erlotinib combination treatments compared with either agent alone. In contrast in the normal fibroblast cell line GM-38, monensin alone or in combination with erlotinib treatment did not show significant effects on cell viability as assessed by the MTT assay (Fig. 2C, conditions identical to Fig. 1C).

**Monensin enhances the erlotinib inhibition of EGFR activation**

The synergistic effects of lovastatin in combination with EGFR–TKIs that we previously reported were due
to its ability to inhibit EGFR activity (30) and to induce the integrated stress response; particularly, the ATF3 that regulates the apoptosis induction of this pathway (35, 36). We first evaluated the effect of monensin treatment alone and in combination with erlotinib on ligand-induced EGFR activation and its downstream signaling pathways by Western blot analysis. Phosphorylation status of EGFR and its downstream targets AKT and ERK proteins were assessed in serum starved SCC9 cells treated with 10 μmol/L lovastatin for 24 hours (Fig. 3A, left) or 1 μmol/L monensin for 24 hours (Fig. 3A, right) each compared with untreated control cells and 10 μmol/L erlotinib treatment for 2 hours. In untreated control cells, 50 ng/mL EGF addition for 15 minutes induced significant activation of this pathway as pEGFR, pAKT, and pERK levels were increased. Treatment of SCC9 cells with erlotinib inhibited ligand-induced activation of all three proteins as pEGFR, pAKT, and pERK levels were undetectable by Western blot analysis. Both 10 μmol/L lovastatin and 1 μmol/L monensin 24 hours treatments induced approximately a 50% inhibition of EGF-treated SCC9 cells with respect to pEGFR and its downstream targets pAKT and pERK (Fig. 3A).

Figure 2. A, histograms of propidium iodide flow-cytometry data in SCC25 cells showing, in the top row from left to right, control, 1 and 5 μmol/L monensin. Bottom row, the addition of 10 μmol/L erlotinib and its combination with the above treatments. Combinations of monensin with erlotinib increased apoptosis in SCC25 cells. B, flow-cytometry quantification using a bar diagram that summarizes the data from the flow-cytometry experiments by plotting the percentage of apoptosis (sub-G1 peak) as a function of treatment. Error bars, SD from the mean; n = 3, *P < 0.001 when comparing monensin treatment alone with its combination with erlotinib by student t test analysis. C, GM-38 fibroblasts pretreated for 24 hours with monensin and further treated with increasing concentrations of erlotinib for a further 48 hours assayed for cell viability using MTT assay. Activity presented as the percentage of control using an average of six replicates and error bars represent the SD from the mean.
To determine the effect of monensin treatment on erlotinib-induced EGFR inhibition, we treated both SCC9 and SCC25 cells with a wide range of erlotinib concentrations (0.001–10 µmol/L, 24 hours) alone and in combination with either 0.1 or 1 µmol/L monensin for 24 hours (Fig. 3B). Monensin treatments enhanced the inhibitory effects of erlotinib on ligand-induced EGFR activation as measured by pEGFR levels assessed by Western blot analysis. These results are supported by densitometric quantification shown in Fig. 3C in which pEGFR levels normalized to their corresponding EGFR levels are presented that demonstrate cooperativity of monensin and erlotinib treatments with respect to EGFR inhibition. Both cell lines express wild-type (WT) EGFR at similar levels (29).

Our previous studies demonstrated that lovastatin-induced inhibition of EGFR activity results from its ability to inhibit ligand-induced receptor trafficking and dimerization (30). Monensin is a recognized inhibitor of intracellular trafficking, including the ability to inhibit trafficking of the EGFR (37, 38), thus, may share a common mechanism with lovastatin. To assess the effect of monensin on the trafficking of the EGFR in SCC25 and SCC9 cells, we used an Alexa-488–tagged EGF ligand and followed its trafficking using fluorescent microscopy. SCC25 and SCC9 cells were either untreated, 0.1 to 10 µmol/L erlotinib, 10 µmol/L lovastatin, or 1 µmol/L monensin for 24 hours in serum-free media and then stimulated with the 50 ng/mL of tagged EGF for 15 minutes. As shown in Fig. 4A, following 15 minutes of tagged EGF treatment at 37°C, untreated cells display a uniform punctuate pattern of pEGFR, while both SCC9 and SCC25 treated with 10 µmol/L erlotinib, 0.1 µmol/L lovastatin, or 1 µmol/L monensin show a reduced punctuate pattern of pEGFR, indicating a disrupted trafficking of the EGFR.
distribution pattern corresponding to the ligand–receptor complex trafficking throughout the cell. Addition of increasing concentrations of erlotinib up to 10 μmol/L in both cell lines for 2 hours resulted in absence of fluorescence indicating lack of ligand internalization and trafficking. Labeled ligand at the cell surface was removed by acid washing of these cells in all cases to limit visualization to internalized ligand complexes only. In SCC25 cells, treatment with 10 μmol/L lovastatin or 1 μmol/L monensin for 24 hours lead to accumulation of the ligand adjacent to the cytoplasmic membrane in SCC25 cells and an impairment of EGF internalization in SCC9 cells. Nuclei counterstained with DAPI. Untreated SCC25 cells or SCC25 cells treated with 1 μmol/L monensin for 24 hours treated with 50 ng/mL of Alexa-488-labeled EGF for 15 to 120 minutes to visualize ligand localization following internalization.

Monensin activates pathways implicated in lipid synthesis and apoptosis

To gain further insight on the potential mechanism of action of monensin on EGFR inhibition and enhancement of erlotinib cytotoxicity in SCC25 cells, we performed RNA-seq full transcriptome analysis (40) of untreated controls compared with 24 hours 1 μmol/L monensin-treated SCC25 cells. Significant differences in expression of greater than 2-fold were 618 genes; to narrow the list of hits to analyze, we selected the magnitude of differential expression to greater than 4-fold, limiting the number of differentially expressed genes to 115 targets. Using the DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) to perform functional annotation clustering of this gene set, a significant number of differentially regulated genes following monensin treatment are involved in cholesterol and lipid synthesis pathways (Supplementary Table S2) as well as various apoptosis
regulators. The low-density lipoprotein (LDL) receptor binds and transports LDLs that contain cholesterol and lipids into cells and is a mechanism to maintain cholesterol and cellular lipid pools (41). Previous studies have shown that monensin treatment can also inhibit LDL receptor trafficking (41), and we assessed the effect of monensin on LDL uptake in SCC25 cells that were treated with 0 and 1 μmol/L monensin for 48 hours. To visualize LDL receptor trafficking, we added Alexa-488–tagged LDL for 15 minutes at 37°C. We found that monensin treatment inhibited the uptake of LDL, indicating that in SCC25 cells monensin treatment inhibits the cellular trafficking of LDL (Fig. 5A).

We next used quantitative RT-PCR (qRT-PCR) to validate the differential expression identified by RNA-seq in monensin-treated SCC25 cells (1 μmol/L, 24 hours). Because of the fact that lovastatin also targets cholesterol synthesis and induces apoptosis in SCC25 cells, we focused on the cholesterol synthesis enzymes HMG-CoA synthase, HMG-CoA reductase (target of statins) and INSIG1 and the apoptosis regulators BNIP3 (42) and DDIT4 (43). All of these genes were significantly upregulated in SCC25 with 1 μmol/L 24 hours monensin treatment cells as predicted in our RNA-seq analysis (Fig. 5B). However, when the expression of these genes was evaluated in SCC9 cells, similar monensin treatments failed to demonstrate the induction of these genes, including the apoptosis regulators (Fig. 5B). Because both lovastatin and monensin induce apoptosis in SCC9 cells, we evaluated the expression of ATF3 in both SCC25 and SCC9 cells following monensin treatment. Monensin induced a potent induction of ATF3 in SCC9 but not in SCC25 under these conditions (Fig. 5B), suggesting that monensin can induce apoptosis through different and independent mechanisms in these two cell lines.

**Treatment of ex vivo tumor tissue samples with monensin**

To assess the potential of monensin to induce either HMG-CoA (Fig. 6A) reductase (Fig. 6A) and/or ATF3 (Fig. 6B) mRNA expression in a more clinically relevant model, we assessed *ex vivo* HNSCC tumor tissue in culture from 4 different patients with HNSCC undergoing surgery at our Institute (HN01-04), a noncancerous hyperplastic epithelial lesion (HN05) and an oropharynx lymphoma (HN06). We also similarly evaluated 2 lung cancer and 2 ovarian cancer patient tumor tissues. Tissue samples were processed following surgical excision into 2 × 1-mm slices, randomized with three tissue slices evaluated per treatment (Fig. 6C) and analyzed by qRT-PCR performed in triplicate. In our previous studies using lovastatin treatments, HMG-CoA reductase induction was generally to a lower extent than ATF3 (29, 35).

In the *ex vivo* tissue samples evaluated, seven of 10 showed induction of greater than 2-fold of HMG-CoA reductase mRNA levels following 1 and 10 μmol/L monensin treatments for 48 hours. This included three of the four HNSCC, the hyperplastic lesion, and the lymphoma head and neck samples as well as one of two lung adenocarcinomas and one of two ovarian mucinous tumors (Fig. 6A). For ATF3 expression, five of 10 showed induction of greater than 4-fold of ATF3 mRNA levels following monensin treatments as above. This included two of the four HNSCC and the hyperplastic lesion as well as one of two lung adenocarcinomas and one of two ovarian mucinous tumors.
mucinous tumors (Fig. 6B). As in the SCC9 and SCC25 cell lines, monensin treatments also displayed disparate results with the induction patterns of the mRNA expression of these two genes. For example, HMG-CoA reductase (HN02, HN04, and HN06) or ATF3 (HN01) were induced alone, in concert (HN03, HN05, LU02, and OV01) or showed no change in expression (LU01 and OV02). In this limited cohort of two HNSCC cell lines and a variety of ex vivo tissue evaluated that included four HNSCC, a mucosal hyperplasia and an oropharynx lymphoma, two lung adenocarcinomas (LU), and two ovarian mucinous carcinomas ex vivo tissues. Fold changes were calculated following normalization to gapdh levels (ΔΔCt) and expressed as means (±SD; n = 3). Error bars, SD from the mean of three replicates (n = 3). C, schematic representation of ex vivo tumor tissue processing and evaluation.

Figure 6. Levels of HMG-CoA reductase (A) and ATF3 (B) mRNA were analyzed by real-time qRT-PCR following solvent control, 1 and 10 μmol/L monensin in six HN tissues, including four HNSCC, a mucosal hyperplasia and an oropharynx lymphoma, two lung adenocarcinomas (LU), and two ovarian mucinous carcinomas ex vivo tissues. Fold changes were calculated following normalization to gapdh levels (ΔΔCt) and expressed as means (±SD; n = 3). Error bars, SD from the mean of three replicates (n = 3). C, schematic representation of ex vivo tumor tissue processing and evaluation.
Discussion

The use of erlotinib in treatment regimens of cancers with EGFR-activating mutation such as in 10% of patients with NSCLC has demonstrated a promising although modest improvement in patient outcomes (15, 16). In these patients, erlotinib shows enhanced binding affinity to the EGFR kinase domain demonstrating a more robust inhibition of this receptor (15, 16). In tumor types like HNSCC that overexpress WT EGFR to enhance pathway activation, erlotinib treatments have not demonstrated significant clinical activity (6, 10). This is likely the result that erlotinib as a single agent may not inhibit WT EGFR sufficiently to invoke a clinical response in these patients. Combining erlotinib with agents that can co-operate to target EGFR may enhance activity and uncover a novel therapeutic approach. To this end, our previous work has identified the mevalonate pathway as an important regulator of EGFR activity and that inhibiting the rate-limiting enzyme of this pathway with statins inhibits EGFR activation and can induce synergistic cytotoxicity with erlotinib in WT EGFR possessing HNSCC and NSCLC cells (29, 30). In fact, a retrospective analysis of the BR.21 phase III clinical trial of NSCLC treated with erlotinib as a single agent showed a trend to better OS in patients on statins for hypercholesterolemia treatment (30). This has led to a phase I study at our Institute combining rosuvastatin and erlotinib in combination. Although durable stable disease was induced in 20% of patients, these high rosuvastatin doses also produced significant muscle toxicities, thereby limiting the efficacy of this approach (ClinicalTrials.gov Identifier: NCT00966472; G. Goss and colleagues, unpublished data). Thus, identifying more refined therapeutic approaches that maintain the efficacy without statin-induced toxicities is warranted. In this study, we identified monensin as an enhancer of the erlotinib-induced HNSCC cell cytotoxicity. We also demonstrated a similar mechanism of action with respect to EGFR inhibition as statin treatments. As such, monensin treatment impairs EGFR cellular trafficking, induced expression of similar apoptotic markers, induced synergistic cytotoxicity in combination with erlotinib, and importantly activates pathways implicated in lipid and cholesterol synthesis.

Monensin has been previously shown to induce cytotoxicity in a variety of cancer-derived cell lines, including prostate, colon, and leukemias (19–22). Moreover, monensin’s cytotoxicity appeared to be cancer-specific as monensin differential sensitivity was observed between sensitive malignant versus resistant nonmalignant prostate-derived cell lines (23). In this study, we have shown that monensin mimics the inhibitory effects of lovastatin on EGFR activity. Monensin can inhibit cellular trafficking of both the EGFR and LDL receptor (37, 41) and in this study, we confirmed this inhibition in HNSCC cells. Monensin treatments showed aberrant EGF trafficking in both HNSCC cell lines and is a known inhibitor of trafficking on many levels due to its influence on the endoplasmic reticulum and golgi bodies, pH of endosomes, and protein glycosylation (44, 45).

Monensin is used extensively in poultry and cattle feed as it can increase muscle mass in livestock (18). Our RNA-Seq analysis demonstrated its ability to induce expression of cholesterol and lipid synthesis pathways as well as apoptosis regulating pathways that was confirmed by qRT-PCR. Although monensin and lovastatin exert pleiotropic effects on a variety of cellular pathways, there is overlap with respect to their effects on EGFR function and cholesterol and lipid metabolism regulation. Monensin also exhibited a significant induction of apoptosis with the induction of disparate pathways that was cell line dependent. In the SCC25 cell line, monensin-induced apoptosis is likely regulated by BNIP3 and DDIT4 that was associated with cholesterol pathway induction, including HMG-CoA reductase, whereas in the SCC9 cell line, the effect of monensin is likely regulated by ATF3 induction. Remarkably, the ex vivo tissue samples from HNSCC, a hyperplastic lesion, a lymphoma, lung, and ovarian cancers also demonstrate disparate responses to these pathways. These tissue samples showed either no effect of monensin treatment on HMG-CoA reductase or ATF3 expression, the induction of only ATF3 or HMG-CoA reductase alone or the induction of both genes in the same sample. Of interest, both the hyperplastic noncancerous cells and the nonepithelial lymphoma cells were responsive to monensin treatment that requires further study to determine the scope of this response. Furthermore, the heterogeneity of the tumor cells within these tissues, including the presence of stromal cells, may account for the upregulation of both HMG-CoA reductase and ATF3 expression in four of the 10 tissues evaluated following monensin treatment also requires further study.

The mechanism by which monensin enhances erlotinib cytotoxicity in HNSCC cells is unknown. It may involve its ability to further inhibit EGFR in an unrelated mechanism to erlotinib enhancing EGFR inhibition and cytotoxicity as suggested in this study. Monensin may also enhance erlotinib activity through its ability to inhibit autophagy, a strategy that has demonstrated the potential to enhance erlotinib cytotoxicity in NSCLC cells (46). Furthermore, lipoprotein lipolysis can lead to the induction of ATF3 resulting in apoptosis (47) and the potential of monensin to target lipid homeostasis suggest this as an alternative mechanism. Thus, combining monensin with erlotinib requires further study to delineate the mechanism of their synergistic cytotoxic response in HNSCC and to determine their utilization as a potential novel therapeutic strategy.

Disclosure of Potential Conflicts of Interest
P.J. Villeneuve was a consultant/participant in an Advanced NSCLC Multidisciplinary Meeting for Pfizer and received travel/course reimbursement for training on Matrix rib fixation system from DePuy Synthes. No potential conflicts of interest were disclosed by the other authors.

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