TARGETING THE ONCOGENIC MUC1-C PROTEIN INHIBITS MUTANT EGFR-MEDIATED SIGNALING AND SURVIVAL IN NON-SMALL CELL LUNG CANCER CELLS

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Abbreviations: NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; MUC1, mucin 1; MUC1-C, MUC1 C-terminal subunit; MUC1-CD, MUC1 cytoplasmic domain; TKI, tyrosine kinase inhibitor.

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Abstract

Purpose: Non-small cell lung cancers (NSCLC) that express the EGF receptor (EGFR) with activating mutations frequently develop resistance to EGFR kinase inhibitors. The mucin 1 (MUC1) heterodimeric protein is aberrantly overexpressed in NSCLC cells and confers a poor prognosis; however, the functional involvement of MUC1 in mutant EGFR signaling is not known.

Experimental Design: Targeting the oncogenic MUC1 C-terminal subunit (MUC1-C) in NSCLC cells harboring mutant EGFR was studied for effects on signaling, growth, clonogenic survival and tumorigenicity.

Results: Stable silencing of MUC1-C in H1975/EGFR(L858R/T790M) cells resulted in downregulation of AKT signaling and inhibition of growth, colony formation and tumorigenicity. Similar findings were obtained when MUC1-C was silenced in gefitinib-resistant PC9GR cells expressing EGFR(delE746_A750/T790M). The results further show that expression of a MUC1-C(CQC→AQA) mutant, which blocks MUC1-C homodimerization, suppresses EGFR(T790M), AKT and MEK→ERK activation, colony formation and tumorigenicity. In concert with these results, treatment of H1975 and PC9GR cells with GO-203, a cell-penetrating peptide that blocks MUC1-C homodimerization, resulted in inhibition of EGFR, AKT and MEK→ERK signaling and in loss of survival. Combination studies of GO-203 and afatinib, an irreversible inhibitor of EGFR, further demonstrate that these agents are synergistic in inhibiting growth of NSCLC cells harboring the activating EGFR(T790M) or EGFR(delE746-A750) mutants.

Conclusions: These findings indicate that targeting MUC1-C inhibits mutant EGFR signaling and survival, and thus represents a potential approach alone and in combination for the treatment of NSCLCs resistant to EGFR kinase inhibitors.
**Statement of Translational Relevance**

Patients with non-small cell lung cancer (NSCLC) harboring activating EGFR mutations invariably develop resistance to EGFR tyrosine kinase inhibitors (TKIs) that is often associated with acquisition of the secondary T790M mutation. Here in NSCLC EGFR(T790M) cells, we demonstrate that the oncogenic MUC1-C protein interacts with EGFR and that targeting MUC1-C suppresses EGFR activation and the downstream AKT and MEK pathways. Consistent with the dependence of these NSCLC cells on EGFR(T790M) signaling, targeting MUC1-C was also associated with inhibition of colony formation and tumorigenicity. Afatinib is an irreversible EGFR inhibitor that is approved for the treatment of NSCLCs with EGFR exon 19 deletions or L858R mutations. Our results further show that targeting MUC1-C is synergistic with afatinib in inhibiting the growth of NSCLC cells with EGFR(T790M) and other activating EGFR mutations. These findings indicate that MUC1-C is a potential target for the treatment of patients with NSCLCs resistant to EGFR inhibitors.
Introduction

Mutations in the gene encoding the epidermal growth factor receptor (EGFR) are detectable in 10-30% of tumors from patients with non-small cell lung cancer (NSCLC) (1). Activating EGFR point mutations in exon 21, such as L858R, and exon 19 deletions are effective predictors of response to EGFR tyrosine kinase inhibitor (TKI) therapy with gefitinib or erlotinib (1). However, the majority of patients with NSCLCs harboring these activating EGFR mutations relapse within 10 to 16 months of treatment with EGFR TKIs (1, 2). In over half of these patients, resistance to EGFR TKI therapy is associated with the acquisition of a secondary T790M mutation in the EGFR TK domain that alters interaction of reversible TKIs with the ATP-binding pocket (3, 4). These findings have led to the development of irreversible EGFR TKIs, such as HKI-272, BIBW2992 (afatinib) and PF00299804, that covalently interact with the EGFR TK domain at Cys-797 and are effective in blocking EGFR T790M-driven signaling pathways in preclinical models (5-8). Among the irreversible EGFR TKIs, afatinib has been shown to increase progression-free survival (PFS) compared with placebo in a randomized Phase III trial of patients with NSCLC who had failed EGFR TKIs, indicating that afatinib may have inhibited the selective expansion of EGFR(T790M) clones (9). Afatinib treatment is also associated with prolongation of PFS when compared to chemotherapy in patients with advanced NSCLC and EGFR exon 19 or L858R mutations (10). Other studies have demonstrated that dual inhibition of mutant EGFR with afatinib and the anti-EGFR antibody, cetuximab, can overcome T790M-mediated resistance in preclinical models and potentially in patients (11, 12). Moreover, AZD9291, a third generation inhibitor of mutant EGFR may be more active than the afatinib/cetuximab combination (13). Despite these promising findings, NSCLCs with EGFR mutations eventually develop resistance to irreversible TKI therapy, emphasizing the need to identify other targets for treatment in this setting.

The oncogenic mucin 1 (MUC1) protein is aberrantly overexpressed in NSCLCs and confers a poor prognosis (14, 15). The functional significance of MUC1 overexpression in NSCLCs may be related in part to the findings that MUC1 associates with EGFR at the cell membrane in other types of cancer cells (16-18). Of importance to MUC1-EGFR interactions, MUC1 is translated as a single polypeptide, undergoes autocleavage and is expressed as a non-covalent heterodimer of N-
terminal (MUC1-N) and C-terminal (MUC1-C) subunits (19). MUC1-N contains glycosylated tandem repeats that are characteristic of mucins and is positioned extracellularly in a complex with the transmembrane MUC1-C subunit (19). MUC1-C also functions as a cell surface receptor that interacts with galectin-3 and thereby associates with EGFR (20). The MUC1-C cytoplasmic domain of 72 amino acids contains a YEKV motif that serves as a substrate for EGFR phosphorylation and in turn a SRC SH2 binding site (16). The MUC1-C cytoplasmic domain also contains a YHPM motif that, following phosphorylation, functions as a binding site for PI3K and thereby activation of the AKT pathway (21-23). In addition and when phosphorylated on tyrosine, a YTNP site in the MUC1-C cytoplasmic domain interacts with GRB2, linking MUC1-C to SOS and activation of the RAS→MEK→ERK pathway (23-25). The overexpression of MUC1, as found in NSCLCs, is also associated with targeting of MUC1-C to the nucleus, where it contributes to activation of the Wnt/β-catenin/TCF4 (26, 27), NF-κB RelA (28) and STAT1/3 (29, 30) pathways. In this context, other work has shown that MUC1-C induces gene signatures that are highly predictive of decreases in disease-free and overall survival of patients with NSCLC (14, 15).

The present studies have investigated the potential involvement of MUC1-C in NSCLC cells that harbor activating EGFR mutations. The results show that MUC1-C interacts with EGFR and that targeting MUC1-C inhibits EGFR activation, results in suppression of the downstream AKT and MEK pathways, and inhibits tumorigenicity. We also demonstrate that targeting MUC1-C is synergistic in combination with afatinib in inhibiting mutant EGFR signaling and thereby NSCLC cell survival.
Materials and Methods

Cell culture. H1975, H1299, PC9GR, H1650 and HCC827 NSCLC cells (ATCC) were grown in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 μg/ml streptomycin, 100 units/ml penicillin and 2 mM L-glutamine. H1975 and PC9GR cells were infected with lentiviral vectors expressing a MUC1 shRNA (Sigma) or a scrambled control shRNA (CshRNA; Sigma). H1975 cells were stably transfected with a pHR-CMV vector expressing MUC1-C or MUC1-C(CQC→AQA). Cells were treated with the MUC1-C inhibitor GO-203 (Genus Oncology) (22) and/or the irreversible EGFR inhibitor, afatinib (Selleck Chemicals).

Immunoprecipitation and immunoblot analysis. Cell lysates were prepared as described (22). Soluble proteins (1 mg) were immunoprecipitated with anti-EGFR (Cell Signaling Technology) or a control IgG. The precipitates and lysates not subjected to immunoprecipitation were analyzed by immunoblotting with anti-MUC1-C (31), anti-p-EGFR(Tyr-1148) (Cell Signaling Technology), anti-EGFR (Abcam), anti-p-AKT(Ser-473), anti-AKT, anti-p-MEK(Ser-217/221), anti-MEK, anti-p-ERK(Thr-202/Tyr-204), anti-ERK (Cell Signaling Technologies) or anti-β-actin (Sigma) as described (22). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Promoter-reporter assays. Cells (5 x 10^4) growing in 6-well plates were transfected with 1 μg of the pGL4.32-Luc plasmid containing NF-κB-activated sequences upstream to luciferase (pGL4.32/luc2P/NF-kB-RE/Hygro; Promega) or pGL3-basic vector (Promega) and 20 ng of SV-40-Renilla-Luc in the presence of Superfect (Quiagen). Cells (5 x 10^4) growing in 6-well plates were also transfected with 1 μg TOPFlash or FOPFlash (Addgene) and 20 ng of SV-40-Renilla-Luc in the presence of Superfect. At 48 h after transfection, the cells were lysed in passive lysis buffer. Lysates were analyzed for fire-fly and Renilla luciferase activities with the Dual-Luciferase assay kit (Promega).
Colony formation assays. Cells were seeded in 6-well plates for 24 h and then left untreated or treated with inhibitor. After 7-14 d, the cells were washed and stained with 0.5% crystal violet in 25% methanol. Colonies >30 cells were counted in triplicate wells.

NSCLC xenograft models. Four- to 6-week old BALB/c nu/nu mice were injected subcutaneously with 4 x 10^6 cells in the flank. Tumor volumes were calculated using the formula V=(L x W^2)/2, where L and W are the larger and smaller diameters, respectively. In xenograft models, mice with established H1975 tumors (90-120 mm^3) were randomized and treated intraperitoneally each day with vehicle control, 12.5 mg/kg GO-203 (dissolved in 5% acetic acid and diluted in PBS), 10 mg/kg afatinib (dissolved in DMSO and diluted in PBS) or both GO-203 and afatinib. Tumors were measured every other day with calipers, and tumor volumes were calculated as above. Frozen tumor tissues were thinly sliced and disrupted using a Dounce homogenizer in 3 ml RIPA lysis buffer/protease inhibitor cocktail (Roche) per gram of tissue at 4°C. Tissue suspensions were cleared at 10,000 x g for 20 min at 4°C. The supernatant was used as lysate of soluble proteins.

Determination of IC50 values and isobologram analysis. Cells were seeded on 96-well plates in 100 μl growth medium at a density of 1000-2000 cells per well. After 24 h, the cells were exposed to GO-203 and/or afatinib for an additional 72 h. Cell viability was assessed using the alamar blue viability assay (Invitrogen). Triplicate wells for each treatment were analyzed and each experiment was performed three times. The IC50 was determined by nonlinear regression of the dose-response data using Prism 5.0 for Mac OSX (GraphPad Software). Cells were exposed to 1:1 ratios of the respective IC50 values for GO-203 and afatinib at ¼ x IC50, ½ x IC50, IC50, 2 x IC50 and 4 x IC50. The assessment of synergy was performed using CalcuSyn software (Biosoft). The combination index (CI) was calculated to assess synergism (CI<1) or antagonism (CI>1).
Results

**MUC1-C regulates H1975 cell EGFR(L858R/T790M)-driven signaling and growth.** Coimmunoprecipitation studies performed on lysates from H1299 NSCLC cells that express wild-type EGFR demonstrated that MUC1-C associates with EGFR (Fig. 1A). H1975 NSCLC cells harbor EGFR with the L858R/T790M mutations. A similar analysis of H1975 cell lysates further demonstrated that MUC1-C interacts with EGFR in NSCLC cells that express the wild-type or mutant forms (Fig. 1A). To assess potential involvement of MUC1-C in EGFR(L858R/T790M) signaling, H1975 cells were infected with lentiviruses expressing a control CshRNA or one targeting MUC1-C (MUC1shRNA). Stable silencing of MUC1-C was associated with increases in phosphorylation of EGFR on Tyr-1148 (Fig. 1B). Previous work had shown that silencing MUC1-C in breast cancer cells is associated with suppression of galectin-3, which facilitates the interaction between MUC1-C and EGFR (20). However, silencing MUC1-C in H1975 cells had no effect on galectin-3 levels, indicating that MUC1-C-induced regulation of galectin-3 is cell context dependent (Fig. 1B). Previous work also showed that MUC1-C interacts with PI3K and promotes activation of the AKT → mTOR pathway (22). In concert with those findings, silencing MUC1-C was associated with suppression of p-AKT levels (Fig. 1C). We also found that MUC1-C silencing results in increased MEK phosphorylation, and has little if any effect on p-ERK levels (Fig. 1C). Similar results were obtained when H1975 cells were silenced for MUC1-C with another MUC1shRNA (Supplemental Figs. S1B, left and right), providing evidence that the observed changes in EGFR and AKT signaling are in response to MUC1-C silencing and not off-target effects of the MUC1shRNAs. H1975 cells are dependent on EGFR(L858R/T790M) activation for growth and survival (4). Moreover, survival of NSCLC cells with activating EGFR mutations is dependent, at least in part, on NF-κB signaling (32). EGFR and AKT have also been linked to activation of both the NF-κB and WNT/β-catenin pathways in NSCLC cells (33, 34). Consistent with these findings and the demonstration that silencing MUC1-C suppresses AKT, we found downregulation of these nuclear signaling pathways (Supplemental Figs. S2A and S2B), indicating that targeting of MUC1-C in the setting of activated EGFR can extend to the regulation of gene expression. In further support of a functional role, we found that silencing MUC1-C is associated with significant inhibition of H1975 cell growth (Fig. 1D).
In addition, the capacity of H1975 cells to form colonies was substantially decreased by downregulating MUC1-C expression (Fig. 1E, left and right). Growth of H1975 cells as tumor xenografts in nude mice was also markedly inhibited by MUC1-C silencing (Fig. 1F). These findings indicate that H1975 cells are, at least in part, dependent on MUC1-C for their growth and survival, and that stable silencing of MUC1-C is associated with downregulation of AKT activation and a compensatory upregulation of EGFR→MEK signaling.

Silencing MUC1-C suppresses the proliferative capacity of gefitinib-resistant PC9GR EGFR(delE746_A750/T790M) cells. Selection of PC9/EGFR(delE746_A750) NSCLC cells for gefitinib resistance (PC9GR) is associated with emergence of the T790M mutation (35). As found with H1975 cells, MUC1-C silencing in PC9GR was associated with increases in p-EGFR (Fig. 2A). In addition, downregulation of MUC1-C decreased p-AKT and increased p-MEK levels (Fig. 2B). We also found that silencing MUC1-C slows PC9GR cell growth (Fig. 2C) and substantially inhibits their capacity for forming colonies (Fig. 2D, left and right). In addition, silencing MUC1-C suppressed the growth of PC9GR tumors in nude mice (Fig. 2E). These findings thus corroborated those obtained with H1975 cells and support the functional importance of MUC1-C in NSCLC cells expressing EGFR with the T790M mutation.

Expression of a MUC1-C(CQC→AQA) mutant inhibits growth of NSCLC EGFR(T790M) cells. The MUC1-C cytoplasmic domain contains a CQC motif (Fig. 3A) that is necessary for the formation of MUC1-C homodimers and thereby the interaction between MUC1-C and cell surface RTKs (19, 36, 37). Accordingly, we stably overexpressed MUC1-C and the MUC1-C(CQC→AQA) mutant in H1975 cells to further assess the involvement of MUC1-C in EGFR(L858R/T790M) signaling (Fig. 3B). In contrast to MUC1-C, coimmunoprecipitation studies demonstrated that the association with EGFR is attenuated by MUC1-C(CQC→AQA) (Fig. 3C). Expression of the MUC1-C(CQC→AQA) mutant was associated with downregulation of p-EGFR levels (Fig. 3D). In addition, H1975/MUC1-C(CQC→AQA) cells exhibited suppression of p-AKT, p-MEK and p-ERK (Fig. 3D), consistent with MUC1-C-mediated activation of these pathways. MUC1-C(CQC→AQA) expression also resulted in inhibition of H1975 cell proliferation (Fig. 3E),
colony formation (Fig. 3F), and growth as tumor xenografts (Fig. 3G), providing further support for involvement of MUC1-C in NSCLC cells that express EGFR(T790M).

**Targeting MUC1-C with the GO-203 inhibitor suppresses mutant EGFR signaling.** The demonstration that the MUC1-C CQC motif is of importance for EGFR(T790M) activation prompted studies with GO-203, a cell penetrating D-amino acid peptide that contains the [R], transduction domain linked to CQCRRKN (22). The GO-203 CQCRRKN peptide binds to the corresponding endogenous sequence in the MUC1-C cytoplasmic domain and blocks MUC1-C homodimerization (Fig. 3A) (36). The MUC1-C CQCRRKN motif is not found in other mammalian proteins. Studies were therefore performed with H1975 cells to determine whether targeting MUC1-C with GO-203 is effective in suppressing EGFR activation. Notably, treatment of H1975 cells with GO-203 was associated with the rapid downregulation of p-EGFR levels in the absence of an apparent effect on total EGFR protein (Fig. 4A, left). GO-203 treatment also resulted in the rapid suppression of p-AKT, p-MEK and p-ERK activation (Fig. 4A, left). A longer time-course (24 and 48 h) showed a rebound in p-EGFR, p-AKT and p-MEK levels at 24 h that was suppressed in part by GO-203 retreatment (Fig. 4A, right). In contrast to recovery of p-EGFR and p-MEK at these later time-points, p-ERK levels were suppressed at 24 and 48 h (Fig. 4A, right). Consistent with the dependence of H1975 cells on EGFR(L858R/T790M) activation (4), GO-203 treatment resulted in a marked decrease in H1975 clonogenic survival (Fig. 4B, left and right). Treatment of PC9GR cells with GO-203 was associated with an initial upregulation of p-EGFR, p-AKT, p-MEK and p-ERK levels and then their suppression (Fig. 4C, left). In addition and as found in H1975 cells, we observed recovery of p-EGFR, p-AKT and p-MEK, but not p-ERK, activation at 24 h (Fig. 4C, right). Moreover, PC9GR cell survival, as determined by colony formation assays, was suppressed by GO-203 treatment (Fig. 4D, left and right). PC9GR cells express EGFR with both the delE746-A750 and T790M mutations, indicating that EGFR(delE746-A750) signaling would also be affected by targeting MUC1-C. Therefore, to extend these observations, studies were performed on H1650/EGFR(delE746-A750) cells. As found with H1975 and PC9GR cells, treatment of H1650 cells was similarly associated with suppression of EGFR, AKT, MEK and ERK activation (Fig.
4E, left and right). As might be expected, the kinetics of effector downregulation varied depending on which cells were under study; however, the results are clearly consistent in terms of the GO-203-induced inhibition of EGFR, AKT, MEK and ERK signaling. In addition, GO-203 treatment of H1650 cells was associated with marked inhibition of colony formation (Fig. 4F). These results support the contention that targeting MUC1-C at the CQC motif is sufficient to suppress mutant EGFR-mediated signaling and survival.

Effects of targeting MUC1-C on the response of H1975 cells to the irreversible EGFR inhibitor afatinib. Afatinib is an irreversible inhibitor of EGFR and HER2 that suppresses the activity of EGFR mutants, including those with T790M (8). However, dual inhibition of mutant EGFR with both afatinib and cetuximab was necessary to overcome T790M-mediated resistance (11), indicating that inhibition of EGFR kinase activity by afatinib alone may not be sufficient against certain NSCLC cells expressing EGFR(T790M). Accordingly, we first examined the effects of afatinib alone on EGFR signaling in H1975 cells. Unexpectedly, treatment with 1 μM afatinib, as used in previous studies (8), was associated with an increase in EGFR and MEK phosphorylation that persisted through 24 h (Fig. 5A). To our knowledge, this observation has not been previously reported in that most in vitro studies with afatinib had been performed at 72 h when there is inhibition of EGFR activity (8, 38-40). In contrast, p-AKT and p-ERK levels were suppressed within the first 24 h of afatinib exposure (Fig. 5A). Upregulation of p-EGFR was similarly observed when H1975/CshRNA cells were treated with afatinib for 24 h (Fig. 5B). However, the constitutive upregulation of EGFR signaling in H1975/MUC1shRNA cells (Fig. 1B) was suppressed by afatinib (Fig. 5B), suggesting that targeting MUC1-C may promote afatinib-induced EGFR inhibition. Indeed, treatment of H1975/MUC1-C cells with afatinib was associated with activation of EGFR signaling; however, this response was abrogated in H1975 cells expressing the MUC1-C(CQC→AQA) mutant (Fig. 5C). Based on these results, we assessed the effects of combining afatinib with GO-203 using the Chou-Talalay method and the median effect equation (41). At half-maximal inhibitory concentrations of afatinib (IC50=178 nM), which is in the range (IC50s=99 to 300 nM) of previous reports (8, 40, 42), and GO-203 (IC50=3.1 μM), these agents were tested alone for
effects on H1975 cell growth at 1/8X, 1/4X, 1/2X, 1X, 2X and 4X the IC50 values and at equipotent concentrations at the same ratios in combination. Isobologram analysis of the data at the ED50, ED75 and ED90 values confirmed synergy of the afatinib/GO-203 combination (Fig. 5D, left) with CIs of less than 1 (Fig. 5D, right). Further analysis of the interaction between afatinib (500 nM) and GO-203 (2.5 μM) at synergistic concentrations demonstrated that the combination is more effective in suppressing p-EGFR, p-AKT and p-MEK levels (Fig. 5E) and colony formation (Fig. 5F) than either agent alone. Consistent with these findings, treatment of established H1975 tumor xenografts with the combination of afatinib and GO-203 was also more effective than either agent alone in inhibiting growth (Fig. 5G), without an increase in toxicity (Supplemental Fig. S3), and in suppressing EGFR, AKT, MEK and ERK signaling (Fig. 5H).

**Afatinib and GO-203 are synergistic in the treatment of diverse NSCLC cells with activating EGFR mutations.** As noted above, PC9GR cells harbor EGFR with both the delE746_A750 and T790M mutations and thus differ from H1975/EGFR(L858R/T790M) cells. To determine if combining afatinib with GO-203 is also effective against PC9GR cells, we first defined the half-maximal inhibitory concentrations of these agents when used alone and then performed an isobologram analysis (Fig. 6A, left). Determination of CI values at the ED50, ED75 and ED90 demonstrated that, as found for H1975 cells, the afatinib/GO-203 combination is synergistic (Fig. 6A, right). In concert with these results, treatment of PC9GR cells with synergistic concentrations of these agents was associated with more pronounced suppression of p-EGFR levels (Fig. 6B) and inhibition of colony formation (Fig. 6C) than that obtained with afatinib or GO-203 alone. In extending this analysis to NSCLC cells expressing EGFR with only the activating delE746-A750 mutation, we found that H1650 cells respond to the combination of afatinib and GO-203 with a synergistic inhibition of growth (Fig. 6D, left) and CI values of less than 1 (Fig. 6D, right). Combining afatinib and GO-203 was also more effective in downregulating EGFR signaling (Fig. 6E) and inhibiting colony formation (Fig. 6F) than either agent alone. Similar results were obtained with HCC827/EGFR(delE746-A750) cells (Supplemental Fig. S4A-C), indicating
that targeting MUC1-C promotes the afatinib response of 4 different NSCLC cells with diverse activating EGFR mutations.
Discussion

The MUC1-C transmembrane protein is positioned at the apical borders of normal epithelial cells and is thereby sequestered from RTKs that are expressed at the lateral and basal membranes (19, 43). In contrast, with stress or transformation and loss of polarity, MUC1-C is expressed over the entire cell membrane, where it becomes accessible for forming complexes with RTKs (43). Thus, in cancer cells, MUC1-C has been shown to constitutively interact with EGFR (16, 20, 44), HER2/HER3 (37), FGFR3 (45), MET (46) and PDGFR (47). The available evidence has supported a role for MUC1-C in participating in RTK downstream signaling (43). However, until recently, there was no mechanistic information to support a role for MUC1-C in activation of RTKs themselves (37). The present studies have focused on NSCLC cells that express EGFR with activating mutations as a model to assess the potential role of MUC1-C in these settings. Three approaches for targeting MUC1-C were studied: (i) stable MUC1-C silencing; (ii) expression of a dominant-negative MUC1-C(CQC→AQA) mutant; and (iii) treatment with the GO-203 inhibitor. As expected, the responses to targeting MUC1-C varied under these different experimental conditions; however, we found that all three approaches were effective in inhibiting mutant EGFR signaling that is necessary for the growth and survival of these NSCLC cells. Silencing MUC1-C in H1975/EGFR(L858R/T790M) cells resulted in downregulation of AKT, slowing of growth, loss of clonogenic survival and inhibition of tumorigenicity, supporting the potential importance of MUC1-C in cells dependent on EGFR(T790M). Curiously, we found that MUC1-C silencing is also associated with increases in p-EGFR(L858R/T790M) and p-MEK levels. Similar results were obtained with gefitinib-resistant PC9GR/EGFR(delE746_A750/T790M) cells, indicating that this response to MUC1-C silencing is consistent in different NSCLC cell types. The present findings thus differ from those obtained in breast cancer cells dependent on HER2 activation (37). In those studies, silencing MUC1-C was associated with loss of growth and survival, and marked downregulation of HER2 activation (37). Nonetheless, the present results from MUC1-C silencing in NSCLC cells lent support to the contention that MUC1-C is of importance to EGFR(T790M) signaling and that the upregulation of p-EGFR(T790M)→p-MEK is a compensatory mechanism.
feedback response to suppression of AKT signaling, which has been observed for ERBB receptors (48).

The MUC1-C cytoplasmic domain contains a CQC motif that is necessary and sufficient for the formation of MUC1-C homodimers (36, 49). Notably, expression of a MUC1-C(CQC→AQA) mutant in MUC1-null HCT116 colon cancer cells is associated with decreases in anchorage-independent growth and tumorigenicity, indicating that MUC1-C(CQC→AQA) functions in inhibition of the malignant phenotype (49). In the present studies, expression of MUC1-C(CQC→AQA) in H1975 cells decreased MUC1-C/EGFR complexes, and resulted in marked downregulation of p-EGFR, p-AKT, p-MEK and p-ERK levels. In contrast to MUC1-C silencing, the MUC1-C(CQC→AQA) mutant functions as a dominant-negative, such that MUC1-C(CQC→AQA) is effective in blocking the compensatory feedback response to EGFR and MEK. Moreover, MUC1-C(CQC→AQA) inhibited H1975 cell growth, clonogenic survival and tumorigenicity. These results provide further support for a model in which MUC1-C contributes to EGFR(T790M) activation. In this way, MUC1-C(CQC→AQA) was functional in suppressing p-EGFR activation through genetic abrogation of the capacity for MUC1-C to form homodimers. These results further indicated that targeting the MUC1-C CQC motif could be effective in suppressing mutant EGFR signaling. To address this possibility, we treated H1975 cells with GO-203, a cell penetrating peptide that blocks MUC1-C function and has little if any effect on growth of normal lung epithelial cells (22). In concert with the effects of MUC1-C(CQC→AQA), H1975 cells responded to GO-203 with suppression of p-EGFR, p-AKT, p-MEK and p-ERK levels. PC9GR cells also responded to GO-203 with downregulation of p-EGFR signaling. To confirm these observations, studies were also performed with H1650/EGFR(delE746-A750) cells, which again showed inhibition of EGFR, AKT, MEK and ERK signaling. In addition, treatment of these different NSCLC cells with GO-203 was associated with marked cell death. These findings indicate that NSCLC cells with activating EGFR mutations respond to targeting MUC1-C homodimerization with suppression of EGFR signaling and loss of survival.
Given the effects of targeting MUC1-C on EGFR function in NSCLC cells, we reasoned that GO-203 might be effective in combination with other agents that inhibit EGFR mutants, particularly those that harbor the T790M mutation. Afatinib is an irreversible EGFR inhibitor that was recently approved for the treatment of patients with NSCLCs that express EGFR with exon 19 deletions and L858R substitution mutations. In preclinical models, dual inhibition of mutant EGFR with afatinib and cetuximab was effective in decreasing levels of both p-EGFR and total EGFR (11). In the present work, we found that afatinib treatment of H9175 cells is associated with increases in p-EGFR levels at 3-9 h that persist through 24 h. Notably, this compensatory response of p-EGFR activation was attenuated by silencing MUC1-C or expressing the MUC1-C(CQC→AQA) mutant, indicating that targeting MUC1-C might be effective in combination with afatinib. Indeed, studies of H1975 cells demonstrated that combining GO-203 and afatinib is synergistic in inhibiting growth and that GO-203 potentiates the inhibitory effects of afatinib on EGFR activation. Similar findings in PC9GR, H1650 and HCC827 cells indicate that such responses to the combination of GO-203 and afatinib are not restricted to EGFR with a specific activating mutation, and thus may be observed in other settings of TKI resistance. The present studies have examined AKT, MEK and ERK activation as read-outs of GO-203-induced inhibition of mutant EGFR. Afatinib has been effectively combined with other agents, such as the mTOR inhibitor rapamycin (8). MUC1-C also contributes to activation of the PI3K→AKT→mTOR pathway (43). In this context, the effectiveness of the GO-203/afatinib combination could be related to GO-203-induced inhibition of mTOR (22). Moreover, MUC1-C contributes to activation of nuclear signaling pathways, such as NF-κB and WNT/β-catenin, that reside further downstream of RTK, AKT and ERK (43). Therefore, we do not exclude the possibility that effectiveness of the GO-203/afatinib combination is also a consequence of inhibiting EGFR, AKT and ERK driven nuclear signals. Other studies have shown that HER2 amplification is associated with acquired resistance to EGFR TKIs in the absence of the T790M mutation (50). In this context, targeting MUC1-C has been shown to be highly effective in suppressing HER2 activation in HER2-overexpressing breast cancer cells (37), invoking the possibility that combining GO-203 with afatinib, which is a dual EGFR/HER2 inhibitor, could also be effective in TKI-resistant, HER2
amplified NSCLC cells. Finally, a Phase I trial of GO-203 has been completed in patients with refractory solid tumors and which has defined a dose for studying the effectiveness of this agent in treating NSCLC. Studies will also be needed that assess the activity of combining GO-203 with afatinib as compared to that found for afatinib/cetuximab (11, 12) or AZD9291 (13) against NSCLC with TKI-resistant mutant EGFR. In summary, the present work provides the first mechanistic evidence that MUC1-C contributes to the activation of mutant EGFR signaling and that targeting MUC1-C alone or in combination with afatinib represents a potential approach for the treatment of patients with NSCLC resistant to EGFR kinase inhibitors.
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Figure Legends

**Figure 1. Silencing MUC1-C in H1975 cells inhibits AKT, growth, survival and tumorigenicity.** A. Lysates from H1299 and H1975 cells were subjected to precipitation with anti-EGFR or a control IgG. The precipitates were immunoblotted with the indicated antibodies. Lysates not subjected to immunoprecipitation were used as input controls. B and C. H1975 cells were stably infected with lentiviruses expressing a control scrambled shRNA (CshRNA) or a MUC1 shRNA. Lysates were immunoblotted with the indicated antibodies. D. H1975/CshRNA and H1975/MUC1shRNA cells were plated at 5 x 10^4 cells/well. The results (mean±SD of three replicates) are expressed as cell number on day 4. E. H1975/CshRNA and H1975/MUC1shRNA cells were seeded at 1000 cells/well (6-well plate), grown for 10 days and stained with crystal violet (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right). F. H1975/CshRNA (solid bars) and H1975/MUC1shRNA (open bars) cells (4 x 10^6) were injected subcutaneously in the flanks of female nude mice. Tumor volumes were determined on the indicated days after injection. The results are expressed as tumor volumes (mean±SEM for 3 mice).

**Figure 2. PC9GR EGFR(delE746_A750/T790M) cells respond to downregulation of MUC1-C with inhibition of survival and tumorigenicity.** A and B. PC9GR cells were stably infected with lentiviruses expressing a control scrambled shRNA (CshRNA) or a MUC1 shRNA. Lysates were immunoblotted with the indicated antibodies. C. PC9GR/CshRNA and PC9GR/MUC1shRNA cells were plated at 5 x 10^4 cells/well. The results (mean±SD of three replicates) are expressed as cell number on day 4. D. PC9GR/CshRNA and PC9GR/MUC1shRNA cells were seeded at 1000 cells/well (6-well plate), grown for 14 days and stained with crystal violet (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right). E. PC9GR/CshRNA (solid bars) and PC9GR/MUC1shRNA (open bars) cells (4 x 10^6) were injected in the flanks of female nude mice. Tumor volumes were determined on the indicated days after injection. The results are expressed as tumor volumes (mean±SEM for 3 mice).

**Figure 3. Expression of a MUC1-C(CQC→AQA) mutant suppresses the EGFR→MEK pathway and survival.** A. Schema of the MUC1-C subunit with
the 58 aa extracellular domain (ED), 28 aa transmembrane domain (TM) and the 72 aa sequence of the cytoplasmic domain (CD). The CQC motif is necessary for MUC1-C homodimerization and is the target for GO-203 treatment. Also highlighted are the binding sites that link the MUC1-C cytoplasmic domain to activation of the PI3K→AKT and MEK→ERK pathways. B. H1975 cells were stably transfected with vectors expressing MUC1-C or the mutant MUC1-C(CQC→AQA) [designated MUC1-C(AQA) in figures]. Lysates were immunoblotted with the indicated antibodies. C. Lysates from H1975/MUC1-C and H1975/MUC1-C(CQC→AQA) cells were immunoprecipitated with anti-EGFR or a control IgG. The precipitates were blotted with the indicated antibodies. D. Lysates from H1975/MUC1-C and H1975/MUC1-C(CQC→AQA) cells were immunoblotted with the indicated antibodies. E. H1975/MUC1-C and H1975/MUC1-C(CQC→AQA) cells were plated at 5 x 10⁴ cells/well. The results (mean±SD of three replicates) are expressed as cell number on day 4. F. H1975/MUC1-C and H1975/MUC1-C(CQC→AQA) cells were seeded at 1000 cells/well (6-well plate), grown for 12 days and stained with crystal violet. Colony number (>30 cells) is expressed as the mean±SD of three replicates (right). G. H1975/MUC1-C (solid bars) and H1975/MUC1-C(CQC→AQA) (open bars) cells (4 x 10⁶) were injected in the flanks of female nude mice. Tumor volumes were determined on the indicated days after injection. The results are expressed as tumor volumes (mean±SEM for 3 mice).

**Figure 4.** Targeting MUC1-C with GO-203 inhibits EGFR→MEK signaling and induces NSCLC cell death. A. H1975 cells were treated with 5 μM GO-203 at 0 and 24 h. Lysates were immunoblotted with the indicated antibodies (left and right). B. H1975 cells were seeded at 1000 cells/well in 6-well plates and left untreated (Control) or treated with 5 μM GO-203 each day for 4 days. Colonies were stained with crystal violet on day 10 after treatment (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right). C. PC9GR cells were treated with 5 μM GO-203 at 0 and 24 h. Lysates were immunoblotted with the indicated antibodies (left and right). D. PC9GR cells were seeded at 1000 cells/well in 6-well plates and left untreated (Control) or treated with 5 μM GO-203 each day for 4 days.
Colonies were stained with crystal violet on day 15 after treatment (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right). E. H1650 cells were treated with 5 μM GO-203 at 0 and 24 h. Lysates were immunoblotted with the indicated antibodies (left and right). F. H1650 cells were seeded at 1000 cells/well in 6-well plates and left untreated (Control) or treated with 5 μM GO-203 each day for 4 days. Colonies were stained with crystal violet on day 15 after treatment (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right).

Figure 5. Interaction between GO-203 and afatinib treatment in H1975 EGFR(L858R/T790M) cells. A. H1975 cells were treated with 1 μM afatinib at 0 and 24 h. Lysates were immunoblotted with the indicated antibodies (left and right). B. H1975/CshRNA and H1975/MUC1shRNA cells were left untreated (-) or treated with 1 μM afatinib (+) for 48 h. Lysates were immunoblotted with the indicated antibodies. C. H1975/MUC1-C and H1975/MUC1-C(CQC→AQA) were left untreated (-) or treated with 1 μM afatinib (+) for 48 h. Lysates were immunoblotted with the indicated antibodies. D. H1975 cells were treated with (i) fixed IC50 ratios of GO-203 alone on days 0, 1, 2 and 3, (ii) fixed IC50 ratios of afatinib alone on day 0, or (iii) the GO-203/afatinib combination. The multiple effect-level isobologram analyses on day 4 are shown for the ED50 (X), ED75 (+) and ED90 (●) values. E. H1975 cells were left untreated or treated with 2.5 μM GO-203, 0.5 μM afatinib, or the GO-203/afatinib combination for 24 h. Lysates were immunoblotted with the indicated antibodies. F. H1975 cells were seeded at 1000 cells/well in 6-well plates and left untreated or treated each day with 2.5 μM GO-203 alone, 0.5 μM afatinib alone on day 0, or the GO-203/afatinib combination for 4 d. Colonies were stained with crystal violet on day 10 after treatment (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right). G. Mice with established H1975 tumors were treated with vehicle control (closed squares), GO-203 (closed diamonds), afatinib (open triangles) or the GO-203/afatinib combination (open circles). Tumor volumes were determined on the indicated days of treatment. The results are expressed as tumor volumes (mean±SEM for 6 mice). Statistical analysis
was performed on day 12 using the student’s t-test. The results obtained with the GO-203/afatinib combination was significantly different as compared to the control (p=0.0025), the GO-203-treated group (p=0.028) and the afatinib-treated group (p=0.038). H. Lysates from tumors isolated on day 12 from mice in the different treatment groups were immunoblotted with indicated antibodies.

**Figure 6. GO-203 is synergistic with afatinib in the treatment of PC9GR/EGFR(delE746_A750/T790M) and H1650/EGFR(delE746_A750) cells.**

A. PC9GR cells were treated with (i) fixed IC50 ratios of GO-203 alone on days 0, 1, 2 and 3, (ii) fixed IC50 ratios of afatinib alone on day 0, or (iii) the GO-203/afatinib combination. The multiple effect-level isobologram analyses on day 4 are shown for the ED50 (X), ED75 (+) and ED90 (Θ) values. B. PC9GR cells were left untreated (Control) or treated with 2.5 μM GO-203, 0.5 μM afatinib, or the GO-203/afatinib combination for 24 h. Lysates were immunoblotted with the indicated antibodies. C. PC9GR cells were seeded at 1000 cells/well in 6-well plates and left untreated or treated each day with 2.5 μM GO-203 alone, 0.5 μM afatinib alone on day 0, or the GO-203/afatinib combination for 4 d. Colonies were stained with crystal violet on day 12 after treatment (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right). D. H1650 cells were treated with (i) fixed IC50 ratios of GO-203 alone on days 0, 1, 2 and 3, (ii) fixed IC50 ratios of afatinib alone on day 0, or (iii) the GO-203/afatinib combination. The multiple effect-level isobologram analyses on day 4 are shown for the ED50 (X), ED75 (+) and ED90 (Θ) values. E. H1650 cells were left untreated or treated with 2.5 μM GO-203, 0.5 μM afatinib, or the GO-203/afatinib combination for 24 h. Lysates were immunoblotted with the indicated antibodies. F. H1650 cells were seeded at 1000 cells/well in 6-well plates and left untreated or treated each day with 2.5 μM GO-203 alone, 0.5 μM afatinib alone on day 0, or the GO-203/afatinib combination for 4 d. Colonies were stained with crystal violet on day 10 after treatment (left). Colony number (>30 cells) is expressed as the mean±SD of three replicates (right).
Figure 2ABCDE

**A.** PC9GR

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**C.** PC9GR

Cell Number x 10^4

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**E.** PC9GR

Tumor Volume (mm^3)

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p = 0.001, 0.003
Figure 4ABCDDEF

A. H1975

B. H1975

C. PC9GR

D. PC9GR

E. H1650

F. H1650
Targeting the oncogenic MUC1-C protein inhibits mutant EGFR-mediated signaling and survival in non-small cell lung cancer cells.


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