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

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Running Title: Monensin enhances erlotinib activity

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Abbreviations: RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor; HNSCC, head and neck squamous cell carcinomas; HMG-CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; ATF, activating transcription factor; TKI, tyrosine kinase inhibitor; LDL, low density lipoprotein; MTT Assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay; CI, Combination Index.

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Abstract

Targeting the epidermal growth factor receptor (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 rosvastatin and erlotinib, statin induced muscle pathology limited the usefulness of this approach. To overcome these toxicity limitations, we sought to uncover other potential combinations employing a 1200 compound screen of approved FDA 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 signalling. RNA–seq analysis of monensin treated SCC25 cells demonstrated a wide array of cholesterol and lipid synthesis genes up regulated 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 where HMG-CoA reductase expression and ATF3 were either not induced, induced singly or both induced together in a cohort of 10 patient samples including 4 HNSCC. These results suggest the potential clinical utility of combining monensin with erlotinib in HNSCC patients.
Introduction

Squamous cell carcinoma of the head and neck (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 tumours are treated with radiation or surgery (2) and in advanced cases a multimodal approach is utilized often incorporating chemotherapy (3). Unfortunately, long-term survival remains relatively unchanged (4) and new therapeutic options are required in order to enhance over-all survival of patients with HNSCC. Expression of the Epidermal Growth Factor Receptor (EGFR) is associated with HNSCC pathogenesis and is up regulated in 90% of these tumours (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 signalling 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 NSCLC patients 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 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 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 where 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 et al. compared the cytotoxic effect of monensin between malignant versus non-malignant cell lines and concluded that non-malignant cell lines are more than 20 fold more resistant than their malignant counterparts (23). 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 tumour specific apoptosis particularly in various paediatric malignancies, acute myeloid leukemias and HNSCC cells (25-27). This led to a Phase I study in SCC 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 of lovastatin to target the activity of EGFR and other RTKs enhancing the cytotoxicity of EGFR-TKIs in HNSCC cells (30, 31). These studies eventually led to a Phase I clinical trial of rosvastatin 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 targeting while minimizing the side effects of the treatment regimen. In this study, we performed a high throughput analysis of 1200 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 American Type Culture Collection ATCC (Rockville, MD). The SCC9 and SCC25 cells lines were authenticated employing short tandem repeat genetic profiling (Centre for Applied Genomics, Toronto, ON). PCR analysis confirmed that all cell lines showed undetectable mycoplasma contamination employing routine methodology as outlined (32). All experiments were performed within 10 passages of
SCC9 and SCC25 cells and within 3 passages of the GM-38 cells. Cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro, Manassas, VA, USA) supplemented with 1% (v/v) penicillin/streptomycin (Sigma Aldrich, St Louis, MO, USA) 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 (Mountain View, CA) and resuspended and diluted to 10mM stock in dimethyl sulfoxide (DMSO). Lovastatin was obtained from Apotex (Toronto, ON) and suspended in ethanol to 10mM. Sodium salt of monensin was purchased from Sigma-Aldrich (St Louis, MO, USA) and suspended in ethanol to 10mM solution.

**High Throughput Chemical Library Screen**

The SCC9 and SCC25 cell lines were treated with a chemical library of 1200 FDA-approved compounds (Prestwick Chemical, Illkirch, FRA) (33). All compounds were supplied in a 10mM stock diluted in DMSO and were used at a final concentration of 1µM. Base-line cytotoxicity was evaluated for the drug library alone for both the 48 and 72hr time-points to be compared to the combination treatments. Two combination screen were performed (1) SCC9 and SCC25 pre-treated with 10µM lovastatin for 24hrs then exposed to the drug library at 1µM for a further 48hrs, and (2) SCC9 and SCC25 pre-treated with the drug library for 24hrs at 1µM followed by 10µM erlotinib treatment for 48hrs. 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, Corning, NY, USA) at a density of 5,000 cells/well. The next day, cells were pre-treated with lovastatin or erlotinib (0, 1, 5, or 10µM) for 24hrs
followed by monensin (0-5 µM) for a further 48hrs or pre-treated with monensin (0-5 µM) for 24hrs followed by erlotinib (0, 1, 5, or 10µM) for 48hrs. This sequence of treatments recapitulate the chemical library screen employed in this study. After 48 hours, 10µg/ml MTT reagent (Sigma-Aldrich) dissolved in phosphate buffered saline (PBS) was added 2 hours followed by cell lysis and MTT solubilization with 0.005N HCl (in 10% sodium dodecyl sulphate (SDS) for 24hrs. MTT activity was measured using a BioTek Synergy MX plate reader at 570nm and analyzed using Gen5 software (BioTek, Winooski, VT). The combination effects of lovastatin or erlotinib in combination with monensin were determined by the Chou-Talalay method (34) using CalcuSyn computer software (Biosoft, Cambridge, GBR). The dose-effect curves of each drug alone, and in combination, were produced by MTT assay. This data was 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 10cm plates and incubated overnight to allow for attachment and recovery. The following day, cells were pre-treated with 0, 1 or 5µM monensin or 10µM lovastatin for 24hrs then treated with 1 or 10µM erlotinib alone or in combination with monensin or lovastatin for a further 24hrs. Adherent and cells in suspension were collected by centrifugation and fixed in 3ml of cold 80% ethanol overnight at -20°C. Prior to analysis, cell pellets were washed with PBS resuspended in staining buffer containing 25µg/ml propidium iodide (Sigma-Aldrich) and 40µg/ml RNase A (BioShop, Burlington, Ontario, Canada) and incubated for a minimum of 1 hour in the dark at room temperature. Data (10000 events) was acquired on an EPICS XL flow
cytometer (Beckman Coulter, Brea, California, USA) and analyzed with Modfit software (Verity Software House, Topsham, ME, USA).

**Western Blotting**

SCC9 and SCC25 cells were pre-treated with 0, 0.1 or 1µM monensin (Sigma-Aldrich, St-Louis, USA), for 22hrs followed by 2hrs of treatment with control, 10, 1, 0.1, 0.01 and 0.001µM erlotinib under serum free conditions. Cells were then stimulated for 15min with 50ng/ml Epidermal Growth Factor (EGF) before lysis with RIPA buffer (50mM Tris-Cl, 150mM NaCl, 1mM EDTA, 1% (v/v) Triton X-100, 0.25 % (w/v) sodium deoxycolate and 0.1% (w/v) SDS and pH 7.5) supplemented with protease inhibitor cocktail (Sigma Aldrich), 17.5 mM beta-glycerophosphate and 0.2 mM Na₃VO₄ (Sigma-Aldrich, St-Louis, MO, USA). The samples’ protein contents were quantified using the Pierce BCA protein assay protocol (Pierce, Rockford, IL, USA). Protein samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA). Blocking the membrane was performed with 5% BSA (Bovine Serum Albumin) (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 Signalling Technology, Davers, MA, USA) overnight at 4°C and diluted 1/1000, pY20 (BD Biosciences) overnight at 4°C and diluted 1/2000, and actin antibody (Sigma Aldrich) for 1 hour at room temperature and diluted 1/10000. 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 Horse Radish Peroxidase (HRP)-tagged secondary antibody (anti-rabbit 1/2500 and anti-mouse 1/300 Jackson
ImmunoResearch, West Grove, PA, USA). Supersignal west pico chemiluminescence substrate (Pierce) was applied to the blot and developed using Syngene bioimaging system (Syngene Bio-imaging, Frederick, MD).

Densitometric analysis was performed using the GeneTools software from Syngene (Syngene Bio-imaging). The density of the non-phosphorylated EGFR antibody was normalized with that of the actin antibody, then the density of the pY20 antibody of the 170kDa band was divided by the normalized density of the non-phosphorylated 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 x 10^5 cells/well in complete media and incubated overnight and replaced by serum free media containing 0, 0.1, 1, 10µM erlotinib, 10µM lovastatin or 1µM monensin for 24hrs. The following day the cells were treated with 100 ng/ml EGF that is conjugated to the Alexa-488 fluorophore (Molecular probes, Eugene, Oregon, USA) 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 15-120 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 M acetic acid and 0.5 M 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 mM MgCl₂, 1.25 mM 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, Burlingame, CA, USA).
The slides were then examined under a Zieiss inverted microscope (Ziess, Oberkochen, Germany) using oil immersion microscopy. A similar procedure was followed for the LDL internalization employing 1μg/mL LDL-Alexa 488 (Molecular Probes).

**Transcriptome Analysis (RNAseq)**

Total RNA was isolated employing 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 x 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 89-90% of reads mapped back to the genome. The Illumina CASAVA pipeline was used to generate fastq sequence files. TopHat/Cufflink 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 1μM monensin or control for 24hrs and mRNA extracted using the RNeasy® kit (Qiagen, Germantown, MD, USA) 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, Winooski, VT). 1μg 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, Foster City, USA). The synthesized cDNA was used to carry out real-time polymerase chain reaction (RT-PCR). The total reaction volume of 20µl contained 2µl of cDNA, 1µl TaqMan Gene Expression Assay Primer/Probe (20x), 10µl of TaqMan Universal PCR Master Mix (2x) (Applied Biosystems, 4304437) and 7µl of RNase-free water. The endogenous control for the assay was the housekeeping gene, human GAPDH (20x) (Applied Biosystems, HS4333764-F). The reaction was performed in a 7500 Real-Time PCR system (Applied Biosystems). TaqMan Primers/Probes (ATF3, HS00231069); DDIT4: HS011111686_G1; HMGCS1: HS00940429_M1; INSIG1: HS01650977_G1; HMGCR: HS00168352_M1; BNIP3: HS00969291_M1)

**Ex-Vivo Tumour 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 in this study that was approved by the Ottawa Hospital Research Ethics Board (Protocol# 20120559-01H). Areas containing tumour were identified by gross pathological examinations. Approximately 2mm cores were obtained using a sterile biopsy punch that were further sliced with a scalpel to obtain approximately 2x1mm tumour 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-units/ml-antibiotic/antimycotic solution (Sigma). After 48h drug treatments, the tumour slices were processed for RNA extraction and RT-PCR analysis of HMG-CoA
reductase and ATF3 mRNA levels as described above in triplicate. Initial tumour 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 (Winooski, VT) 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 1200 FDA approved compounds all at a dose of 1μM, alone or in combination with either 10μM lovastatin or 10μM erlotinib and assayed for changes in cell viability employing the MTT assay. Monensin, employed as an antibiotic in animal feed (18), enhanced the cytotoxic action of both lovastatin and erlotinib treatments in both cell lines examined (Supplemental Table 1). Interestingly, the EGFR TKIs gefitinib and erlotinib were also enhancers of lovastatin treatment, while 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 (Figure 1A) and erlotinib cytotoxicity (Figure 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 effect (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 (Figure 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 pre-treated with 1 or 5µM monensin for 24hrs, followed treatment with 10µM erlotinib alone or in combination with monensin for 24hrs; 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 (Figure 2A). Untreated cells displayed 2.5% apoptosis; 48hrs treatment with 1µM monensin showed 4.5% while 5µM monensin for 48hrs induced a greater apoptotic response (16.4%). The erlotinib treatment response for 24hrs at a 10µM dose was similar to control (3.4%), however, pre-treatment with either 1 or 5µM monensin for 24hrs followed by 10µM erlotinib treatment for another 24hrs resulted in a marked increases in apoptotic events (14.6% and 38.7%, respectively) when compared to either monensin or erlotinib treatments alone. Additionally, combination of 5µM monensin with 10µM erlotinib showed the highest percentage of apoptosis (38.7%). These histograms also show an increase in S phase suggesting the potential for monensin to induce an S phase cell cycle arrest as well as inducing apoptosis in SCC25 cells. The
results of these flow cytometric analyses are tabulated in Figure 2B showing a statistically significant induction of apoptosis in the monensin and erlotinib combination treatments compared to 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 (Figure 2C, conditions identical to Figure 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 activating transcription factor 3 (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 signalling 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\(\mu\)M lovastatin for 24hrs (Figure 3A, left panel) or 1\(\mu\)M monensin for 24hrs (Figure 3A, right panel) each compared to untreated control cells and 10\(\mu\)M erlotinib treatment for 2hrs. In untreated control cells, 50ng/ml EGF addition for 15min 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\(\mu\)M lovastatin and 1\(\mu\)M monensin 24hr treatments induced approximately a 50% inhibition of EGF treated SCC9 cells with respect to pEGFR and its downstream targets pAKT and pERK (Figure 3A).
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 (0.001-10μM, 24hrs) alone and in combination with either 0.1 or 1μM monensin treatments in combination for 24hrs (Figure 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 Figure 3C where pEGFR levels normalized to EGFR levels are presented that demonstrate co-operativity of monensin and erlotinib treatments with respect to EGFR inhibition. Both cell lines express wild-type 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 utilized an Alexa-488 tagged EGF ligand and followed its trafficking using fluorescent microscopy. SCC25 and SCC9 cells were either untreated, 0.1-10μM erlotinib, 10μM lovastatin or 1μM monensin for 24hrs in serum free media and then stimulated with the 50ng/ml of tagged EGF for 15min. As shown in Figure 4A, following 15min of tagged EGF treatment at 37°C, untreated cells display a uniform punctuate distribution pattern corresponding to the ligand-receptor complex trafficking throughout the cell. Addition of increasing concentrations of erlotinib up to 10μM in both cell lines for 2hrs resulted in absence of fluorescence indicating lack of
ligand internalization and trafficking. Labelled ligand at the cell surface was removed by acid washing of these cells in all cases to limit visualization of internalized ligand complexes only. In SCC25 cells, treatment with 10µM lovastatin or 1µM monensin lead to accumulation of the Alexa-488 tagged EGF ligand near the cytoplasmic membrane indicating an inhibitory effect on its intracellular trafficking (Figure 4A). In SCC9 cells by contrast, similar lovastatin and monensin treatments showed inhibition of ligand internalization as intracellular fluorescence was significantly reduced (Figure 4A). To determine if the inhibitory effect of monensin on EGF trafficking persists in SCC25 cells, we evaluated later internalization times of up to 120min. In control untreated SCC25 cells, EGF fluorescence was predominately located at the cell surface at 15min but showed perinuclear localization at the later time points clearly evident at 60min EGF treatment (Figure 4B). This likely demonstrates early endosome to lysosomal trafficking of this ligand as previously reported (39). In 1µM 24hr monensin treated SCC25 cells, the inhibitory effects on EGF trafficking are present up to 30min following ligand internalization but progress to the typical perinuclear localization and later time points (Figure 4B)

Monensin Activates Pathways Implicated in Lipid Synthesis and Apoptosis

In order 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 to 24hr 1µM monensin treated SCC25 cells. Significant differences in expression of greater than two fold was 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. Employing 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 (Supplemental Table 2) 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 1µM monensin for 48hrs. To visualize LDL receptor trafficking, we added Alexa-488 tagged LDL for 15min 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 (Figure 5A).

We next employed quantitative RT-PCR to validate the differential expression identified by RNA-seq in monensin treated SCC25 cells (1µM, 24hrs). Due to the fact that lovastatin also targets cholesterol synthesis and induces apoptosis in SCC25 cells, we focussed 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 up regulated in SCC25 with 1µM 24hr monensin treatment cells as predicted in our RNA-seq analysis (Figure 5B). However when the expression of these genes were evaluated in SCC9 cells, similar monensin treatments failed to demonstrate the induction of these genes including the apoptosis regulators (Figure 5B). Since 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 (Figure 5B) suggesting that monensin can induce apoptosis through different and independent mechanisms in these two cell lines.

**Treatment of Ex-Vivo Tumour Tissue Samples with Monensin**

To assess the potential of monensin to induce either HMG-CoA (Figure 6A) reductase (Figure 6A) and/or ATF3 (Figure 6B) mRNA expression in a more clinically relevant model, we assessed *ex-vivo* HNSCC tumour tissue in culture from 4 different HNSCC patients undergoing surgery at our Institute (HN01-04), a non-cancerous hyperplastic epithelial lesion (HN05) and an oropharynx lymphoma (HN06). We also similarly evaluated 2 lung cancer (LU) and 2 ovarian (OV) cancer patient tumour tissues. Tissue samples were processed following surgical excision into 2x1mm slices, randomized with 3 tissue slices evaluated per treatment (Figure 6C) and analyzed by quantitative RT-PCR performed in triplicate. In our previous studies employing lovastatin treatments, HMG-CoA reductase induction was generally to a lower extent than ATF3 (29, 35).

In the ex-vivo tissue samples evaluated, 7 of 10 showed induction of greater than 2 fold of HMG-CoA reductase mRNA levels following 1 and 10μM monensin treatments for 48hrs. This included 3 of the 4 HNSCC, the hyperplastic lesion and the lymphoma HN samples as well as 1 of 2 lung adenocarcinomas and 1 of 2 ovarian mucinous tumours (Figure 6A). For ATF3 expression, 5 of 10 showed induction of greater than 4 fold of ATF3 mRNA levels following monensin treatments as above. This included 2 of the 4 HNSCC and the hyperplastic lesion as well as 1 of 2 lung adenocarcinomas and 1 of...
2 ovarian mucinous tumours (Figure 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, HN06) or ATF3 (HN01) were induced alone, in concert (HN03, HN05, LU02, OV01) or showed no change in expression (LU01, OV02). In this limited cohort of 2 HNSCC cell lines and a variety of ex-vivo tissue evaluated that included 4 HNSCC tumours, no associations with clinical parameters and response to monensin was evident (Supplemental Table 3, Patient Characteristics).

Discussion

The use of erlotinib in treatment regimens of cancers with EGFR activating mutation such as in 10% of NSCLC patients 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 tumour types like HNSCC that over-express wild type 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 wild type 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 wild type EGFR possessing HNSCC and NSCLC cells (29, 30). In fact, a retrospective analysis of the
Phase III clinical trial of NSCLC treated with erlotinib as a single agent showed a trend to better overall survival in patients on statins for hypercholesterolemia treatment (30). This has led to a Phase I study as our Institute combining rosuvastatin and erlotinib in combination. While 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) (Goss, G et al, manuscript in preparation). 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 non-malignant 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 ER 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 quantitative RT-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 dependant. 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 while 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 non-cancerous cells and the non-epithelial lymphoma cells were responsive to monensin treatment that requires further study to determine the scope of this response. Furthermore, the heterogeneity of the tumour cells within these tissues including the presence of stromal cells may account for the up-regulation of both HMG-CoA reductase and ATF3 expression in 4 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.

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Figure Legends

Figure 1. A, Lovastatin pre-treated cells for 24hrs combined with increasing concentration of monensin for a further 48hrs assayed for cell viability employing MTT assay. Activity presented as % of control employing an average of six replicates and error bars represent the SD from the mean. B, Monensin pre-treated cells for 24hrs combined with increasing concentration of erlotinib for a further 48hhrs and assayed by MTT as above. C, Fraction Affected/ Combination Index Plots show the synergistic effect of monensin in combination with lovastatin (top row) or erlotinib (bottom row of panels) in SCC25 and SCC9 cells. Combination Indices (CI)<1 are considered synergistic, CI>1 are considered antagonistic, and CI=1 are considered additive.

Figure 2. A, Histograms of propidium iodide flow cytometry data in SCC25 cells showing, in the top row of panels from left to right, control, 1µM monensin and 5µM monensin. The bottom row of panels includes the addition of 10µM 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 % of apoptosis (subG1 peak) as a function of treatment. Error bars represent the SD from the mean; n=3 and *P<0.001 when comparing monensin treatment alone with its combination with erlotinib by Student T-test analysis. C, Monensin pre-treated GM-38 fibroblasts for 24hrs combined with increasing concentration of erlotinib for a further 48hrs assayed for cell viability employing MTT assay. Activity presented as % of control employing an average of six replicates and error bars represent the SD from the mean.
Figure 3. A, Western Blot analysis of control, 10μM erlotinib, and 10μM lovastatin and their combination (left panel) and control, 10μM erlotinib and 1μM monensin (right panel) in SCC25 cells treated for 24hrs with these agents with or without EGF stimulation (50ng/ml for 15min). Western blots of phospho (p)-EGFR, pAkt, and pERK and their corresponding total levels along with actin as loading controls are depicted. B, Western blot analysis of pEGFR and EGFR in SCC9 and SCC25 cells treated with 0, 10, 1, 0.1, 0.01, 0.001μM erlotinib without or with 0.1 or 1μM monensin for 24hrs. In all cases, EGF stimulation (50ng/ml for 15min) was performed prior to protein extraction. C, Densitometry analysis of pEGFR levels normalized to their corresponding EGFR levels. Monensin treatment enhanced erlotinib’s ability to inhibit phosphorylation of the EGFR. Error bars represent SD from the mean of three replicates (n=3).

Figure 4. A, The effect of 24 hours treatment of erlotinib (0.1, 1 and 10μM), 10μM lovastatin and 1μM monensin on the ability of SCC25 and SCC9 cells to uptake Alexa-488 conjugated EGF compared to untreated controls. Cell surface EGF was removed by acid wash prior to visualization. Both treatments with 10μM lovastatin or 1μM monensin for 24hrs 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. B, Untreated or 1μM monensin treated SCC25 cells for 24hrs treated with 50ng/ml of Alexa-488 labelled EGF for 15-120min to visualize ligand localization following internalization.

Figure 5. A, The effect of 1μM monensin treatment for 48 hours on the uptake of LDL in vitro employing the SCC25 cell line. Untreated cells internalized LDL as evidenced by intracellular punctate staining pattern of the labelled LDL, whereas treatment with
monensin inhibited its uptake. Green color represents Alexa-488 tagged LDL and blue is DAPI stain. B, Confirmatory quantitative RT-PCR analysis of 1μM monensin treatment for 24hrs in SCC25 and SCC9 cells. mRNA levels were determined normalized to GAPDH levels. ATF3, HMG-CoA reductase, HMG-CoA synthase, INSIG1, BNIP3 and DDIT4 gene expression was evaluated. The RT-PCR results show that the two cell lines respond differently to monensin treatment as in SCC25 where cholesterol synthesis and BNIP3 and DDIT4 were up-regulated. In contrast, only the stress response gene ATF3 was up regulated in SCC9 cells.

**Figure 6.** Levels of *HMG-CoA reductase* (A) and *ATF3* (B) mRNA were analyzed by real time quantitative RT-PCR following solvent control, 1 and 10μM monensin in 6 head and neck tissues (HN) including 4 HNSCC a mucosal hyperplasia and an oropharynx lymphoma, 2 lung adenocarcinomas (LU) and 2 ovarian mucinous carcinomas *ex-vivo* tissues. Fold changes were calculated following normalization to *gapdh* levels (ΔΔCt) and expressed as means (±SD) (n = 3). The error bars represent SD from the mean of three replicates (n=3). C, Schematic representation of *ex-vivo* tumour tissue processing and evaluation.
Figure 2
Figure 5
Molecular Cancer Therapeutics

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

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