Disulfiram Treatment Facilitates Phosphoinositide 3-Kinase Inhibition in Human Breast Cancer Cells \textit{In vitro} and \textit{In vivo}

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Abstract

Frequent genetic alterations of the components in the phosphoinositide 3-kinase (PI3K)/PTEN/AKT signaling pathway contribute greatly to breast cancer initiation and progression, which makes targeting this signaling pathway a promising therapeutic strategy for breast cancer treatment. In this study, we showed that in the presence of copper (Cu), disulfiram (DSF), a clinically used antialcoholism drug, could potently inhibit breast cancer cell growth regardless of the PIK3CA status. Surprisingly, the treatment with a mixture of DSF and copper (DSF-Cu) led to the decreased expression of PTEN protein and the activation of AKT in a dose- and time-dependent manner in different cell lines with or without PIK3CA mutations. Treatment of breast cancer cell lines with a combination of DSF-Cu and LY294002, a pan-PI3K inhibitor, resulted in the significant inhibition of cell growth when compared with either drug alone. In addition, the combined treatment of DSF and LY294002 significantly inhibited the growth of the breast tumor xenograft in nude mice induced by MDA-MB-231 cells expressing mutant PIK3CA-H1047R and PIK3CA-E545K, whereas neither DSF nor LY294002 alone could significantly retard tumor growth. Finally, the observed \textit{in vivo} inhibitory effects are found associated with aberrant signaling alterations and apoptosis-inducing activities in tumor samples. Thus, our finding shows for the first time that treatment of breast cancer with DSF results in a novel feedback mechanism that activates AKT signaling. Our study also suggests that the combination of DSF and a PI3K inhibitor may offer a new combinational treatment model for breast cancer, particularly for those with PIK3CA mutations. Cancer Res; 70(10); 3996–4004. ©2010 AACR.

Introduction

The phosphoinositide 3-kinase (PI3K)/PTEN/AKT signaling pathway has been firmly established for its role in multiple cellular activities, including cell proliferation, survival, metabolism, cytoskeleton reorganization, and membrane trafficking (1–3). The abnormal activation of this signaling pathway leads to various diseases such as diabetes, autoimmunity, and cancer. PIK3CA, the gene encoding the α form of class IA PI3Ks, is amplified and overexpressed in an assortment of human cancers, including breast, ovarian, head and neck, urinary tract, cervical, and thyroid cancers (4–8). Recently, somatic mutations of PIK3CA were identi-
metastatic melanoma and refractory solid tumors involving the liver, its exact mechanism of action has not been well characterized.

In this report, we investigated the effect of DSF-Cu on the AKT signaling pathway, a well-established survival signaling pathway in breast cancer. We found that DSF-Cu treatment on several breast cancer cell lines led to the downregulation of PTEN protein expression and activation of pAKT along with the induction of cell death. This observation prompted us to test the possible synergistic effects of DSF-Cu and the PI3K inhibitor, LY294002. In these breast cancer cell lines, as loss of PTEN and activation of AKT may make cells more dependent on the PI3K/AKT pathway, and consequently more sensitive to PI3K inhibition. Indeed, our in vitro study showed that the combination of DSF-Cu and LY294002 suppressed the growth of various breast cancer cells more effectively than either treatment alone. In addition, the effect was more significant in breast cancer cells with PIK3CA mutations than in breast cancer cells with wild-type-PIK3CA. When given to mice with a human breast cancer cell line MDA-MB-231 expressing mutant PIK3CA, the combined treatment of DSF and LY294002 showed more significant tumor growth inhibition than either single treatment. Moreover, the in vivo inhibitory effects were found associated with signaling alterations, proteasome inhibition, and apoptosis activation, as shown by the accumulation of p27 and BAX proteins, poly ADP ribose polymerase (PARP) cleavage, and apoptotic nuclei formation detected by multiple assays using tumor samples. Taken together, the information from this study provides a basis for developing a promising therapeutic model in which a PI3K inhibitor and a proteasome inhibitor may be combined to effectively treat breast cancer.

Materials and Methods

Cell culture and protease inhibitor experiment. Human breast cancer cell lines MDA-MB-157, MDA-MB-231, BT20, MCF7, T47D, and BT47D were obtained from the American Tissue Culture Collection and grown at 37°C in humidified 5% CO₂ in DMEM (Invitrogen) supplemented with 10% fetal bovine serum. MDA-MB-157 and MDA-MB-231 are cells that express wild-type PIK3CA, whereas the MCF7 cell line contains a PIK3CA-E545K mutation. The T47D cell line contains a PIK3CA-H1047R mutation and the BT20 cell line contains two PIK3CA mutations (H1047R and P539R). All MDA-MB-157 and MDA-MB-231-derived cells were cultured with the same medium. The MCF10A and MCF10A-derived cell lines were cultured in serum-free medium with insulin, hydrocortisone and epidermal growth factor as described in ref. 25. Caspase-3 inhibitor III was purchased from Calbiochem. Protease inhibitors N-acetyl-L-Leucyl-L-methioninal, trans-epoxysuccinyl-L-leucylamido-(4-guanidin) butane (E64), N-ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), Np-tosyl-L-lysinechloromethyl ketone, and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Sigma. All these reagents were prepared according to the manufacturer’s instructions and were used to pretreat cells for 4 hours, followed by treatment with DSF-Cu for 6 hours. Protein lysates were then collected for the Western blot.

Construction of recombinant lentivirus and establishment of stable cells expressing mutant PIK3CA. The method was done as described (25).

Western blotting. For immunoblotting, we used the following primary antibodies: rabbit polyclonal antibodies against pAKT (Ser473), AKT, phosphorylated extracellular signal-regulated kinase (pERK), ERK, p-p70s6K, p70s6K, ubiquitin, PARP (Cell Signaling), p27 (Santa Cruz Biotechnology), and BAX (Sigma-Aldrich); and mouse monoclonal antibodies against V5 (Invitrogen), and β-actin (Sigma-Aldrich). A Western blot process was carried out as described (25).

Cell proliferation assay. Combinational treatment was performed by incubating cells with LY294002 for 2 days, adding DSF-Cu, and incubation for 1 additional day. After treatment, an MTT assay was performed as described in the protocol (Promega).

Human breast tumor xenograft experiments. Five-week-old female athymic nude mice were purchased from Taconic Research Animal Services and housed under pathogen-free conditions. Xenograft experiments were performed in compliance with the relevant laws and guidelines set forth by the Institutional Laboratory Animal Care and Use Committee of Wayne State University. MDA-MB-231/PIK3CA-H1047R and MDA-MB-231/PIK3CA-E545K cells (3 × 10⁶) were injected s.c. into one flank of each mouse. Tumor size was measured with calipers every other day. Tumor volume (V) was determined by the equation V = (L × W)²/2, in which L is the length and W is the width of the tumor. When xenografts reached volumes of ~200 mm³, the mice showing tumors were randomly assigned to a control group (n = 7), a DSF only group (n = 6), a PIK3CA inhibitor LY294002 only group (n = 6), and a combination group (DSF+LY294002; n = 6). These groups received the following treatments thrice weekly by s.c. injection on a different flank of the cell injection: solvent control (PBS/cremophor/DMEM/ethanol, 7.5:1.5:0.5:0.5), DSF (30 mg/kg), LY294002 (70 mg/kg), or DSF (30 mg/kg) plus LY294002 (70 mg/kg), respectively. The experiment was terminated and the mice were sacrificed when the control tumors reached ~1000 mm³ (on day 29). The tumors were removed and photographed, and then used for multiple assays including Western blotting and immunohistochemistry (IHC).

H&E staining, IHC, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. For H&E staining, paraffin-embedded sample slides were deparaffinized, hydrated, and then stained with hematoxylin for 1 minute. After rinsing, the slides were stained with eosin for 1 minute, rinsed, and sealed with coverslips using Permount. For immunohistochemical staining, mouse monoclonal anti-p27 antibody (1:40; Vector Laboratory) and rabbit polyclonal anti-BAX antibody (1:50; Zymed Labs, Inc.) were used. The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed using the ApopTag Peroxidase In situ Apoptosis Detection kit (Chemicon).
**Statistical analysis.** Evaluation of the synergism of two drugs DSF and LY294002 on cell proliferation for each cell line was carried out using a two-way ANOVA F test on the interaction of two factors: DSF concentration levels and presence/absence of LY294002. The evaluation of the synergism of DSF at a single concentration level and LY294002 on tumor burden in vivo was carried out using a balanced 2×2 factorial design with six replicates in each category. The drug effect of DSF + 10 μmol/L LY294002 is compared between mutated and wild-type cell lines using t test. All tests apply a significance level of 0.05 without adjustment for multiple tests. All the above-specified analyses were performed in the statistical software R.

**Results**

**The effect of DSF-Cu on breast epithelial cells with or without PIK3CA mutation.** In an effort to investigate whether DSF-Cu has different effects on breast cancer cell lines with different PIK3CA status, we treated BT20 cells (with P539R and H1047R mutations) and MDA-MB-231 cells (with wt-PIK3CA) with various doses of DSF-Cu under various time courses. There was no observable difference in the dose- or time-dependent effects of DSF-Cu treatment on proliferation in these two cell lines. In addition, DSF-Cu treatment induced significant accumulation of ubiquitinated proteins and PARP cleavage in both breast cancer cell lines (Fig. 1). Interestingly, treatment with the authentic proteasome inhibitor MG132, which was used as a control, generated a p85 PARP cleavage fragment in both cell lines, whereas DSF-Cu treatment resulted in a p85 fragment in MDA-MB-231 cells and a p65 fragment in BT20 cells (Fig. 1). Taken together, these data suggests that inhibition of cell proliferation induced by DSF-Cu is a common phenomenon in breast cancer cell lines regardless of PIK3CA status.

**DSF-Cu treatment results in PTEN protein decrease and AKT activation.** We next investigated the effect of DSF-Cu on the AKT signaling pathway, which plays a critical role in cell survival and proliferation. We first treated MDA-MB-231 cells with a mixture containing copper and either DSF, clioquinol (CQ), or tetrathiomolybdate (TM). CQ is a therapeutic agent for Alzheimer’s disease and has antiproteasomal, androgen receptor–suppressive, proapoptotic, and antitumorigenic activities in human breast and prostate cancer cells in the presence of copper (26, 27). TM is a strong...
copper chelator currently being tested in clinical trials (28, 29) and was used as a control here. To our surprise, we found that treatment with 10 or 20 μmol/L DSF-Cu or 20 μmol/L CQ-Cu mixtures could significantly inhibit PTEN protein expression, whereas TM-Cu at both concentrations had no effect (Fig. 2A).

A kinetic experiment using MDA-MB-231 cells showed a time-dependent decrease in PTEN protein expression with DSF-Cu treatment, which correlated with an initial increase in pAKT levels that decreased at later time points. The total AKT signal was not changed in this treatment (Fig. 2B). To confirm that this phenomenon was not cell line specific, we treated BT20 cells that harbor PIK3CA mutations with 10 μmol/L DSF-Cu and showed a significant time-dependent decrease in PTEN protein expression in these cells as well. Again, p-AKT was abruptly increased with DSF-Cu treatment (0.5–2 h) and then decreased to normal levels (after 2 h; Fig. 2C). A similar phenomenon was also observed with a low level of DSF-Cu treatment (0.5 μmol/L DSF) in BT20 cells, although the decrease of PTEN protein expression was postponed until after 24 hours of treatment and pAKT activation did not last longer than 24 hours (Fig. 2D).

We next sought to explore the mechanism underlying the decrease in expression of PTEN upon the treatment of DSF-Cu. Reverse transcription-PCR analysis showed no significant change in PTEN mRNA level after DSF-Cu treatment in cultured cells. As shown in Fig. 3B, decreased PTEN protein expression induced by DSF-Cu treatment was attenuated in BT20 cells pretreated with two cysteine protease inhibitors NEM and TPCK.

However, when MDA-MB-231 cells were tested, three protease inhibitors, E64, NEM, and PMSF, showed effect in blocking PTEN degradation induced by DSF-Cu (Fig. 3C). In contrast, as shown in Fig. 3D, a caspase-3 inhibitor could not rescue the PTEN degradation in BT20 cells treated with DSF-Cu. Therefore, caspase-3 does not play a critical role in PTEN degradation in DSF-Cu–treated cells.

To investigate whether other authentic proteasome inhibitors could also cause a decrease in PTEN protein expression, we used MG132 and PS341, two well-recognized proteasome inhibitors, on breast cancer cell lines with different dose and time courses. The result showed that both MG132 and PS341 treatments led to the activation of pAKT and apparent decrease of PTEN protein in BT20 cells. The effects were less apparent in MDA-MB-231 cells (Supplementary Data S1). These data suggest that AKT activation and PTEN protein decrease may be common to all the proteasome inhibitors.

**Combined treatment of LY294002 and DSF-Cu inhibits cell growth in vitro.** AKT is a major cell survival molecule and activation of pAKT is a common cellular response to proapoptotic signaling. PTEN antagonizes PI3K and negatively regulates the AKT signaling pathway (30–32). Suppression of PTEN expression in breast cancer cells leads to AKT activation and confers sensitivity to inhibitors of mammalian target of rapamycin and AKT (33). In addition, breast cancer cell lines with PIK3CA mutation are more sensitive to PI3K inhibitor than cells with wt-PIK3CA, as evidenced by several reports including our own using newly established cell lines (Supplementary Data S2–4).

Considering that DSF-Cu treatment leads to multiple biological effects such as decreasing PTEN and activating AKT, along with its cell death induction function, we investigated whether the combined treatment of DSF-Cu with LY294002 would show an additive or a synergistic effect in killing breast cancer.
cancer cells and also whether this effect would become more significant in cells with PIK3CA mutation. First, we performed in vitro cell proliferation assays on BT20, MCF7, T47D, and MDA-MB-231, four cell lines with different PIK3CA mutation statuses. The combination of DSF-Cu and LY294002 treatment led to a more significant decrease in cell proliferation than each single treatment alone in all four cell lines (Fig. 4A). Specifically, the effect in BT20 cells was more significant than in MDA-MB-231 cells at three concentrations ($P = 0.007, 0.001, \text{and} <0.001$). MCF7 and T47D cells had very similar effect in response to the combinational treatment (Supplementary Data S5). Statistical analysis indicated that synergism of the two drug treatments was found in all four cell lines (Supplementary Data S5; Fig. 4B). In addition, we performed a similar treatment on a set of isogenic cell models: MDA-MB-231/vector cells, MDA-MB-231/PIK3CA-H1047R, and MDA-MB-231/PIK3CA-E545K (Fig. 4C). We found that combined treatment led to increased proliferation inhibition compared with each single treatment alone in all three cell lines. The effect was more significant in MDA-MB-231/PIK3CA-H1047R and MDA-MB-231/PIK3CA-E545K cells than in MDA-MB-231/vector cells with $10 \mu$mol/L LY294002 plus $1 \mu$mol/L DSF-Cu (Fig. 4C), although no significant synergism was found with indicated treatment conditions (Fig. 4D). Moreover, the effect of MDA-MB-231/PIK3CA-H1047R and MDA-MB-231/PIK3CA-E545K cells responding...
to the combinational treatment is similar (Fig. 4C). We also observed similar results in MDA-MB-157 and MCF10A-derived model cells (Supplementary Data S6).

Combined treatment of LY294002 and DSF synergistically inhibits tumorigenesis of breast cancer cells in vivo. To further test our hypothesis in vivo, four different treatments were delivered to a xenograft mouse model created by s.c. injection with MDA-MB-231/PIK3CA H1047R cells. We found that combined treatment, as well as treatment with DSF or LY294002 alone, inhibited tumor growth by 74.5% \( (P = 0.02) \), 35% \( (P = 0.18) \), and 27% \( (P = 0.21) \), respectively, compared with the solvent control (Fig. 5A). Furthermore, tumor burden in the DSF and LY294002 single-treatment groups was reduced by 25% \( (P = 0.3) \) and 16.7% \( (P = 0.4) \), respectively, whereas in the combined treatment group, tumor burden was reduced by 58.3% \( (P = 0.02) \), compared with the control group (Fig. 5B). Two-way ANOVA test was performed and showed synergism of the two drugs DSF and LY294002 on tumor burden in vivo \( (P = 0.002) \); Supplementary Data S7). Similarly, in contrast to either single treatment, only the combined treatment could significantly retard the growth of the xenograft tumor induced by the MDA-MB-231/PIK3CA-E545K cells (Supplementary Data S8). Taken together, the in vivo data are consistent with in vitro data and support the hypothesis that the combined treatment of DSF and LY294002 has a more significant effect in targeting breast cancer cells. In addition, the mutation in the helical and kinase domain had no significant difference in response to the combined treatment.

To further investigate whether the observed inhibitory effects of the combined treatments and single treatments are associated with signaling alterations or apoptosis induction, tumor samples from xenograft mice were subjected to Western blotting and IHC assays. Western blots using the tumor extracts showed that DSF treatment slightly increased pAKT, whereas LY294002 treatment decreased pAKT and the combined treatment led to a relatively low-level of pAKT compared with control. No significant changes in p70S6K or p-p70 S6K were observed (Fig. 5C). In addition, combined treatment led to significant PARP cleavage compared with single treatment alone or control. Subtle accumulation of ubiquitinated protein was also observed in DSF-, LY294002-, and combination-treated tumors (Fig. 5D). An increase in the levels of the proteasome-targeting proteins p27 and BAX were also observed in the combination-treated sample (Fig. 5D) and confirmed by IHC (Fig. 6A), indicating proteasomal inhibition in these tumor samples. Moreover, apoptosis in tissues derived from xenografts that received the combined treatment occurred about thrice more frequently than in tissues derived from xenografts treated with DSF or LY294002 alone as shown by the TUNEL assay (Fig. 6A; positive control was shown in Supplementary Data S9). Apoptosis induction in tumor samples was further observed by the H&E staining results. In contrast, apoptosis occurrence was not apparent in
the control xenograft tumors (Fig. 6A). Overall, the combined treatment of DSF and LY294002 showed significant inhibition of breast tumor growth in nude mice, and this inhibition was associated with aberrant signaling alterations, proteasome inhibition, and induction of apoptosis.

Discussion

Although DSF is clinically used as an antialcoholism drug, our group, along with several others, reported that DSF could be used as an anticancer drug (22). One possible mechanism is that DSF may interact with elevated levels of endogenous copper in tumor tissues (34–36) and form DSF-Cu complexes with active antitumor properties, specifically proteasomal inhibition and apoptotic induction. Consistent with previous observations, our current study found that DSF treatment induced an accumulation of many ubiquitinated proteins in different cell lines regardless of the \( \text{PIK3CA} \) status. We also showed that a low dose of DSF-Cu could induce the accumulation of ubiquitinated proteins and activate AKT, although it did not significantly inhibit tumor growth \textit{in vivo}. This observation prompted us to speculate that a combination of low-dose DSF with reagents harboring different mechanisms could result in high therapeutic effects and low toxicity \textit{in vivo}.

In this study, we showed that DSF-Cu treatment decreased the expression of PTEN protein (but not RNA) levels in different breast cancer cell lines in a time- and dose-dependent manner. Therefore, inhibition of protein synthesis and/or increase of protein degradation could be the mechanisms underlying this serendipitous observation. However, because decreased expression of PTEN protein upon DSF-Cu treatment was observed in the very early hours, we thus focused on the protein degradation mechanism. Our study showed that different protease inhibitors could reverse the PTEN degradation induced by DSF-Cu in two different cell lines and the cysteine protease inhibitor NEM commonly works in two different cell lines. This result not only confirmed
our hypothesis but also is consistent with several previous reports that protease expression could be regulated by a feedback mechanism in response to some proteasome inhibitors (37, 38). Therefore, in our study, the DSF treatment might lead to the upregulation of some protease activity, which in turn may lead to the degradation of the PTEN protein.

In the past several years, numerous studies have identified a high frequency of PIK3CA somatic mutations in cancer cell lines and tumor samples, and have clarified the oncogenic properties of mutant PIK3CA in cancer tumorigenesis (39, 40). More importantly, 80% of the PIK3CA somatic mutations were identified in two hotspots, which are separately located in the helical and kinase domains of the PIK3CA molecule. Several recent studies suggest that the mutations in the helical and kinase domains trigger a gain of function through different mechanisms and speculate the contrasting roles of p85 and Ras-GTP in these two mutations (41–43). In addition, these two mutations were also reported to be associated with different prognoses for disease-free survival (44). However, the results of several previous studies using different mutant model cell lines indicated that H1047R and E545K, two mutations located separately in the kinase and helical domains, have very similar response to a pan-PI3K inhibitor LY294002 (13, 25, 45). The in vitro and in vivo assay results of the current study further support this notion, suggesting that the inhibition of the ATP binding site of PI3Ks is a feasible strategy in targeting cancer cells with different PIK3CA mutations.

Due to the structural similarity between the isoforms of PI3Ks, a specific inhibitor targeting PIK3CA is still unavailable and, as a result, most developed PI3K inhibitors are pan-PI3K inhibitors. Meanwhile, accumulating evidence has shown that various other PI3K isoforms (e.g., β, δ, and γ) are also involved in tumorigenesis (46–49). Therefore, a pan-PI3K isoform inhibitor may offer enhanced therapeutic benefits compared with an isoform-specific inhibitor. This notion has been further supported by a recent report that shows that although p110α activation is required to sustain the proliferation of established PIK3CA-mutant tumors, PTEN-deficient tumors depend instead on p110β signaling (50). In this regard, a pan-PI3K inhibitor is expected to have the broadest clinical application, as it will target the growth of both PIK3CA mutations and PTEN-null cancers. In our current study, DSF treatment led to decreased PTEN protein and activation of AKT in both cell types with and without PIK3CA mutations. Therefore, we expect that the combination of a pan-PI3K inhibitor with DSF would be more effective than an isoform-specific inhibitor.

Many newly identified drugs tested in research or preclinical settings are common apoptosis inducers and the mechanisms of these drugs on cells are not entirely clarified. This drawback limits their transition from research to clinical applications. In our current study, we show for the first time that DSF-Cu treatment on breast cancer cell lines leads to a decrease in the PTEN protein level and an increase in the pAKT level. Both changes are believed to ultimately lead to the activation of the AKT signaling pathway. Therefore, DSF seems to have two mechanisms of action. On one hand, DSF-Cu inhibits the proteasome, which in turn triggers the death pathway and induces apoptosis. On the other hand, DSF-Cu also activates the PI3K/PTEN/AKT survival signaling pathway (Fig. 6B). Consistent with this observation, our study showed that the PI3K inhibitor, LY294002, combined with DSF treatment, not only effectively inhibited cell proliferation in vitro, but also significantly decreased the tumor formation in vivo. Taken together, our current observations reinforce the notion that effective therapy could result from combinations of therapeutic agents with different functional mechanisms. More significantly, our results suggest that a potential proteasome inhibitor and a pan PI3K inhibitor could be a novel and promising combined treatment option for breast cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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