Oncogenic MUC1-C Promotes Tamoxifen Resistance in Human Breast Cancer

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Abstract
Tamoxifen resistance of estrogen receptor-positive (ER+) breast cancer has been linked in part to activation of receptor tyrosine kinases, such as HER2, and the PI3K-AKT pathway. Mucin 1 (MUC1) is aberrantly overexpressed in about 90% of human breast cancers, and the oncogenic MUC1-C subunit is associated with ERα. The present studies using HER2 overexpressing BT-474 breast cancer cells, which are constitutively resistant to tamoxifen, demonstrate that silencing MUC1-C is associated with (i) downregulation of p-HER2 and (ii) sensitivity to tamoxifen-induced growth inhibition and loss of clonogenic survival. In contrast, overexpression of MUC1-C in tamoxifen-sensitive MCF-7 breast cancer cells resulted in upregulation of p-AKT and tamoxifen resistance. We show that MUC1-C forms complexes with ERα on the estrogen-responsive promoter of Rab31 and that MUC1-C blocks tamoxifen-induced decreases in ERα occupancy. MUC1-C also attenuated tamoxifen-induced decreases in (i) recruitment of the coactivator CREB binding protein, (ii) Rab31 promoter activation, and (iii) Rab31 mRNA and protein levels. The importance of MUC1-C is further supported by the demonstration that targeting MUC1-C with the cell-penetrating peptide inhibitor, GO-203, sensitized tamoxifen-resistant cells to tamoxifen treatment. Moreover, we show that targeting MUC1-C in combination with tamoxifen is highly synergistic in the treatment of tamoxifen-resistant breast cancer cells. Combined, these findings indicate that MUC1-C contributes to tamoxifen resistance. Mol Cancer Res; 11(7); 714–23. ©2013 AACR.

Introduction
The estrogen receptor (ER)-α signaling pathway contributes to the development and progression of human breast cancers. More than 70% of all breast cancers express ERα with a somewhat higher frequency in tumors from postmenopausal women (1). Endocrine therapy of patients with ER+ breast cancer has included (i) blocking estrogen binding with selective ER modulators, such as tamoxifen, (ii) decreasing ER expression with fulvestrant, and (iii) inhibiting estrogen synthesis with aromatase inhibitors. These endocrine therapies have had a major impact on the natural history of hormone-dependent breast cancer; however, their effectiveness is often limited by intrinsic or acquired resistance (1, 2). For example, adjuvant therapy of ER+ breast cancers with tamoxifen is associated with recurrent disease in about one-third of patients (2). One mechanism of acquired tamoxifen resistance is the downregulation of ERα expression, although this response has been observed in only 15% to 20% of breast cancers (3). Tamoxifen resistance has also been linked to cross-talk between ERα and receptor tyrosine kinases (RTK), specifically EGF receptor (EGFR), EGFR2 (HER2/ERBB2), and insulin-like growth factor receptor (IGFI-R; refs. 1, 2). In this context, amplification and overexpression of HER2 have been associated with endocrine resistance (4–6). However, only about 10% of ER+ breast cancers overexpress HER2, indicating that additional mechanisms confer tamoxifen resistance in the majority of these tumors. Other work has shown that hyperactivation of the phosphoinositide 3-kinase (PI3K) pathway confers resistance to endocrine therapy through both direct and indirect ERα interactions (7). Accordingly, PI3K pathway inhibitors are being evaluated for the treatment of patients with tamoxifen-resistant ER+ breast cancer (8). Nonetheless, new therapeutic targets are needed for the treatment of tamoxifen-resistant disease.

The mucin 1 (MUC1) heterodimeric protein is aberrantly overexpressed in about 90% of human breast cancers (9). The two MUC1 subunits are generated by autocleavage of a single polypeptide and, in turn, form a stable noncovalent complex (9, 10). The MUC1 N-terminal (MUC1-N) subunit is the heavily glycosylated mucin component of the heterodimer. MUC1-N is positioned extracellularly in a complex at the cell membrane with the MUC1 C-terminal

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(MUC1-C) transmembrane subunit (10). MUC1-C functions as an oncoprotein by interacting with RTKs, such as EGFR and HER2, at the breast cancer cell surface and by contributing to their downstream signaling pathways (9).

In this regard, the 72 amino acid MUC1-C cytoplasmic domain acts as a substrate for EGFR and other RTKs. The MUC1-C cytoplasmic domain also contains a Tyr-His-Pro-Met (YHPM) motif, that when phosphorylated on tyrosine, functions as a binding site for PI3K src homology 2 (SH2) domains and thereby activation of the PI3K→AKT pathway (11, 12). The MUC1-C subunit is, in addition, targeted to the nucleus where it interacts with certain transcription factors (10). Of relevance to breast cancer, MUC1-C associates with ERα and this interaction is stimulated by 17β-estradiol (E2; ref. 13). MUC1-C binds directly to the ERα DNA-binding domain and stabilizes ERα by blocking its ubiquitination and degradation. MUC1-C also enhances ERα promoter occupancy, increases recruitment of coactivators, and stimulates ERα-mediated transcription (13).

Notably, tamoxifen has no effect on MUC1-C/ERα complexes, and MUC1-C antagonizes the inhibitory effects of tamoxifen on ERα-mediated transcription (13). In other studies, a MUC1-C–induced 38-gene set was applied to the analysis of a database on tumors treated with tamoxifen and (i) showed a strong association with tamoxifen resistance; however, there has been no direct evidence to date for such an association.

The present studies using loss and gain of MUC1-C function show that MUC1-C is sufficient to confer tamoxifen resistance in breast cancer cells. The mechanistic basis for these results is supported by the demonstration that MUC1-C (i) contributes to HER2 and AKT activation and (ii) blocks tamoxifen-induced decreases in ERα occupancy on an estrogen-responsive promoter. The results also show that targeting MUC1-C is synergistic with tamoxifen in the treatment of tamoxifen-resistant breast cancer cells.

Materials and Methods

Cell culture

Human HER2-overexpressing BT-474 breast cancer cells (American Type Culture Collection; ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM)/Ham F12 medium (1:1 ratio), 10% heat-inactivated FBS (HI-FBS), 100 μg/mL streptomycin, 100 U/mL penicillin, and 2 mmol/L L-glutamine. BT-474 cells were infected with lentiviruses expressing a MUC1 short hairpin RNA (shRNA; Sigma) or a scrambled control shRNA (CshRNA; Sigma). Human MCF-7 breast cancer and 293T renal cells (ATCC) were maintained in DMEM, 10% HI-FBS, antibiotics, and L-glutamine. MCF-7 cells were transfected to stably express a control pHR-CMV-GFP vector or one expressing MUC1-C. For certain experiments carried out in the absence of estrogen stimulation, cells were grown in phenol red-free Iscove’s modified Eagle’s medium (IMEM), 10% charcoal-stripped serum (CSS), antibiotics, and L-glutamine. Cells were treated with tamoxifen (Sigma-Aldrich) or 4-hydroxytamoxifen (OHTAM; Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) and, as a control, with a corresponding dilution of DMSO. The cells were also treated with the MUC1-C inhibitor GO-203 (Genus Oncology; ref. 12) or the PI3K inhibitor LY294002 (Sigma).

Immunoblot analysis

Cell lysates were analyzed by immunoblotting with anti-MUC1-C (15), anti-ERα (Santa Cruz Biotechnology, Cat. #SC-8005), anti-p-HER2 (Cell Signaling Technology, Cat. #D66B7), anti-HER2 (Cell Signaling Technology, Cat. #2242), anti-p-AKT (Cell Signaling Technology, Cat. #473), anti-AKT (Santa Cruz Biotechnology, Cat. #C67E7), or anti-β-actin (Sigma, Cat. #A5441) as described (12, 13). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Chromatin immunoprecipitation assays

Soluble chromatin was prepared as described (16) and precipitated with anti-ERα (2 μg; Neomarkers, Cat. #TE111.5D11) or a control nonimmune immunoglobulin G (IgG). For re-ChIP assays, complexes from the initial chromatin immunoprecipitation (ChIP) were eluted and reprecipitated with anti-MUC1-C (Neomarkers, Cat. #HM-1630-PABX) as described (16). For PCR, 2 μL from a 50 μL DNA sample was used with Rab31 promoter primers (16) and 25 to 35 cycles of amplification. Fold enrichment was calculated as described (17).

Promoter-reporter assays

Control pGL3 or pRab31-Luc constructs (16) were transfected into cells with the Renilla plasmid in the presence of Superfect (Qiagen). Luciferase activity was measured using the Promega Dual Glo kit as described (16).

qRT-PCR

Total RNA was isolated from cells using a RNeasy Mini Kit (Qiagen). cDNAs were synthesized from RNA using the first-strand cDNA synthesis kit (Invitrogen) as described (16). The SYBR green qPCR assay kit (Applied Biosystems) was used with 5 μL of 20-fold diluted cDNA. The samples were amplified with the ABI Prism 7300 machine (Applied Biosystems). Rab31 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used
for quantitative real-time PCR (qRT-PCR) are listed in Supplementary Table S1.

Results
Silencing MUC1-C confers sensitivity of BT-474 cells to tamoxifen treatment

BT-474 breast cancer cells overexpress HER2, are ER\(^+\), and are resistant to tamoxifen (18, 19). Immunoblot analysis further showed that BT-474 cells express MUC1-C (Fig. 1A, left). To determine whether MUC1-C plays a role in tamoxifen resistance, we transduced cells with a lentiviral vector expressing a scrambled CshRNA or one expressing a MUC1 shRNA (Fig. 1A, left). Compared with wild-type BT-474 cells and those stably expressing the CshRNA, there was downregulation of MUC1-C in the cells expressing the MUC1shRNA (Fig. 1A, left). As a control, the partial silencing of MUC1-C had little, if any, effect on ER\(\alpha\) levels (Fig. 1A, left). MUC1 interacts with HER2 and promotes HER2-mediated signaling (20, 21). In this context, partial silencing of MUC1-C in BT-474 cells was associated with downregulation of p-HER2 and no detectable effect on HER2 levels (Fig. 1A, right). With regard to tamoxifen resistance, growth of BT-474/CshRNA cells was unaffected by the addition of tamoxifen as compared with that obtained with untreated cells (Fig. 1B). In contrast, proliferation of BT-474/MUC1shRNA cells was partially slowed as compared with BT-474/CshRNA cells and was clearly further inhibited by tamoxifen treatment (Fig. 1B). To exclude an off-target effect of the MUC1shRNA, we infected BT-474 cells with a lentivirus expressing another MUC1 shRNA, designated MUC1shRNA(#2). Studies of BT-474/MUC1shRNA(#2) cells also showed (i) downregulation of MUC1 abundance (Supplementary Fig. S1A, left), (ii) decreases in p-HER2 levels (Supplementary Fig. S1A, right), and (iii) tamoxifen-induced growth inhibition (Supplementary Fig. S1B), confirming the effects of silencing MUC1 on reversal of tamoxifen resistance. In concert with these results, BT-474/MUC1shRNA cells exhibited a marked loss of viability in response to tamoxifen as compared with that obtained for BT-474/CshRNA cells (Fig. 1C, left). Moreover, similar results were obtained when the cells were treated with OHTAM, the active metabolite of tamoxifen (Fig. 1C, right). Plating efficiency of BT-474/MUC1shRNA cells was also significantly decreased compared with BT-474/CshRNA cells (Fig. 1D, left and right). As expected, tamoxifen had little, if any, effect on the ability of BT-474/CshRNA to form colonies (Fig. 1E, left). Notably, however, tamoxifen treatment was associated with a marked decrease in BT-474/MUC1shRNA cell colony formation (Fig. 1E, right). These findings indicate that MUC1-C contributes to tamoxifen resistance in BT-474 cells.

Figure 1. Resistance of HER2-overexpressing BT-474 cells to tamoxifen is conferred by MUC1-C expression. A, lysates from wild-type (WT) BT-474 cells, BT-474/CshRNA, and BT-474/MUC1shRNA cells were immunoblotted with the indicated antibodies (left and right). B, control BT-474/CshRNA (squares) and BT-474/MUC1shRNA (diamonds) cells were left untreated. BT-474/CshRNA (triangles) and BT-474/MUC1shRNA (circles) were also treated with 5 \(\text{m mol/L}\) tamoxifen on days 0 and 2. Cell number is expressed as the mean \(\pm\) SD of 3 replicates. C, control BT-474/CshRNA (solid bars) and BT-474/MUC1shRNA (open bars) cells were treated with 5 \(\mu\text{mol/L}\) tamoxifen (left) or 10 nmol/L OHTAM (right) on days 0 and 2. The results (mean \(\pm\) SD of 3 replicates) are expressed as the percentage of cell death as determined by Trypan blue staining on the indicated days (left) or on day 4 (right). D, BT-474/CshRNA and BT-474/MUC1shRNA cells were seeded at 1,000 cells/well (6-well plate), grown for 14 days, and then stained with crystal violet (left). Colony number (30 cells) is expressed as the mean \(\pm\) SD of 3 replicates. E, BT-474/CshRNA (1,000 cells/well; left) and BT-474/MUC1shRNA (2,000 cells/well; right) cells were seeded in 6-well plates and left untreated (control) or treated with 5 \(\mu\text{mol/L}\) tamoxifen (TAM) every other day for 14 days. Colony number (30 cells) is expressed as the mean \(\pm\) SD of 3 replicates.
Overexpression of the MUC1-C subunit confers resistance of MCF-7 cells to tamoxifen

In contrast to BT-474 cells, MCF-7 breast cancer cells are ER⁺ and sensitive to tamoxifen. To extend the analysis of MUC1-C involvement in tamoxifen resistance, MCF-7 cells were stably transfected with a control vector or one expressing MUC1-C (Fig. 2A). Overexpression of MUC1-C resulted in a modest increase in ERα levels (Fig. 2A). In addition and consistent with the reported effects of MUC1-C on upregulation of the PI3K→AKT pathway (11, 12), overexpression of MUC1-C in MCF-7 cells was associated with a marked induction of p-AKT activation (Fig. 2A).

Growth of MCF-7/vector cells was inhibited by tamoxifen (Fig. 2B). Significantly, however, tamoxifen treatment had little effect on proliferation of MCF-7/MUC1-C cells (Fig. 2B). The MCF-7/MUC1-C cells were also less sensitive to tamoxifen- and OHTAM-induced loss of viability as compared with MCF-7/vector cells (Fig. 2C, left and right). Plating efficiency of MCF-7/MUC1-C cells was substantially increased compared with MCF-7/vector cells (Fig. 2D, left and right). Moreover, tamoxifen was effective in decreasing formation of MCF-7/vector cell colonies (Fig. 2E, left), but not clonogenic survival of MCF-7/MUC1-C cells (Fig. 2E, right). To determine whether activation of AKT contributes to resistance of MCF-7/MUC1-C cells to tamoxifen, we incubated these cells with a sublethal concentration of the PI3K inhibitor, LY294002 (22). As expected, treatment with LY294002 was associated with downregulation of p-AKT levels (Supplementary Fig. S2A). We also found that LY294002 partially reversed the sensitivity of MCF-7/MUC1-C cells to tamoxifen (Supplementary Fig. S2B). These findings indicate that overexpression of MUC1-C in MCF-7 cells confers tamoxifen resistance and that this effect is conferred in part by AKT activation.

MUC1-C confers MCF-7 cell growth in the absence of estrogen

Growth of MCF-7 cells is dependent on estrogen (23). The demonstration that MUC1-C confers resistance to tamoxifen prompted studies to determine whether MCF-7/MUC1-C cells are also estrogen independent. Culture of MCF-7/vector cells in estrogen-depleted IMEM/CSS medium was associated with inhibition of growth (Fig. 3A). Strikingly, however, proliferation of MCF-7/MUC1-C cells was readily apparent in the setting of estrogen depletion (Fig. 3A). Loss of MCF-7/vector cell viability in the absence of estrogen stimulation was also abrogated by MUC1-C overexpression (Fig. 3B). In addition, the plating efficiency of

Figure 2. Overexpression of MUC1-C in MCF-7 cells is associated with tamoxifen resistance. A, lysates from wild-type (WT) MCF-7 cells, MCF-7/vector, and MCF-7/MUC1-C cells were immunoblotted with the indicated antibodies. B, control MCF-7/vector (diamonds) and MCF-7/MUC1-C (triangles) cells were untreated. MCF-7/vector (circles) and MCF-7/MUC1-C (squares) cells were also treated with 5 μmol/L tamoxifen on days 0, 2, and 4. Cell number is expressed as the mean ± SD of 3 replicates. C, MCF-7/vector (open bars) and MCF-7/MUC1-C (solid bars) cells were treated with 5 μmol/L tamoxifen on days 0, 2, and 4 (left) or 10 nmol/L OHTAM on days 0 and 2 (right). The results (mean ± SD of 3 replicates) are expressed as the percentage of cell death as determined by Trypan blue staining on the indicated days (left) or on day 4 (right). D, MCF-7/vector and MCF-7/MUC1-C cells were seeded at 500 cells/well (6-well plate), grown for 7 days, and then stained with crystal violet (left). Colony number (>30 cells) is expressed as the mean ± SD of 3 replicates (right). E, MCF-7/vector (2,000 cells/well; left) and MCF-7/MUC1-C (500 cells/well; right) cells were seeded in 6-well plates and left untreated (control) or treated with 5 μmol/L tamoxifen (TAM) every other day for 7 days. Colony number (>30 cells) is expressed as the mean ± SD of 3 replicates.
MCF-7/MUC1-C cells in IMEM/CSS medium was substantially greater than that found for MCF-7/vector cells (Fig. 3C, left and right). These findings show that overexpression of MUC1-C in MCF-7 cells confers estrogen independence.

MUC1-C abrogates effects of tamoxifen on ERα-mediated gene transcription

To study the effects of MUC1-C on the response of an estrogen-responsive gene to tamoxifen, we first examined ERα and MUC1-C occupancy of the Rab31 promoter by ChIP analysis (16). In this context, recent work showed that MUC1-C forms a complex with ERα on the Rab31 promoter and activates Rab31 gene transcription in an estrogen-dependent manner (16). Tamoxifen treatment of MCF-7/vector and MCF-7/MUC1-C cells had little, if any, effect on ERα levels (Supplementary Fig. S3). However, in MCF-7/vector cells, tamoxifen treatment was associated with decreased ERα occupancy of the Rab31 promoter (Fig. 4A, left). In contrast, tamoxifen treatment of MCF-7/vector and MCF-7/MUC1-C cells had little, if any, effect on ERα occupancy (Fig. 4A, right). In re-ChIP studies, occupancy of the Rab31 promoter by ERα and MUC1-C was also decreased by tamoxifen in MCF-7/vector (Fig. 4B, left), but not MCF-7/MUC1-C cells (Fig. 4B, right). The CREB-binding protein (CBP) is a histone acetyltransferase that is recruited to ligand-activated DNA-bound ERα, and enhances ERα-mediated gene transcription (24, 25). Recruitment of CBP to the Rab31 promoter in MCF-7/vector cells was decreased by tamoxifen treatment (Fig. 4C, right); however, tamoxifen had no significant effect on CBP occupancy in MCF-7/MUC1-C cells (Fig. 4C, right). To extend these results, we studied activation of the Rab31 promoter using a Rab31 promoter-luciferase reporter construct. Tamoxifen treatment was associated with a decrease in Rab31 promoter activity in MCF-7/vector, but not in MCF-7/MUC1-C cells (Fig. 4D). In concert with these results, tamoxifen-induced downregulation of Rab31 mRNA levels as observed in MCF-7/vector cells was attenuated in MCF-7/MUC1-C cells (Fig. 4E). Moreover, Rab31 protein was decreased by tamoxifen treatment of MCF-7/vector, but not MCF-7/MUC1-C cells (Fig. 4F). These findings show that MUC1-C blocks the inhibitory effects of tamoxifen on ERα occupancy, CBP recruitment, and Rab31 promoter activity.

Tamoxifen-resistant breast cancer cells are sensitive to MUC1-C inhibition

The finding that MUC1-C confers tamoxifen resistance invoked the possibility that MUC1-C inhibitors could be effective in tamoxifen-resistant cells. Accordingly, we treated BT-474 cells with the MUC1-C inhibitor GO-203, a cell-penetrating d-amino acid peptide (fR)9-CQCRRKN (ref. 12). GO-203 treatment of BT-474 cells was associated with marked downregulation of p-HER2 and p-AKT (Fig. 5A). In addition, treatment of BT-474 cells with GO-203 was associated with marked inhibition of growth (Fig. 5B, left). The effects of GO-203 on BT-474 growth were more pronounced than that obtained with partial silencing of MUC1-C in these cells (Fig. 5B, right), consistent with the...
potential of GO-203 to effectively block MUC1-C homo-
dimerization and thereby function (26). As found with
silencing of MUC1 in BT-474 cells, GO-203 treatment
also resulted in a substantial loss of clonogenic survival (Fig.
5C). In contrast, GO-203 had little, if any, effect on survival
of MUC1-negative 293T cells (Supplementary Fig. S4).
GO-203 treatment of MCF-7/MUC1-C cells also inhibited
AKT activation (Fig. 5D), growth (Fig. 5E), and colony
formation (Fig. 5F). Moreover, inhibition of MUC1-C with
GO-203 decreased recruitment of ERα
237
and CBP to the
Rab31 promoter (Fig. 5G). These results indicate that
tamoxifen-resistant BT-474 and MCF-7/MUC1-C cells are
dependent on MUC1-C function for their growth and
survival.

Synergy between GO-203 and tamoxifen in tamoxifen-resistant breast cancer cells

Our above findings show that silencing MUC1-C in BT-474 cells results in tamoxifen sensitivity. To further sub-
stantiate that MUC1-C confers tamoxifen resistance, we
silenced MUC1-C in MCF-7/MUC1-C cells (Fig. 6A). Notably, the MCF-7/MUC1-C/MUC1shRNA cells regained sensitivity to tamoxifen treatment (Fig. 6B). These results suggested that targeting MUC1-C in tamoxifen-resistant cells could potentiate tamoxifen treatment. To address this line of reasoning, we used the Chou–Talalay
method for evaluating drug combinations (27, 28). For the
tamoxifen-resistant BT-474/CshRNA cells, we had to select
a concentration of tamoxifen for the combination studies.
Accordingly, we used the IC50 (16.6 μmol/L) obtained for
the tamoxifen-sensitive BT-474/MUC1shRNA cells, based
on the reasoning that targeting MUC1-C in BT-474 cells
reverses tamoxifen resistance. Using the half-maximal inhib-
itory concentration for GO-203 (3.9 μmol/L), GO-203 and
tamoxifen were tested alone for their effects on BT-474/
CshRNA cell growth at 1/8X, 1/4X, 1/2X, 1X, 2X, and 4X
IC50 values. GO-203 and tamoxifen were also tested at
equipotent concentrations at the same ratios in combination.
Isobologram analysis at the ED50, ED75, and ED90 values
showed synergy for the GO-203/tamoxifen combination
(Fig. 6C) with combination indices (CI) of less than 1 (ED50
= 0.81, ED75 = 0.56, and ED90 = 0.43). A synergistic
interaction between GO-203 and tamoxifen with CIs of less
than 1 (ED50 = 0.79, ED75 = 0.57, ED90 = 0.50) was also
observed in the tamoxifen-sensitive BT-474/MUC1shRNA
cells (Supplementary Fig. S5A). In assessing the combined
effects of GO-203 and tamoxifen against MCF-7/vector
cells, we found that the activity of GO-203 and tamoxifen is

Figure 4. MUC1-C blocks the effects of tamoxifen on occupancy and activation of the Rab31 promoter. A–C, MCF-7/vector (left) and MCF-7/MUC1-C (right)
cells were left untreated (control) or treated with 5 μmol/L tamoxifen (TAM) for 2 days. A, soluble chromatin was precipitated with anti-ERα or a control IgG.
The final DNA samples were analyzed for Rab31 promoter estrogen-responsive element or control region sequences (16). The results (mean ± SD of 3
determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control. B and C, In re-ChiP experiments, the
anti-ERα precipitates were released, reimmunoprecipitated with anti-MUC1-C (B) or anti-CBP (C) and a control IgG, and then analyzed for Rab31
promoter sequences. The results (mean ± SD of 3 determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG
control. D, MCF-7/vector and MCF-7/MUC1-C cells were transfected with the pGL3 vector or pGL3-Luc and renilla plasmid as an internal control. The
blocks were then left untreated (control) or treated with 5 μmol/L tamoxifen for 2 days and then assayed for luciferase activity. The results are expressed as
the mean ± SD of 2 experiments each carried out with 3 determinations. E, MCF-7/vector and MCF-7/MUC1-C cells were left untreated (control) or
injected with 5 μmol/L tamoxifen for 2 days, Rab31 and GAPDH mRNA levels were determined by qRT-PCR. The results (mean ± SD of 3 determinations) are
expressed as relative Rab31 mRNA levels as compared with that obtained for GAPDH. F, MCF-7/vector and MCF-7/MUC1-C cells were left untreated (control)
and treated with 5 μmol/L tamoxifen for 2 days. Lysates from the indicated cells were immunoblotted with anti-Rab31 and anti-β-actin.
synergistic at the ED50 (CI = 0.69) and ED75 (CI = 0.88), and additive at the ED90 (CI = 1.12; Supplementary Fig. S5B). To assess these drug interactions in the tamoxifen-resistant MCF-7/MUC1-C cells, we used the half-maximal inhibitory concentration identified for tamoxifen in the treatment of MCF-7/vector cells, based on the demonstration that targeting MUC1-C in MCF-7/MUC1-C cells reverses tamoxifen resistance. Under these experimental conditions, a synergistic interaction between GO-203 and tamoxifen was observed for MCF-7/MUC1-C cells with CIs of less than 1 (ED50 = 0.65, ED75 = 0.56, and ED90 = 0.49; Fig. 6D). These results and those obtained with BT-474 cells show that GO-203 and tamoxifen are synergistic in the treatment of both tamoxifen-sensitive and -resistant cells.

Discussion

The overexpression of HER2 in breast cancers has been linked to tamoxifen resistance (4). The present studies provide evidence that the MUC1-C oncoprotein promotes resistance to tamoxifen in the HER2-overexpressing BT-474 breast cancer cell model. Previous work had shown that MUC1-C forms a complex with HER2 and contributes to heregulin-induced downstream signals (20, 21). In concert
with those findings, silencing MUC1-C in BT-474 cells was associated with downregulation of p-HER2 levels. Moreover, silencing MUC1-C and thereby suppressing HER2 activation reversed the resistance of BT-474 cells to tamoxifen, consistent with cross-talk between HER2 signaling and the ER pathway. In further support of a role for MUC1-C in resistance of HER2-overexpressing BT-474 cells to tamoxifen, we found that treatment with the MUC1-C inhibitor, GO-203, suppresses HER2 activation and confers sensitivity to tamoxifen-induced inhibition of growth and colony formation. GO-203 disrupts MUC1-C homodimerization and blocks the interaction of MUC1-C with HER2 at the cell membrane (9). In this way, silencing MUC1-C or blocking its function with an inhibitor results in HER2 downregulation and reversal of tamoxifen resistance. In addition to HER2, activation of EGFR and IGFI-R can confer tamoxifen resistance (2). Moreover, like EGFR and IGFI-R, HER2 activates downstream signals that confer phosphorylation of ER and can result in tamoxifen-mediated activation or ligand independence (1, 6, 29). Activation of pathways downstream to these RTKs can also contribute to tamoxifen resistance (2). For example, signaling by the PI3K→AKT→mTOR pathway as a consequence of HER2 overexpression or loss of PTEN can regulate responsiveness to tamoxifen (6, 30–33). However, the precise mechanisms that confer tamoxifen resistance have not been fully defined and may involve activation of mitogenic and antiapoptotic pathways (1, 2).

Our studies further show that overexpression of MUC1-C in MCF-7 cells induces tamoxifen resistance. MCF-7 cells constitutively express MUC1; however, MUC1-C levels are not sufficient to activate the PI3K→AKT pathway. Indeed, as has been shown in other cell types (11, 12), overexpression of MUC1-C in MCF-7 cells was associated with marked upregulation of AKT activation. In this way, MUC1-C interacts directly with PI3K through binding of PI3K SH2 domains to a consensus pYHPM motif in the MUC1-C cytoplasmic domain and activates the PI3K→AKT pathway (11, 12, 34). The PI3K pathway is hyperactivated in response to the development of resistance to estrogen deprivation (23). In addition, activation of PI3K signaling has been linked to antiestrogen resistance in breast cancer cells (7). In concert with these findings, MCF-7 cells that overexpress MUC1-C were found to be resistant to estrogen deprivation. Moreover, MUC1-C overexpression was sufficient to confer resistance to tamoxifen-induced loss of proliferation and clonogenic survival. Notably, treatment of MCF-7/MUC1-C cells with the MUC1-C inhibitor GO-203 was associated with a block in AKT activation, consistent with MUC1-C function in activating the PI3K→AKT pathway. Treatment with GO-203 or the PI3K→AKT inhibitor LY294002 was also associated with reversal of MUC1-C–induced tamoxifen resistance. These findings and those obtained in the BT-474 model of HER2 overexpression indicate that MUC1-C is sufficient to confer tamoxifen resistance by contributing, at least in
part, to signaling pathways, such as AKT, that have been linked to ER activity. Other models of tamoxifen resistance have been described that are induced by chronic tamoxifen exposure (35). As such, further studies will be needed to determine whether MUC1-C plays a role in this setting of induced tamoxifen resistance.

Previous findings have shown that MUC1-C binds directly to the ERα DNA-binding domain and associates with ERα on estrogen-responsive promoters (13). MUC1-C was also found to enhance ERα promoter occupancy and increase recruitment of coactivators (13). In the present work, we studied the effects of MUC1-C on ERα occupancy in the response to tamoxifen treatment. MUC1-C forms a complex with ERα on the ERα-responsive Rab31 promoter and activates Rab31 gene transcription in an estrogen-dependent manner (16). Treatment with tamoxifen was associated with a decrease in ERα occupancy on the Rab31 promoter and this response was blocked by overexpression of MUC1-C. Tamoxifen competes with estrogen for binding to ERα and induces changes in ERα conformation that block recruitment of coactivators (36, 37). In this context, tamoxifen treatment was associated with decreases in recruitment of CBP to the Rab31 promoter, and this response was also attenuated by a MUC1-C-dependent mechanism. MUC1-C contributes to the availability of ERα/E2 complexes for occupancy of estrogen-responsive elements (13). In addition, through a direct interaction with ERα and increasing ERα/E2 complexes, MUC1-C attenuates the competitive effects of tamoxifen on estrogen binding (13). These findings further indicated that overexpression of MUC1-C promotes the transcription of ER-dependent genes and thereby survival of ER− breast cancer cells (13). Thus, the available evidence indicates that, in concert with MUC1-C–induced regulation of the HER2 and AKT pathways, binding of MUC1-C to ERα may also contribute to attenuating the effects of tamoxifen.

The MUC1-C cytoplasmic domain contains a Cys-Glu-Cys (CQC) motif that is necessary for its homodimerization and nuclear localization (38). Accordingly, cell-penetrating peptides and small molecules have been developed to block the CQC dimerization motif (39, 40). In this way, treatment with MUC1-C inhibitors abrogates the formation of MUC1-C homodimers and thereby MUC1-C function in breast cancer cells (26, 39). The present work shows that treatment of tamoxifen-resistant BT-474 cells with the MUC1-C inhibitor, GO-203, is associated with inhibition of growth and loss of clonogenicity, supporting a lack of cross-resistance to targeting MUC1-C. MCF-7 cells with induced resistance to tamoxifen as a result of overexpressing MUC1-C were also sensitive to GO-203 treatment. These findings invoked the possibility that MUC1-C–induced tamoxifen resistance could be reversed by targeting MUC1-C. Tamoxifen-resistant cells were therefore treated with GO-203 in combination with tamoxifen. The demonstration that GO-203 and tamoxifen are highly synergistic against tamoxifen-resistant cells provided further support that MUC1-C is of importance to tamoxifen resistance. By extension, GO-203 and tamoxifen were also found to be synergistic in the treatment of tamoxifen-resistant sensitive cells. These results lend support to the concept that targeting MUC1-C could be effective in the treatment of patients with breast cancers that develop resistance to tamoxifen. A phase I trial of GO-203 is presently underway for patients with refractory solid tumors to identify a maximum tolerated dose for phase II studies. On the basis of the present findings, this agent may be a candidate for evaluation in the treatment of tamoxifen-resistant breast cancers.

Disclosure of Potential Conflicts of Interest
D. Kufe is employed as a director, has ownership interest (including patents), and is a consultant/advisory board member of Genus Oncology. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Kharbanda, H. Rajabi, C. Jin, D. Raina
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Kharbanda, H. Rajabi
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