Breast Cancer Res Treat (2009) 118:113–124
DOI 10.1007/s10549-009-0412-3

PRECLINICAL STUDY

MUC1* is a determinant of trastuzumab (Herceptin) resistance in breast cancer cells

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Received: 30 December 2008 / Accepted: 21 April 2009 / Published online: 5 May 2009
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Abstract In the United States, 211,000 women are diagnosed each year with breast cancer. Of the 42,000 breast cancer patients who overexpress the HER2 growth factor receptor, <35% are responsive to treatment with the HER2-disabling antibody, called trastuzumab (Herceptin). Despite those statistics, women diagnosed with breast cancer are now tested to determine how much of this important growth factor receptor is present in their tumor because patients whose treatment includes trastuzumab are three-times more likely to survive for at least 5 years and are two-times more likely to survive without a cancer recurrence. Unfortunately, even among the group whose cancers originally respond to trastuzumab, 25% of the metastatic breast cancer patients acquire resistance to trastuzumab within the first year of treatment. Follow-on "salvage" therapies have prolonged life for this group but have not been curative. Thus, it is critically important to understand the mechanisms of trastuzumab resistance and develop therapies that reverse or prevent it. Here, we report that molecular analysis of a cancer cell line that was induced to *acquire* trastuzumab resistance showed a dramatic increase in the amount of the cleaved form of the MUC1 protein, called MUC1*. We recently reported that MUC1* functions as a growth factor receptor on cancer cells and on embryonic stem cells. Here, we show that treating trastuzumab-resistant cancer cells with a combination of MUC1* antagonists and trastuzumab, reverses the drug resistance. Further, HER2-positive cancer cells that are *intrinsically* resistant to trastuzumab became trastuzumab-sensitive when treated with MUC1* antagonists and trastuzumab. Additionally, we found that tumor cells that had acquired Herceptin resistance had also acquired resistance to standard chemotherapy agents like Taxol, Doxorubicin, and Cyclophosphamide. Acquired resistance to these standard chemotherapy drugs was also reversed by combined treatment with the original drug plus a MUC1* inhibitor.

Keywords Breast cancer · Herceptin · Drug resistance · MUC1 · Chemotherapy · Trastuzumab

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Introduction

Trastuzumab (Herceptin) is a molecularly targeted drug that is used to treat breast cancers that overexpress the cell surface receptor HER2. HER2 is a single spanning transmembrane protein that is a member of the ErbB (EGF) family of receptors, which also includes HER1 (EGFR), HER3, and HER4. In 25–30% of breast cancers, HER2 is overexpressed and the degree of expression has been shown to correlate with the severity of the disease [1]. The mechanism by which the HER2 receptor mediates cell growth is complex. Ligand binding reportedly induces the formation of heterodimeric signaling complexes comprised of HER2 and another ErbB family member [2–4]. Trastuzumab is an antibody that binds to HER2 and interferes with the transduction of growth signals. In HER2-positive metastatic breast cancers, treatment with trastuzumab is the standard of care [5, 6] although response rates are low unless it is combined with chemotherapy [6, 7]. For this group, treatment that included trastuzumab and whose cancers responded to the drug, showed compelling survival statistics. In one study, a 52% decrease in cancer recurrence was seen when trastuzumab plus a chemotherapy

agent were administered; a 33% decrease in mortality at the 5-year mark was observed when trastuzumab was given post-surgery [8]. Similar findings of enhanced disease free and overall survival have been reported for women with early breast cancer when trastuzumab was added to standard adjuvant chemotherapy [9].

Unfortunately, many breast cancer patients do not respond to trastuzumab therapy, even though their tumors overexpress HER2. These cancers are deemed to be "intrinsically resistant". Additionally, another 25% of the metastatic breast cancer patients, whose cancers initially respond to trastuzumab, acquire resistance within about a year [5, 10]. The treatment options that are available to patients who have failed combination therapy are few and this "salvage" therapy is typically palliative rather than curative, albeit with some recent exceptions [11, 12]. Patients whose cancers are characterized by HER2 overexpression face the prospect of aggressive clinical course of the disease [13], shorter disease-free survival after adjuvant therapy [14], lower response rate to hormone therapy [15] and shorter overall survival times [16].

Previously studies focused on trastuzumab resistance have been carried out by analyzing either excised tumors that had grown resistant or cells that were induced to become trastuzumab resistant in vitro via prolonged exposure to sub-lethal levels of the drug. These studies concluded that trastuzumab resistance could be characterized by: (1) alterations in trastuzumab-HER2 interactions [17]; (2) changes in the expression of regulators of cell cycle [18]; or (3) increases in signaling by other growth factor receptors, co-receptors or their ligands [19–21].

In the present study, we investigated the involvement of MUC1 in trastuzumab resistance. MUC1 has been reported to have anti-apoptotic properties [22–24] as well as pro-growth effects [25, 26]. We recently reported that it is the transmembrane cleavage product of MUC1, MUC1*, that functions as a growth factor receptor [27, 28]. Indeed, transfection of MUC1* into MUC1-negative cells increased growth rate and rendered the cells resistant to a variety of standard chemotherapy drugs [27 and unpublished results]. Here, we specifically examined the role of MUC1* in acquired, as well as intrinsic, trastuzumab resistance and the potential for using MUC1* inhibitors to reverse or prevent it.

Materials and methods

Cell lines and culture

Breast tumor cell lines

T47D, ZR-75-30, and BT474 cells (from ATCC) were cultured in RPMI 1640 medium containing 10% vol/vol HI-FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate.

shRNA-expressing T47D cells

T47D cells expressing the p*Silencer* 3.1-H1 puro plasmid with MUC1-specific, or control shRNA were previously described [27]. The T47D control pool used in these experiments is identical to those used in Mahanta et al. [27]. The T47D MUC1-specific clone is a single cell clone isolated from this pool from cells, sorted into 96-well plates by a BD Aria cell sorter (Becton Dickinson). Cells were cultured in RPMI medium as above, supplemented with 0.5 μg/ml puromycin (Calbiochem 540411).

Trastuzumab-resistant BT474 cells

Two pools of BT474 cells (BTRes1 and BTRes2) were made resistant by culturing in the presence of 1 μg/ml Herceptin (Trastuzumab; Genentech) for 8 weeks.

Cell growth assays

Growth of BT474, BTRes1 and BTRes2 cells in trastuzumab

Cells were plated in 96-well plates at 10,000 cells/well, six wells/condition. The following day, zero hour counts were taken, and medium was changed in the remaining wells to RPMI containing trastuzumab to final concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1, and 3 μg/ml. Three days later, the remaining cells were counted using a hemocytometer.

Percent normalized growth was then calculated: Percent normalized growth = [(Day 3 cell counts with antibody added)−(Zero-day cell counts)]/[(Day 3 cell counts without antibody added)−(Zero-day cell counts)] × 100%.

Growth of BT474 and BTRes1 cells transfected with either a MUC1-specific or a control siRNA in the presence of trastuzumab

BT474 cells were transfected in triplicate with control siRNA (Santa Cruz Biotechnologies; sc-37007), and BTRes1 cells were transfected in triplicate with control or MUC1-specific siRNA (Santa Cruz Biotechnologies sc-35985). About 1 μl of 10 μM siRNA was added to 100 μl of OptiMEM medium (Invitrogen 22600134). About 6 μl of HiPerfect reagent (Qiagen 301704) were then added to each tube. After vortexing, tubes were incubated at room temperature for 20 min. Meanwhile, cells were trypsinized, pelleted and resuspended in fresh RPMI without trastuzumab. 5×10^5 cells in 2.3 ml RPMI

were combined with the OptiMEM with siRNA/HiPerfect complexes and added to a well of a 6-well plate. Two days later, cells were re-transfected by the same method, and plated in 96-well plates, at 10,000 cells/well, five wells/condition. Leftover cells were plated in 6-well plates. The following day, zero hour counts were taken, and medium was changed in the remaining wells to RPMI containing trastuzumab to final concentrations of 0, 0.01, 0.03, 0.1, 0.3, and 1 μg/ml, and cells plated in 6-well plates were harvested and pelleted for Western analysis to evaluate siRNA efficacy. Three days later, cells were counted, and percent normalized growth was calculated.

To visualize the growth of BT474 and BTRes1 cells, 5×10^5 BT474 or BTRes1 cells were plated in duplicate on a 6-well plate. The following day, each cell type was transfected with 10 μl of either 10 μM control siRNA (Santa Cruz Biotechnologies; sc-37007) or MUC1-specific siRNA (Santa Cruz Biotechnologies sc-35985). This was done by adding siRNAs into 100 μl of OptiMEM in microfuge tubes, then 12 μl of HiPerfect reagent (Qiagen 301704). After vortexing, tubes were incubated at room temperature for 20 min. siRNA complexes were then added directly to the cells' culture medium. Cells were re-transfected 3 days later by the same procedure. Two days after this, 5×10^5 cells from each transfection were transferred to duplicate wells of 6-well plates, and simultaneously transfected by adding siRNAs incubated with HiPerfect reagent in OptiMEM prepared as above. Five days later, cells were photographed using a Nikon (Tokyo, Japan) D80 camera, mounted on a Nikon Diaphot microscope.

Growth of trastuzumab-resistant BT474 cells in the presence of trastuzumab and an Anti-MUC1 Fab*

BT474, BTRes1, and BTRes2 cells were plated in 96-well plates at 10,000 cells/well, six wells/condition. The following day, zero hour counts were taken, and medium was changed in the remaining wells to RPMI containing trastuzumab to final concentrations of 0, 0.03, 0.1, and 0.3 μg/ml, in the presence of 2.5 μg/ml Anti-MUC1* Fab (Minerva Biotechnologies [27]; added to BTRes1 and BTRes2), or 2.5 μg/ml Control Fab (Jackson Immunoresearch 315-007-008; added to BT474, BTRes1, and BTRes2). One set of wells was left untreated. Three days later, cells were counted, and percent normalized growth was calculated.

Growth of T47D cells transfected with either a MUC1-specific siRNA or a control siRNA in the presence of trastuzumab

T47D Cells stably expressing control siRNA or MUC1-specific siRNA were plated in 96-well plates at 10,000 cells/well, six wells/condition. The following day, zero hour counts were taken, and medium was changed in the remaining wells to RPMI containing trastuzumab to final concentrations of 0 and 10 μg/ml for control cells, and 0, 1, 5 and 10 μg/ml for MUC1 siRNA-expressing cells. Three days later, cells were counted, and percent normalized growth was calculated.

Growth of T47D and ZR-75-30 cells in the presence of trastuzumab and an Anti-MUC1 Fab*

Cells were plated in 96-well plates at 10,000 cells/well, six wells/condition. The following day, zero hour counts were taken, and medium was changed in the remaining wells to RPMI containing trastuzumab to final concentrations of 0, 5, 10, and 20 μg/ml, in the presence of 12.5 μg/ml Anti-MUC1* Fab, or 12.5 μg/ml Control Fab. One set of wells was left untreated. Three days later, cells were counted, and percent normalized growth was calculated.

Flow cytometry

Monolayers of BT474, T47D, and ZR-75-30 cells were washed in PBS, and cells were resuspended using Versene (Gibco 15040). Cells were washed in PBS, transferred to microcentrifuge tubes, and pelleted at 5000RPM for 3′ at 4°C. Cells were incubated in PBS for 10 min at 4°C, and at 5000RPM for 3′ at 4°C. For detection of HER2 cell surface expression, pellets were resuspended in 20 μl of phycoerythritin-conjugated anti-HER2 antibody (BD Biosciences; 340552), or 20 μl of phycoerythritin-conjugated IgG1 (Santa Cruz Biotechnologies sc-2866), used as an isotype control. After 20 min at 4°C, cells were washed twice in PBS, and fixed in 2% paraformaldehyde (Tousimis 1008C) in PBS. For detection of MUC1* cell surface expression, pellets were resuspended in 40 μl of a 1:50 dilution of rabbit polyclonal Anti-MUC1* antibody (0.75μg/μl; Minerva Biotechnologies), or 40 μl of PBS, for a secondary antibody-only staining control. After 20 min at 4°C, cells were washed twice in PBS, and cell pellets were resuspended in 40 μl of Alexa 488-conjugated anti-rabbit antibody (1:200; Invitrogen A11008). After 20 min at 4°C, cells were washed twice in PBS, and fixed in 2% paraformaldehyde (Tousimis 1008C) in PBS. Cells were analyzed using a FACSCantoII flow cytometer (Becton Dickinson), and FlowJo Software (Treestar).

Western analysis

Cell pellets were lysed in cold radio immuno-precipitation assay (RIPA) buffer plus protease inhibitors. Protein concentration was determined using a BCA Assay Kit (Pierce 23225). Forty (40) μg of lysates were resolved on 6 or 12% wt/vol acrylamide SDS–PAGE gels. Proteins were

transferred to Immobilon P membrane (Millipore IPVH 00010) under semi-dry conditions. Membranes were blocked for 1 h in 5% wt/vol nonfat dry milk in PBST (PBS containing 0.05% Tween-20) and hybridized overnight in 5% wt/vol nonfat dry milk in PBST with rabbit Anti-MUC1* (1:500; Minerva Biotechnologies [27], mouse Anti-MUC1 (1:200; VU4H5, Santa Cruz Biotechnologies sc-7313), mouse anti-HER2 (1:200; Santa Cruz Biotechnologies sc-33684). Membranes were washed three times in PBST, and secondary detection was done using 1:10000 dilutions of HRP-conjugated anti-mouse (Jackson Immunoresearch 115-035-003) or anti-rabbit (Jackson Immunoresearch 111-035-003) antibodies. Membranes were washed three times, and chemiluminescent detection was done using Immun-Star reagent (Bio-Rad 170-5040). Following detection of the protein of interest, blots were stripped in 2% wt/vol SDS, 0.7% vol/vol β-mercaptoethanol, and 63 mM Tris pH 6.8, at 50°C for 20 min. Blots were then probed with antibodies against α-Tubulin or β-Actin (1:5000 and 1:2000, respectively; Neomarkers MS-581-P and RB-9421-P) using methods described above. Expression levels of proteins were quantified by densitometry using a Bio-Rad Versadoc 1000 instrument. Band intensities of α-Tubulin or β-Actin were used to standardize expression levels and allow for comparison among cell populations. The intensity reading of the protein band of interest was divided by the reading for either Tubulin or Actin. The value of BT474 parent cells, divided by that of one of the standards was defined as 100%.

Death assays

Survival of Herceptin-resistant BT474 cells in the presence of chemotherapeutic agents

BT474, BTRes1, and BTRes2 cells were plated at 10,000 cells/well in 96-well plates, four wells/condition. The following day, Cyclophosphamide (Fluka 29875), Taxol (Paclitaxel Sigma T7191), or Doxorubicin (Fisher BP2516) were added in varying concentrations, or left untreated. Two days later, cells were resuspended in 50 µl trypsin, and counted in the presence of trypan blue. Both live and dead (blue) cells were counted. Percent cell death was calculated as percent trypan blue uptake.

Survival of Herceptin-resistant BT474 cells in the presence of chemotherapeutic agents plus Anti-MUC1 Fab*

BT474, BTRes1, and BTRes2 cells were plated at 10,000 cells/well in 96-well plates, four wells/condition. The following day, Cyclophosphamide, Taxol, or Doxorubicin were added in varying concentrations, alone, or in the presence of 10 µg/ml Anti-MUC1* Fab or 10 µg/ml Control Fab. Cells were left untreated, or treated with Fab alone as controls. Two days later, cells were resuspended in 50 µl trypsin, and counted in the presence of trypan blue. Percent cell death was calculated as percent trypan blue uptake.

Results

We induced BT474 cells to acquire Herceptin resistance by culturing the cells in sub-lethal levels of Herceptin for extended periods of time. We chose BT474 cells because it is a breast cancer cell line that overexpresses the HER2 receptor, the target of Herceptin, and also expresses medium to low amounts of the growth factor receptor form of MUC1, called MUC1*. Two pools of BT474 cells, referred to as BTRes1 and BTRes2, were separately cultured in the presence of 1 µg/ml Herceptin for approximately 8 weeks. At that point, cell proliferation experiments showed that both cell populations had become markedly resistant to Herceptin. Treatment with levels of Herceptin that had previously inhibited cell growth by 90–100% had essentially no effect on the growth of the resistant cells, BTRes1 and BTRes2 (Fig. 1).

The resistant cells and the naïve BT474 cells were analyzed to determine whether there were changes in the expression levels of HER2, MUC1-FL (full length) or MUC1* (cleavage product). We previously reported that following MUC1 proteolysis, the low molecular weight transmembrane cleavage product, MUC1*, functions as a growth factor receptor. Ligand-induced dimerization of the truncated extracellular domain activates the MAP kinase signaling pathway and promotes cell growth. Western blot analysis (Fig. 2) revealed that there had been a dramatic

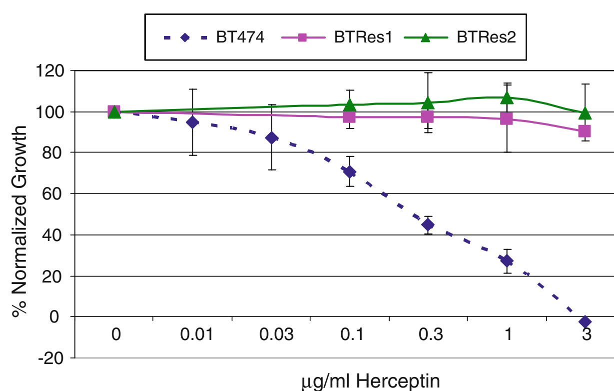

Fig. 1 BT474 breast cancer cells become immune to the therapeutic effects of Herceptin following long-term growth in the presence of 1 µg/ml of the antibody. Treatment of HER2-positive BT474 cells with increasing amounts of Herceptin results in a dose-dependent inhibition of cell growth, as determined by cell counts 3 days post-treatment (*dotted line*). The growth of two separate populations of BT474 cells with induced Herceptin resistance, BTRes1 and BTRes2 (upper two *solid lines*), is unaffected by treatment with Herceptin

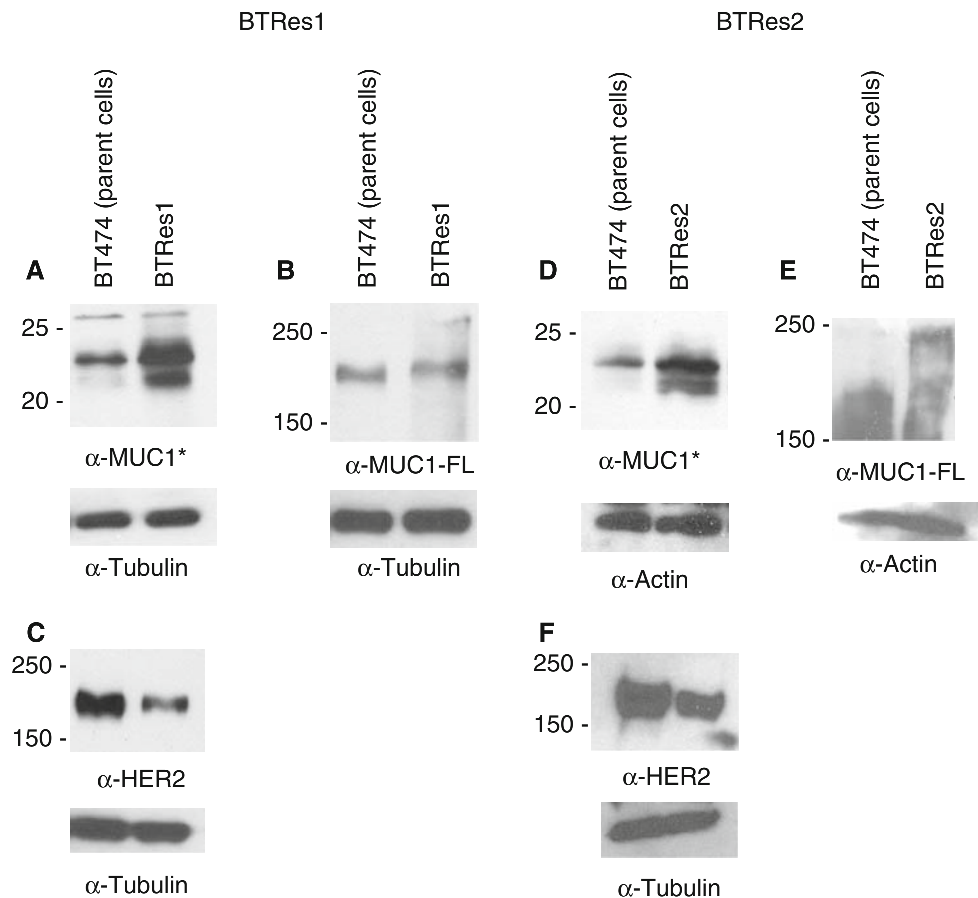

Fig. 2 Western blots of BT474 breast cancer cells and cells that were induced to become Herceptin resistant, BTRes1 and BTRes2, show a dramatic increase in the expression of MUC1*. Levels of MUC1* were 400% that of the parent cells (**a**, **d**), while there was only a modest increase (43 and 15%) in the expression of the full-length MUC1 protein (MUC1-FL; **b**, **e**). Levels of the HER2 receptor, which Herceptin targets, were reduced by 33% in both BTRes1 and BTRes2 (**c**, **f**)

increase in the expression levels of MUC1* in both resistant cell pools. BTRes1 and BTRes2 cells expressed about four-times more MUC1* (Fig. 2a, d) than the parent cell line. However, there was only a modest increase in the amount of full-length MUC1 that was expressed in both resistant cell populations (Fig. 2b, e). HER2 expression was reduced by approximately 33% in both BTRes1 and BTRes2 (Compare Fig. 2c, f).

To determine whether there was a direct correlation between the overexpression of MUC1* and the acquisition of Herceptin resistance, we sought to suppress the expression of MUC1* using MUC1-specific siRNA. Multiple transient transfections of naive BT474 cells as well as resistant population BTRes1 with high levels of MUC1-specific siRNA or a control siRNA were performed. Photos of cell cultures show that BTRes1 cells grew faster and formed bigger colonies than the parent BT474 cells (Fig. 3a, c). A comparison of panels A and B of Fig. 3, shows that the transfection of MUC1-specific siRNA severely inhibited the growth of naïve BT474 cells. BTRes1 cell growth was similarly inhibited by suppression of MUC1* (Fig. 3c, d). We next looked at the effect of MUC1 suppression on Herceptin sensitivity. In this experiment, lower levels of siRNA were used to lessen the growth inhibitory effects of MUC1 suppression so that Herceptin effects could be evaluated. BTRes1 cells that had been transfected with MUC1-specific siRNA were treated with Herceptin, then cultured for 3 days. Western blot analysis shows the degree of MUC1* suppression that was achieved (Fig. 3e). In parallel, naïve cells as well as resistant cells that had been transfected with MUC1-specific siRNA were treated with Herceptin, then cultured for 3 days. When cell number was plotted as a function of Herceptin concentration, it can be seen that MUC1 suppression re-sensitized cells to the inhibitory effects of Herceptin (Fig. 3f). BTRes2 cells responded essentially the same as BTRes1 (data not shown).

We next tested whether MUC1* antagonists could also restore the therapeutic effects of Herceptin on resistant cells. We previously reported that agents that prevented dimerization of MUC1*'s extracellular domain, inhibited the growth of a panel of MUC1*-positive cells [27]. In particular, the Fab of an antibody raised against the short extracellular domain of MUC1* was shown to potently inhibit the growth of both cancer cells and embryonic stem cells [27, 28]. This Fab was shown to have essentially no effect on the growth of MUC1*-negative cells. Thus, we investigated treating Herceptin-resistant cells with the combination of

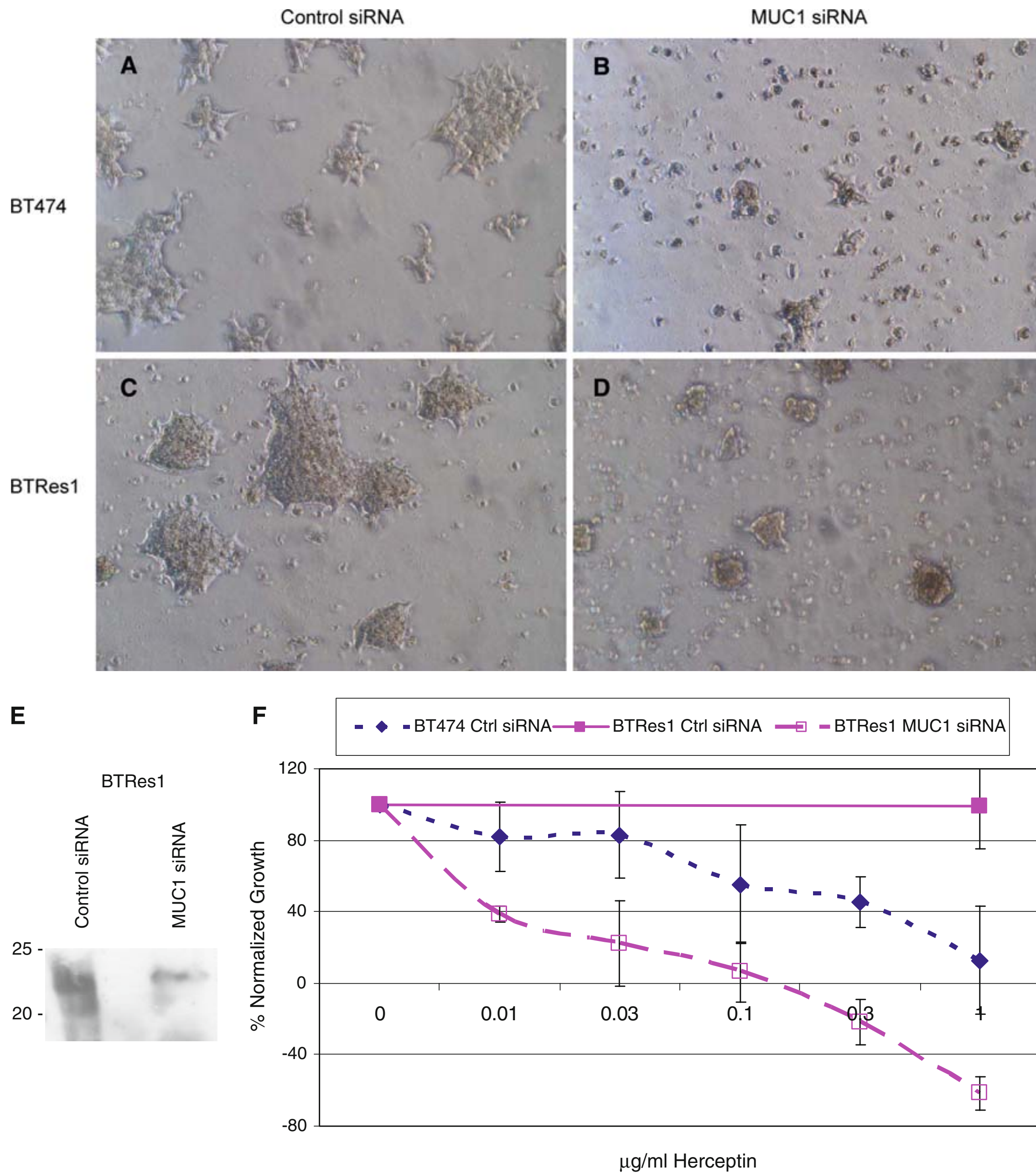

Fig. 3 Down regulation of MUC1 by siRNA sensitizes Herceptin resistant BT474 cells to growth inhibition mediated by Herceptin. BT474 and BTRes1 cells were transfected three times with 10 µl of a 10 µM control siRNA (**a**, **c**) or MUC1 siRNA (**b**, **d**), and placed in culture. Ten days later, cells were photographed, showing inhibitory effects of MUC1 siRNA on proliferation of BT474 and BTRes1 cells. To test effects of MUC1 downregulation on Herceptin sensitivity, BTRes1 cells were transiently transfected twice with 1 µl of a 10 µM MUC1-specific or control siRNA. The effects of siRNA downregulation are shown by Western blot (**e**). Growth of these cells in the presence of Herceptin was compared with growth of BTRes1 cells transfected with control siRNA by cell counts 3 days post-treatment with Herceptin. Growth of BTRes1 cells transfected with control siRNA (*solid line*) is unaffected by Herceptin treatment, but growth of BTRes1 cells transfected with MUC1 siRNA is reduced (*dashed line*). Growth of BT474 cells transiently transfected with a control siRNA (*dotted line*) is essentially unchanged from previous experiments where siRNA was not present (**f**)

Herceptin plus a MUC1* inhibitor. Both resistant cells and the parent BT474 cells were treated with a constant amount of the MUC1* disabling Fab while the concentration of Herceptin was varied. Figure 4 shows that although Herceptin treatment alone did not inhibit the growth of the resistant cells, the combination of Herceptin plus the MUC1* disabling Fab completely reversed the acquired drug resistance. It should be noted that at higher concentrations of the Anti-MUC1* Fab, the dependence on Herceptin for growth inhibition was less pronounced (data not shown).

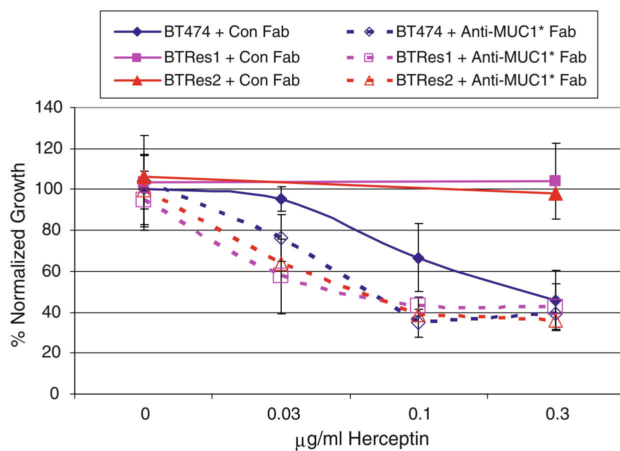

Fig. 4 Disabling MUC1* with an Anti-MUC1* Fab restores the therapeutic effect of Herceptin on BTRes1 and BTRes2. In the presence of 2.5 µg/ml of a control Fab, "Con Fab", (*solid lines*) growth of BTRes1 (*pink*) and BTRes2 (*red*) is unaffected by Herceptin. As expected, Herceptin inhibited the growth of naïve BT474 cells and the control Fab had essentially no added effect (*solid blue line*). In the presence of an Anti-MUC1* disabling Fab (*dotted lines*), growth inhibition mediated by Herceptin is restored for both resistant cell pools

To this point, we have only discussed the reversal of what is termed "acquired" Herceptin resistance. However, there are many instances of HER2-positive breast cancers that do not respond to Herceptin, even initially. We hypothesized that perhaps many other cancers, for which Herceptin is not considered for therapy, could be treated with a combination therapy that combines Herceptin with a MUC1* disabling agent. Some commonly studied breast cancer cell lines express HER2 but are not inhibited by Herceptin and are termed "intrinsically" resistant. Published studies have reported that breast cancer cell lines T47D [29] and ZR-75-30 [30] are resistant to Herceptin in in vitro growth assays. Notably, both these cell lines express high levels of MUC1*. FACS analysis of BT474, T47D, and ZR-75-30 cells, wherein cells were sorted according to HER2 or MUC1* expression (Fig. 5) revealed that BT474 cells express more HER2 than T47D and ZR-75-30, but the latter two express more MUC1* than BT474 cells. MUC1 was suppressed in T47D cells via stable transfection with a MUC1*-specific shRNA. The MUC1-suppressed cells were then assayed for their ability to grow in the presence of Herceptin (Fig. 6a). As can be seen in the figure, Herceptin was able to inhibit the growth of intrinsically resistant T47D cells when MUC1 was suppressed. In a follow-on experiment, T47D cells and ZR-75-30 cells were treated with the MUC1* disabling Fab, at constant concentration, and Herceptin at variable concentration (Fig. 6b, c). These experiments demonstrated that an Anti-MUC1* Fab administered in combination with Herceptin, effectively inhibited the growth of cells previously considered to be Herceptin insensitive. We had previously demonstrated that the Fab of Anti-MUC1*, administered alone at higher concentrations, inhibited the growth of both T47D [unpublished results] and ZR-75-30 [27] cells.

Because Herceptin is typically administered in combination with a cytotoxic chemotherapy agent [6, 8], we looked at whether Herceptin resistant cells had also acquired resistance to other chemotherapeutic drugs. Unexpectedly, we found that our Herceptin resistant pools, BTRes1 and BTRes2, had also become resistant to Taxol, Doxorubicin and Cyclophosphamide. These cytotoxic drugs efficiently killed naïve BT474 cells but had no significant killing effect on the Herceptin-resistant cells that were characterized by an increase in MUC1* expression. This is consistent with a previous study, in which we found that the introduction of MUC1* into MUC1-negative cells induced resistance to several chemotherapeutic drugs [unpublished data and 27]. We therefore sought to reverse this acquired resistance to multiple chemotherapeutic drugs by inhibiting MUC1*. In this set of experiments, naïve BT474 cells as well as cells with induced resistance were treated with the Anti-MUC1* Fab and a chemotherapy drug. Cell death was measured for each condition. Treatment of the resistant cells with the combination of the Anti-MUC1* Fab and the chemotherapy drug, restored the cytotoxic effects of Taxol, Cyclophosphamide and Doxorubicin (Fig. 7 a–c).

Discussion

Here we report that a common HER2-positive breast cancer cell line, BT474, began to overexpress the MUC1* growth factor receptor, when Herceptin resistance was induced by culturing the cells in sub-lethal levels of the drug. We showed that this acquired resistance to Herceptin could be overcome by inhibiting MUC1* using either an Anti-MUC1* Fab or MUC1 specific siRNA. Unexpectedly, we found that these cells had also acquired resistance to a panel of standard chemotherapeutic drugs, in addition to Herceptin. Notably, the cells had become resistant to Taxol and Doxorubicin, which are often given in combination with Herceptin. Resistance to these cytotoxic drugs was similarly reversed by treating the cells with a combination of the drug plus a MUC1* antagonist. Further, we showed that other breast cancer cell lines that had previously been thought to be resistant to the effects of Herceptin, became Herceptin sensitive when MUC1* was inhibited. Strikingly, the growth of breast cancer cell lines T47D and ZR-75-30, was inhibited by Herceptin in a dose-dependent manner when MUC1* antagonists were co-administered.

MUC1* can exert oncogenic effects through pro-growth as well as anti-apoptotic properties, and therefore it is not

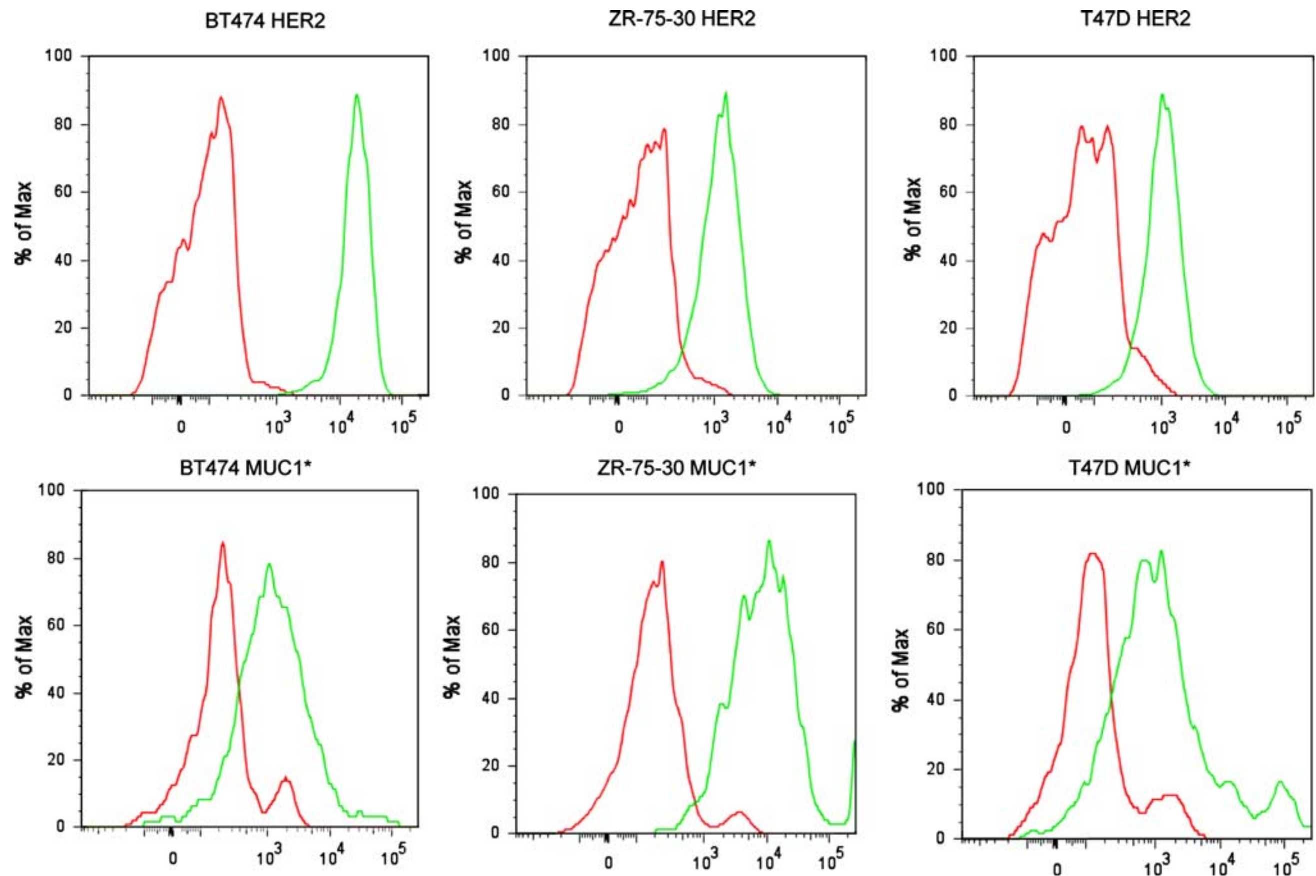

Fig. 5 Breast cancer cell lines T47D and ZR-75-30 express low to intermediate HER2 levels, compared with BT474 cells, but express higher amounts of the MUC1* growth factor receptor. Flow cytometric analysis of surface levels of HER2 show that BT474 cells have a larger amount of surface HER2 than T47D or ZR-75-30 cells. Conversely, T47D and ZR-75-30 cells express more MUC1* than BT474 cells but less HER2. In each case, the *green trace* is the fluorescence intensity of staining with the specific antibody. The *red trace* in the *upper panels* is the intensity of staining with an IgG isotype control antibody. The secondary antibody alone was used as the control for the *lower panels*

surprising that it plays a role in drug resistance. We previously showed that ligand-induced dimerization of the extracellular domain of MUC1* induced ERK2 phosphorylation, cell proliferation and survival [27, 28]. Thus, in Herceptin resistant cells, MUC1* could function independently by homodimerization to increase cell growth and inhibit apoptosis, thereby countering the growth inhibitory effects of Herceptin. In the present study, Herceptin resistant cells also showed resistance to chemotherapeutic agents such as taxol, doxorubicin and cyclophosphamide. The effect of these drugs was essentially restored when given in combination with MUC1* antagonists. This suggests that the anti-apoptotic effect of MUC1* overexpression, in certain resistant cells, could be the primary mechanism by which these cells become resistant to chemotherapeutic agents. MUC1 overexpression has been noted in other contexts of resistance and recurrence. Similar to our study using BT474 cells, it has been reported that the ovarian cancer cell line SKOV-3, also made refractory to Herceptin in vitro, showed MUC1 upregulation [31]. In another study, the only gene out of 26,000 tested, that corresponded to prostate cancer recurrence was MUC1. An increase in the amount of MUC1 mRNA was shown to increase the probability of prostate cancer recurrence [32]. A study of patient matched ovarian tumors, showed that MUC1 was one of 121 genes, out of 21,000 tested, that was upregulated in the post chemotherapy tumor compared with the primary tumor [33]. Notably, MUC1 was apparently the only growth factor receptor in the group of upregulated genes.

It is possible that MUC1* could form signaling heterodimers with HER2 and thereby potentiate its growth promoting signals. In support of this hyptothesis, MUC1 has been shown to associate with HER2 in human breast cancer cells and Heregulin enhanced the interaction [34]. Although the effect of this association on enhancing signaling through HER2 was not addressed in this study, it was shown that this association led to MUC1-dependent accumulation of gamma-catenin in the nucleolus, which is associated with the activation of the Wnt pathway. Thus, it

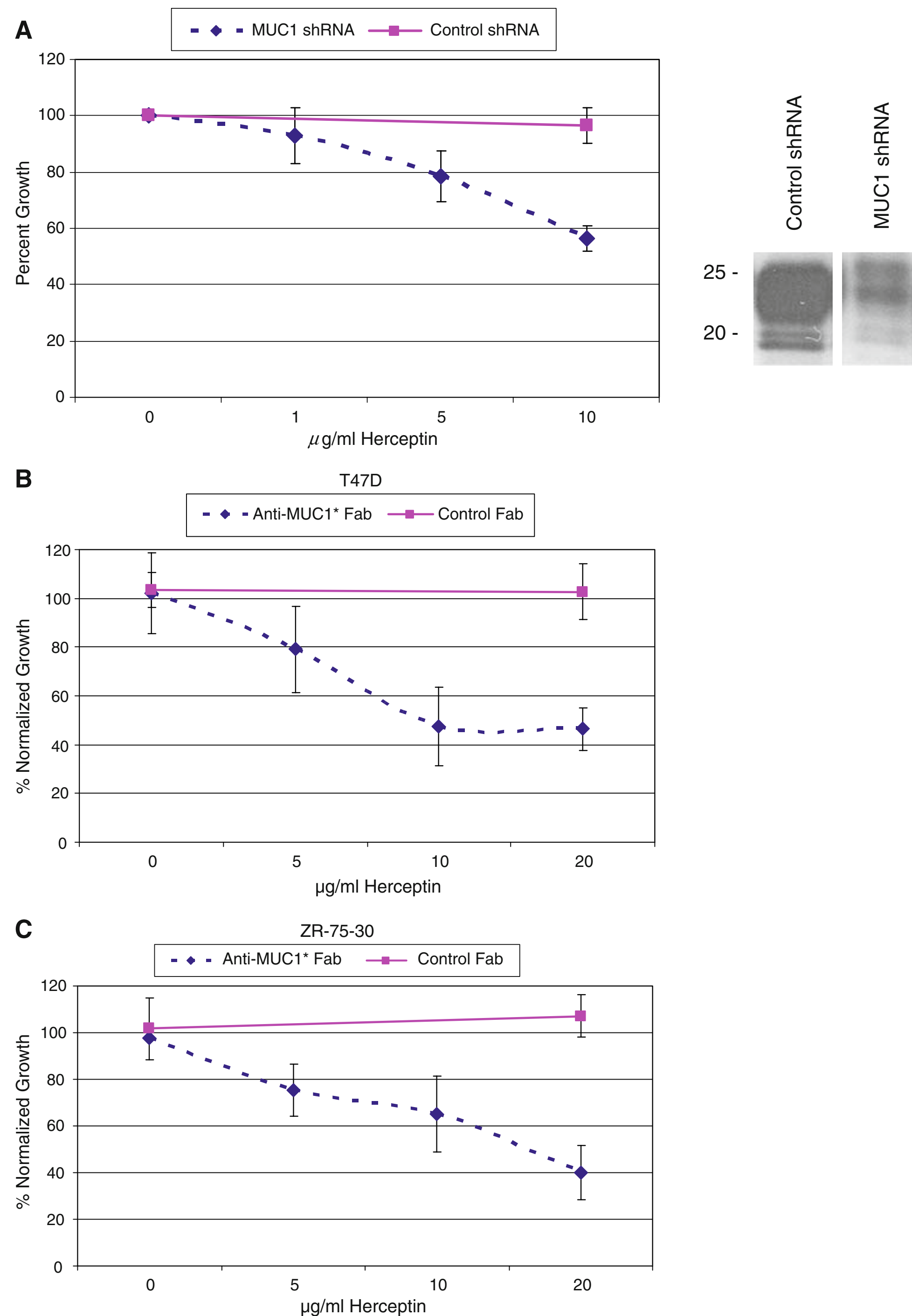

Fig. 6 Suppression of MUC1* sensitizes intrinsically Herceptin-resistant cell lines to Herceptin. **a** Downregulation of MUC1 levels by stable expression of MUC1 shRNA allows growth inhibition of T47D cells mediated by Herceptin. Growth of control shRNA-expressing T47D cells is unaffected by Herceptin treatment (*solid line*), but growth of MUC1 shRNA-expressing cells is reduced in a dose-dependent manner (*dotted line*). The addition of Anti-MUC1* Fab to either **b** T47D or **c** ZR-75-30 cells had a similar effect. In the presence of 12.5 μg/ml control Fab, growth of T47D cells was unaffected by Herceptin (*solid lines*), as determined by cell counts 3 days post-treatment, but in the presence of an identical amount of Anti-MUC1* Fab, the growth of these cells was reduced in response to Herceptin (*dotted lines*)

appears that MUC1 plays a role in cross talk between the ErbB2 and Wnt pathways and raises the possibility that the Wnt pathway could also be mobilized during Herceptin resistance.

This study provides insight into mechanisms that underlie Herceptin resistance. Our results showed that the acquisition of Herceptin resistance was accompanied by a dramatic increase in the expression of MUC1* but the resistance was reversed by treating the cells with the combination of Herceptin plus a MUC1* antagonist. These findings suggest that therapies that include a MUC1* inhibitor could rescue the large percentage of women whose breast cancers initially respond to Herceptin, but later develop resistance to the drug [35, 36]. In addition, our results imply that a much broader subset of cancers, previously thought to be resistant to Herceptin [37], could be successfully treated with Herceptin if combined with a MUC1*-targeting drug. Our results showed that two

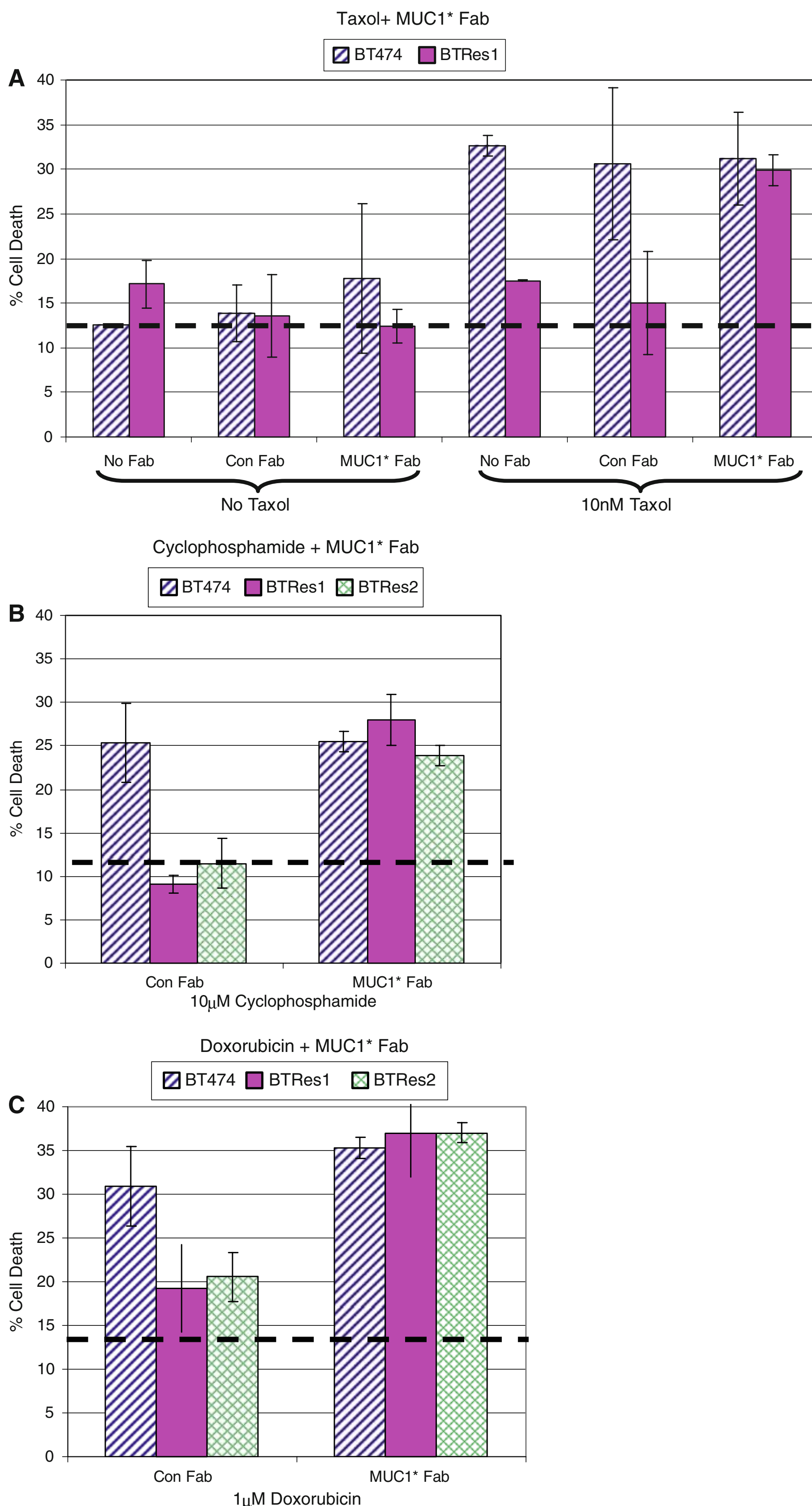

Fig. 7 Herceptin resistant cancer cells are also resistant to standard chemotherapeutic drugs, and this resistance is reversed by an Anti-MUC1* Fab. **a** Naïve BT474 cells are effectively killed by 10nM Taxol (*striped bars*), as determined 2 days post-treatment by *Trypan Blue* exclusion. However, Herceptin resistant cells, BTRes1 (*solid bars*), are essentially unaffected by Taxol when compared with the untreated cells. However, the killing effect of Taxol is restored when the resistant cells, BTRes1, are treated with Taxol and 10 μg/ml of Anti- MUC1* Fab. A control Fab, "Con Fab", at the same concentration had no effect. The *dashed line* indicates the level of cell death measured for untreated cells. **b** Naïve BT474 cells are killed by 10 μM Cyclophosphamide (*striped bars*) but BTRes1 (*solid bars*) and BTRes2 (*hatched bars*) cells are not. However, the cytotoxic effect of the drug was restored when cells were also treated with 10 μg/ml of Anti-MUC1* Fab. **c** Similar results were observed when naïve cells (*striped bars*), BTRes1 (*solid bars*) and BTRes2 cells (*hatched bars*) were treated with 1 μM Doxorubicin, in the presence of an Anti-MUC1* Fab or a control Fab

HER2-positive cell lines that were insensitive to Herceptin, became Herceptin sensitive when treated with Herceptin and a MUC1* inhibitor. The finding that MUC1* overexpression rendered cells resistant to several cytotoxic chemotherapeutic drugs, in addition to Herceptin, raises the possibility that overexpression of MUC1* may be a more generalized mechanism of acquired cancer drug resistance that could also be treated with MUC1* antagonists.

Cancer of any tissue origin is a heterogenous disease at the cellular level. Moreover cancer cells evolve both autonomously and in response to therapy. Therefore, it is to be expected that a number of different proteins will be found to play a role in the development of resistance in these cells of varied origins and evolutionary history. Among these, cell surface receptors that can transduce growth promoting signals are an important class. MUC-4, which heterodimerizes with HER2, is upregulated in some cases of Herceptin resistance [38]. The tumors of patients resistant to Herceptin have been found to overexpress IGF-1R [39] and cells in culture that were made to overexpress IGF-1R by transfection became resistant to Herceptin [40]. Later studies showed a direct interaction between IGF-1R and HER2 and IGF induced phosphorylation of HER2 [20]. Recently, the Met receptor, was shown to be upregulated in response to Herceptin, while and Met antagonists restored sensitivity [41]. Results presented here demonstrate that MUC1* is a novel determinant of Herceptin resistance. Therefore, MUC1* disabling agents have the potential to overcome Herceptin resistance, both intrinsic and acquired. Of the 1.3 million tumors, diagnosed in the US each year, over 60% show tumor-associated aberrant overexpression of MUC1 [24]. Our results suggest that the development of MUC1*-targeting molecules could lead to a promising new class of therapeutics to treat an even broader spectrum of cancers.

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