Activating Transcription Factor 3 as a Novel Regulator of Chemotherapy Response in Breast Cancer[1,2]

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Abstract

Anthracyclines, such as doxorubicin, are used as first-line chemotherapeutics, usually in combination therapies, for the treatment of advanced breast cancer. While these drugs have been successful therapeutic options, their use is limited due to serious drug related toxicities and acquired tumor resistance. Uncovering the molecular mechanisms that mediate doxorubicin's cytotoxic effect will lead to the identification of novel more efficacious combination therapies and allow for reduced doses of doxorubicin to be administered while maintaining efficacy. In our study, we demonstrate that activating transcription factor (ATF) 3 expression was upregulated by doxorubicin treatment in a representative panel of human breast cancer cell lines MCF7 and MDA-MB-231. We have also shown that doxorubicin treatment can induce ATF3 expression in ex vivo human breast and ovarian tumor samples. The upregulation of ATF3 in the cell lines was regulated by multiple cellular mechanisms including the activation of JNK and ATM signaling pathways. Importantly, loss of ATF3 expression resulted in reduced sensitivity to doxorubicin treatment in mouse embryonic fibroblasts. Through a 1200 FDA-approved compound library screen, we identified a number of agents whose cytotoxicity is dependent on ATF3 expression that also enhanced doxorubicin induced cytotoxicity. For example, the combination of the HDAC inhibitor vorinostat or the nucleoside analogue trifluridine could synergistically enhance doxorubicin cytotoxicity in the MCF7 cell line. Synergy in cell lines with the combination of ATF3 inducers and patients with elevated basal levels of ATF3 shows enhanced response to chemotherapy. Taken together, our results demonstrate a role for ATF3 in mediating doxorubicin cytotoxicity and provide rationale for the combination of ATF3-inducing agents with doxorubicin as a novel therapeutic approach.

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Introduction

Breast cancer is the most frequently diagnosed cancer among North American women [1, 2]. Although significant advances have been made in the ability to detect and treat this disease, there remains a poor prognosis for patients who recur with advanced metastatic disease (5-year overall survival of 26%) [3]. Treatment of advanced breast cancer heavily relies upon the utilization of chemotherapeutics, with anthracyclines, such as doxorubicin, being a widely employed class of drugs that represents an important therapeutic option for

these patients [4]. Doxorubicin cytotoxicity is primarily a result of its ability to induce DNA damage, which occurs through the inhibition of topoisomerase IIα resulting in DNA strand breaks, but the downstream mechanisms and cellular pathways responsible for doxorubicin-induced tumor cell death are not well characterized [5]. The overall response rate to doxorubicin treatment in patients with breast cancer is approximately 30%-50% [6]. Acquired resistance and the significant toxicities and side effects associated with doxorubicin treatment, particularly cardiotoxicity, has limited its effectiveness in the clinic [4].

Understanding the molecular mechanisms involved in mediating doxorubicin cytotoxicity may uncover novel therapeutic strategies for the treatment of breast cancer and present novel approaches to overcome these clinical barriers for more efficacious treatment. Doxorubicin is most commonly employed as part of a combination therapy with other chemotherapeutics, such as paclitaxel, docetaxel, cyclophosphamide, and 5-fluorouracil [7]. These combination strategies were developed empirically through combining agents that had demonstrated single agent activity to enhance their efficacies. This strategy has likely reached a therapeutic plateau, and more rational combination therapeutic strategies are urgently required. Improving the efficacy of doxorubicin treatment may be achieved through the identification of the cellular mechanisms regulating doxorubicin cytotoxicity and uncovering novel therapeutic targets. Furthermore, these targets may allow for lower doses of doxorubicin to be administered, maintaining clinical benefit but reducing their associated toxicities.

Activating transcription factor 3 (ATF3), a member of the ATF/ CREB family of transcription factors, is an adaptive responsive gene that is upregulated following a wide range of intra- and extracellular stresses including DNA-damage response [8]. ATF3 homo- or heterodimerizes with other ATF/CREB members to activate or repress transcription and by doing so has been demonstrated to play dual roles in mediating cellular stress response. ATF3 functions by upregulating genes involved in alleviating cellular stress; however, when the stress cannot be overcome, enhanced and sustained expression of ATF3 promotes apoptosis [9–11]. Apoptosis can be initiated through the upregulation of the downstream target of ATF3, DDIT3 (CHOP/GADD153), which upregulates proapoptotic proteins [12–14]. Multiple signaling pathways have been demonstrated to regulate ATF3 expression, including the DNA-damage response (DDR), integrated stress response (ISR), and MAPK signaling pathways [15–17]. Upregulation of ATF3 by all three of these pathways can result in apoptosis.

ATF3 has recently been demonstrated by our group to mediate cisplatin cytotoxicity in non–small cell lung carcinomas (NSCLCs) [18]. Inability to induce ATF3 expression following cisplatin treatment was associated with cisplatin resistance, highlighting its role in regulating its cytotoxicity. We further demonstrated that the combination of other ATF3 inducers with cisplatin enhanced both ATF3 expression and tumor cell cytotoxicity. These results suggest the potential of combining ATF3 inducers as a novel therapeutic strategy. In breast cancer pathology and its treatment, the role of ATF3 remains poorly studied; however, initial data demonstrating survival and apoptotic functions of ATF3 in breast cancer cell lines have been reported [11, 19–21]. A limited number of previous studies have also demonstrated the ability of topoisomerase inhibitors to induce ATF3 expression [22–24], although the significance of ATF3 in doxorubicin-induced tumor cytotoxicity has not been elucidated. In the present study, we aim to delineate the role of ATF3 in mediating doxorubicin

cytotoxicity in order to establish its potential as a therapeutic target. We also evaluated the potential of novel inducers of ATF3 to enhance doxorubicin-induced tumor cell cytotoxicity.

Materials and Methods

Tissue Culture
Human tumor-derived MCF7 and MDA-MB-231 cell lines were obtained from the ATCC (Rockville, MD, USA). The ATF3$^{-/-}$ knockout and paired wild-type counterpart murine embryonic fibroblasts (MEFs) were kindly provided by Dr. T. Hai, (Ohio State University, Columbus, OH). MCF7 and MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM). MDA-MB-231 cells were maintained in low-glucose DMEM (Mediatech, Manassas, VA). All media was supplemented with 10% fetal bovine serum (Medicorp, Montreal, QC, Canada) and 1% penicillin/streptomycin (ThermoFisher Scientific). Frozen aliquots of the cell lines were established upon acquisition and all experimental cells were passaged for fewer than 30 passages. MCF7 and MDAMB231 cell lines were authenticated by STR profiling (The Centre for Applied Genomics, Toronto, ON) and mycoplasma testing was performed through Hoescht staining approximately every 6 months.

Doxorubicin was provided by the pharmacy at the Ottawa Hospital Cancer Centre, Ottawa. Chemical inhibitors for JNK (SP600125), ERK (U0126), p38 (SB203580), and ATM (KU55933) were purchased from Selleck Chem (Houston, TX). Vorinostat was purchased from Calbiochem (Gibbstown, NJ), and trifluridine from Sigma-Aldrich (St. Louis, MO).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay
In 96-well flat bottom plates (Costar, Corning, NY), cells were seeded at a density of 5000 cells/50ul. Cells were incubated overnight and the next day drug treatments were administered up to a final volume of 100ul. For MTT analysis, 42 μl of a 5-mg/ml solution of MTT tetrazolium substrate (Sigma) in phosphate-buffered saline was added and incubated for up to 2 hours at 37 °C. The resulting violet formazan precipitate was solubilized by the addition of 84 μl of 0.01 M HCl in 10% SDS (Sigma-Aldrich, St. Louis, MO) solution at 37 °C overnight. Plates were analyzed on a microplate reader at 570 nm (Synergy Mx Monochromator-Based Multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT).

Western Blot Analysis
Cells were plated at 0.8×10^6/60-mm dish and incubated overnight followed by drug treatment with the indicated drug for 24, 48, or 72 hours. Protein samples were collected in RIPA buffer (50 mM Tris-CL pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1% Triton-X-100, 0.25% sodium deoxycholate, 0.1% SDS) containing the protease inhibitors 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerolphosphate, and 1× Protease Inhibitor Cocktail (Sigma). Protein concentrations were determined using the BCA protein quantification assay (Thermo) following manufacturer's instructions. Western blots were performed as previously described [25]. Antibodies specific for ATF3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Actin from Sigma; ERK, phospho-ERK (Tyr204), Jun, phospho-Jun (Ser73), Hsp27, and phospho-hsp27 (Ser78), PARP from Cell

Signaling Technology (Beverly, MA); and γH2A.X (Ser139) from Millipore. Visualization of protein bands was performed using the Clarity Western ECL substrate (BioRad) and developed using the Syngene Bio-Imaging System (Syngene, Frederick, MD).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (Q-RT-PCR)

Cells plated at 0.6×10^6 cells per 6-cm dish were incubated at 37 °C overnight and then treated with doxorubicin for 24 hours. Total
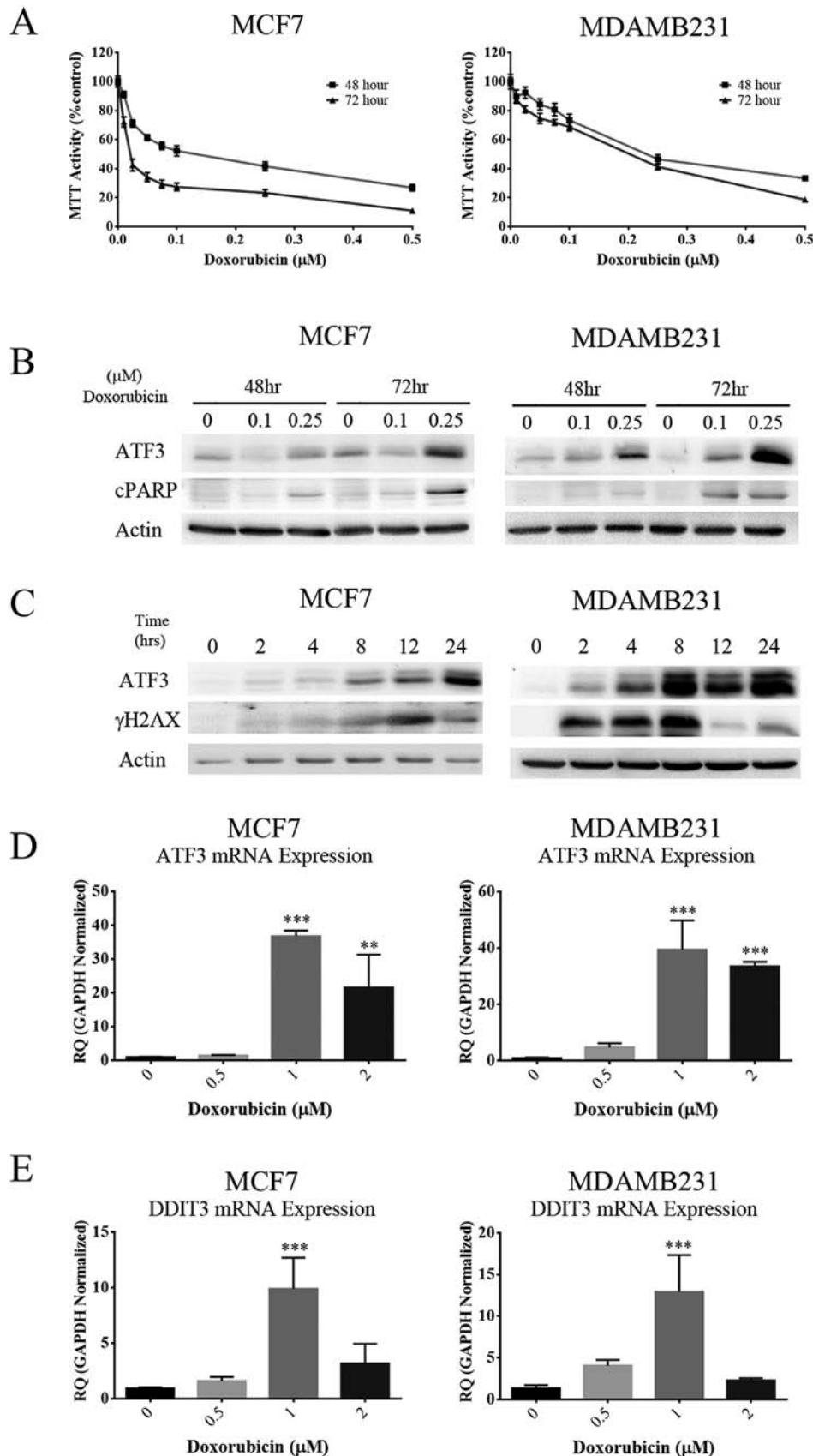
RNA was extracted from cell samples using Trizol (Thermo Scientific). RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). One microgram of total RNA was reverse-transcribed to complementary DNA for RT-qPCR as previously described [26]. The Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to detect amplification. Real-time PCR was performed using Taq Man Gene Expression Assay Primer/Probes for ATF3 (HS00231069), DDIT3 (HS00358796) and the housekeeping gene human GAPDH (HS4333764-F) as per manufacturer's instructions (Applied Biosystems). Three independent experiments were performed to determine the average gene expression and standard deviation.

Ex Vivo Tissue Culture

Patient tumor samples were obtained following surgical resection (Ottawa Hospital Research Ethics Board; Protocol # 20120559-01H). Tumor tissue was processed using a sterile 2-mm biopsy punch followed by slicing with a scalpel to obtain cores that were approximately $2 \times 2 \times 1$ mm in size. Cores were randomized and distributed 3 per well in a 24-well plate containing DMEM supplemented with 10% fetal bovine serum and 100 U/ml antibiotic/antimycotic (Sigma). Cores were drug-treated with doxorubicin at 1 and 10 μM for 48 hours. When a sufficient amount of tissue was obtained, cores were also treated with 5 μM doxorubicin. Following treatment, cores were collected for RNA processing, and RT-qPCR for ATF3, DDIT3, and GAPDH mRNA expression was performed.

High-Throughput Chemical Library Screen

A chemical library of 1200 FDA-approved compounds (Prestwick Chemical, Illkirch, France) was used to treat wild-type and ATF3$^{-/-}$ MEFs. All compounds were provided at an initial stock concentration of 10 mM in DMSO and were used at a final concentration of 5 μM. Cytotoxicity to the test compounds was assessed by MTT assay following 48-hour treatment. A difference in response between the wild-type and ATF3$^{-/-}$ MEFs of greater than 20% was considered a "hit." A second screen was carried out using the MCF7 cell line, where cells were pretreated with 1 μM of the library compound for 24 hours followed by 48-hour treatment with or without 10 nM doxorubicin. Cytotoxicity was evaluated for the library compounds alone, doxorubicin alone, and each combination. A difference of greater than 20% in the combination compared to the library compound and doxorubicin alone was considered a "hit."

Statistical Analyses

Significance between columns for RT-qPCR data was determined by one-way ANOVA using Bonferroni multiple-comparison test. For MTT viability curves, significance was determined by two-way ANOVA employing a Bonferroni multiple-comparison test. For all tests, the criterion for significance was $P < .05$. The combination effect of doxorubicin with vorinostat or trifluridine was calculated using CalcuSyn (Biosoft, Cambridge, UK). Combination index (CI) values were graphed on fraction affected-CI (Fa-CI) plots. A CI < 1 is a synergistic interaction, CI = 1 is additive, and CI > 1 is antagonistic.

Results

Doxorubicin Induces ATF3 Expression in Human Breast Cancer

The role of ATF3 as a potential mediator of doxorubicin's cytotoxicity has not been well defined. In this study, we first investigated the ability of doxorubicin to induce ATF3 expression in human breast cancer cell lines representing two common molecular subtypes based on receptor status of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The luminal breast cancer cell line MCF7 (ER$^+$ PR$^+$ Her2$^+$) and the triple-negative MDA-MB-231 (ER$^-$ PR$^-$ Her2$^-$) were employed in this study. Luminal breast cancers account for approximately 70% of all invasive breast cancers and are considered to be responsive to treatment with chemotherapy [27]. Triple-negative breast cancers account for approximately 15% of invasive breast tumors and often have varying response to treatment with chemotherapy, including doxorubicin [27–29]. The sensitivity of these cell lines to doxorubicin treatment was determined by MTT cell viability assay (Figure 1A). The triple-negative MDA-MB-231 cells were the least sensitive (IC50 of MCF7 = 121.3 nM and MDA-MB-231 = 287.7 nM) to doxorubicin as expected.

We next evaluated the ability of doxorubicin to induce ATF3 expression employing low- (0.1 and 0.25 μM) and high-dose doxorubicin (1 μM) treatment. Treatment with low-dose doxorubicin for 48 and 72 hours and high-dose doxorubicin for 24 hours resulted in induced ATF3 expression (Figure 1, B-D) that was accompanied by an increase in the levels of cleaved PARP, a marker of cellular apoptosis (Figure 1B). Doxorubicin-induced ATF3 expression occurred in a time- and dose-dependent manner with high-dose treatment and was preceded by the accumulation of double-strand DNA breaks, as measured by the expression of γH2AX (Figure 1C). Maximal expression of γH2AX was observed at 8 hours in the MDA-MB-231 and 12 hours in the MCF7, while maximal ATF3 expression was observed at 24 hours (Figure 1C). RT-qPCR demonstrated that high-dose doxorubicin treatment upregulates transcription of ATF3 and its downstream apoptotic effector DDIT3 (Figure 1, D and E). These findings suggest that doxorubicin-induced DNA damage results in the activation of a DNA-damage response involving the upregulation of ATF3.

Patient tumor samples were also used to evaluate ATF3 induction by doxorubicin in a more clinically relevant model. Tumor samples from patients undergoing surgery for breast or ovarian cancer were evaluated. Tissue was processed and treated with doxorubicin for 48 hours. Three breast tumors and two ovarian specimens were tested. In two of three breast and one of two ovarian tumors, we observed an induction of

Figure 1. (A) MTT analysis demonstrating the sensitivity of the human breast cancer cell lines MCF7 and MDA-MB-231 to doxorubicin following 48- and 72-hour treatment. Error bars, SD from the mean of three replicates ($n = 3$). (B) Western blots for ATF3 expression in the breast cancer cell lines following 48- and 72-hour treatment with doxorubicin. Upregulation of the expression of cleaved-PARP was observed to occur alongside the induction of ATF3 expression by doxorubicin treatment. (C) Western blots demonstrating the upregulation of ATF3 by doxorubicin treatment over a 24-hour time course in both cell lines. Doxorubicin-induced DNA DSBs were assessed through the expression of γH2AX. (D and E) RT-qPCR for ATF3 and DDIT3 expression following 24-hour treatment with doxorubicin. Statistical analysis was performed by one-way ANOVA using the Bonferroni multiple-comparison test. **$P \leq .01$, ***$P \leq .001$.
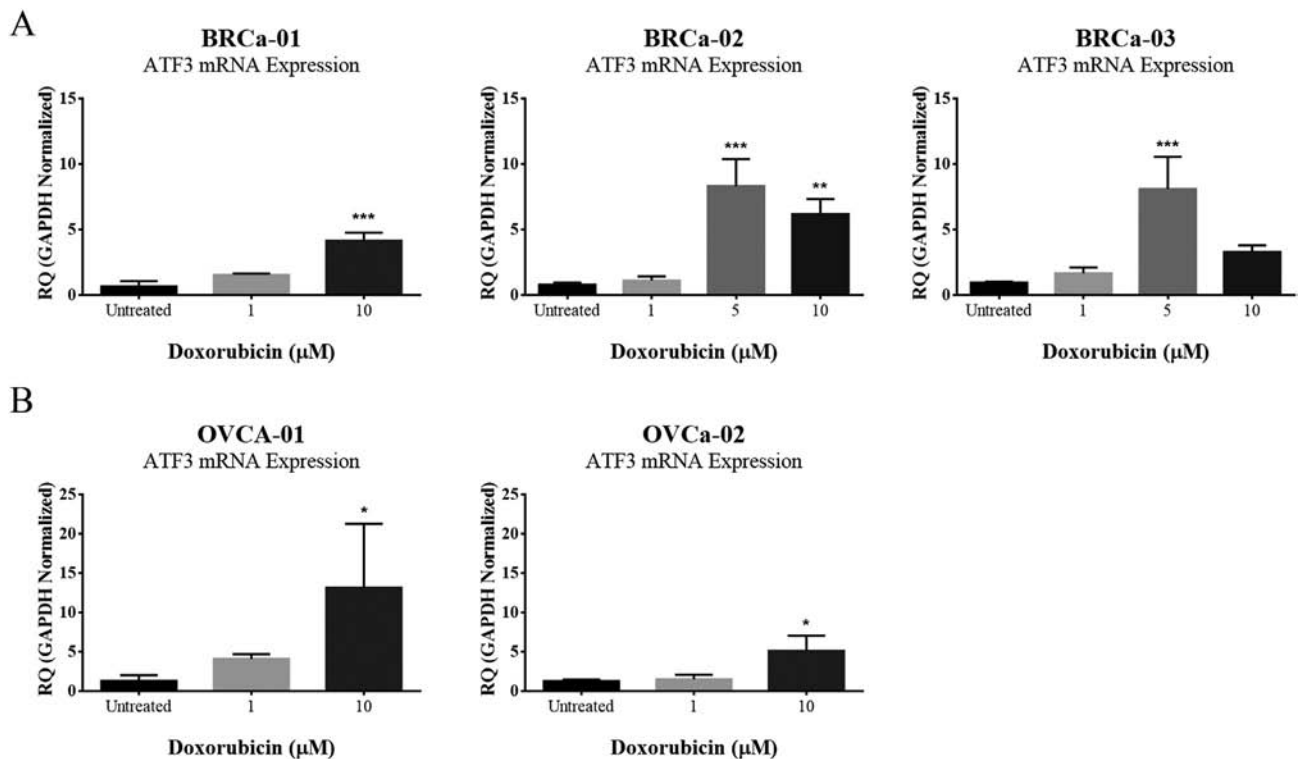
Figure 2. Evaluation of doxorubicin-induced ATF3 expression in human tumor specimens. RT-qPCR for ATF3 expression in (A) three breast and (B) two ovarian tumors treated with doxorubicin for 48 hours. Doxorubicin-induced ATF3 expression of greater than five-fold was observed in two of three breast and one of two ovarian tumors evaluated. Error bars represent standard deviation of three technical replicates from the same patient sample. Statistical analysis was performed by one-way ANOVA using the Bonferroni multiple-comparison test. $*P \le .05$, $**P \le .01$, $***P \le .001$.

ATF3 expression of over five-fold, which was considered to be a significant induction and in agreement with our *in vitro* cell line data (Figure 2). This is the first report that doxorubicin can induce ATF3 expression directly in human tumor tissue.

ATF3: A Novel Mediator of Doxorubicin Cytotoxicity

Stress-induced ATF3 regulates cell apoptosis when its levels are elevated and sustained [17]. We therefore investigated the role of ATF3 in mediating the cytotoxicity of doxorubicin. In order to determine the importance of ATF3 in mediating doxorubicin cytotoxicity, we employed an ATF3 knockout MEF model (ATF3$^{-/-}$). Loss of ATF3 expression in the MEFs resulted in a drastic reduction in sensitivity to doxorubicin treatment compared to the paired wild-type cells (Figure 3A). To ensure that the reduction in sensitivity was in fact attributed to the loss of ATF3 and not a due to overall impaired cell death pathways, we treated the MEFs with docetaxel, which does not induce ATF3, and observed similar cytotoxicity in both the wild-type and knockout cells (data not shown). Cleavage of PARP was clearly evident following doxorubicin treatment in the wild-type MEFs but not in the ATF3$^{-/-}$ MEFs, indicating elevated apoptosis in wild-type MEFs compared to their ATF3–/– counterparts (Figure 3B). Employing the CRISPR/cas9 gene editing methodology, we targeted the ATF3 gene in the MCF7 cell line. Loss of ATF3 in the MCF7 cells resulted in an inhibition of doxorubicin cytotoxicity and apoptosis through reduction in the cleavage of PARP in the knockout cells (Figure 3, *D* and *E*). This inhibition was not as pronounced as in the MEFs likely due to the dysregulation of a variety of cellular signaling pathways inherent in MCF7 transformed cells.

Doxorubicin-Induced ATF3: Activation of the ISR, DDR, and MAPK Signaling

Understanding the cellular mechanisms involved in regulating doxorubicin-induced ATF3 expression may identify new therapeutic targets that can be exploited to enhance doxorubicin efficacy. Doxorubicin treatment results in various cellular stresses which can result in the activation of multiple stress signaling pathways. Upregulation of ATF3 expression is regulated through several signaling pathways following different cellular stresses. The most common pathways include the integrated stress response, DNA-damage response, and MAPK signaling pathways (Figure 3A) [15–17]. The proapoptotic role of ATF3 has been best characterized through its role in the ISR. During the ISR, endoplasmic reticulum stress results in the phosphorylation of eIF2a which upregulates the expression of ATF4 and ATF3. Employing ATF4$^{-/-}$ MEFs and their paired wild-type counterparts, we demonstrated that loss of ATF4 results in attenuated ATF3 expression following doxorubicin treatment (Figure 3C). Additionally, loss of ATF4 resulted in a significant reduction in sensitivity to doxorubicin treatment in the MEFs (Figure 3A). The ability of doxorubicin to initiate the ISR may be in part through the generation of ROS which have been demonstrated to induce endoplasmic reticulum stress. Pretreatment of the breast cancer cell lines with n-acetylcysteine followed by doxorubicin treatment reduced the induction of ATF3 expression (Figure 4B).
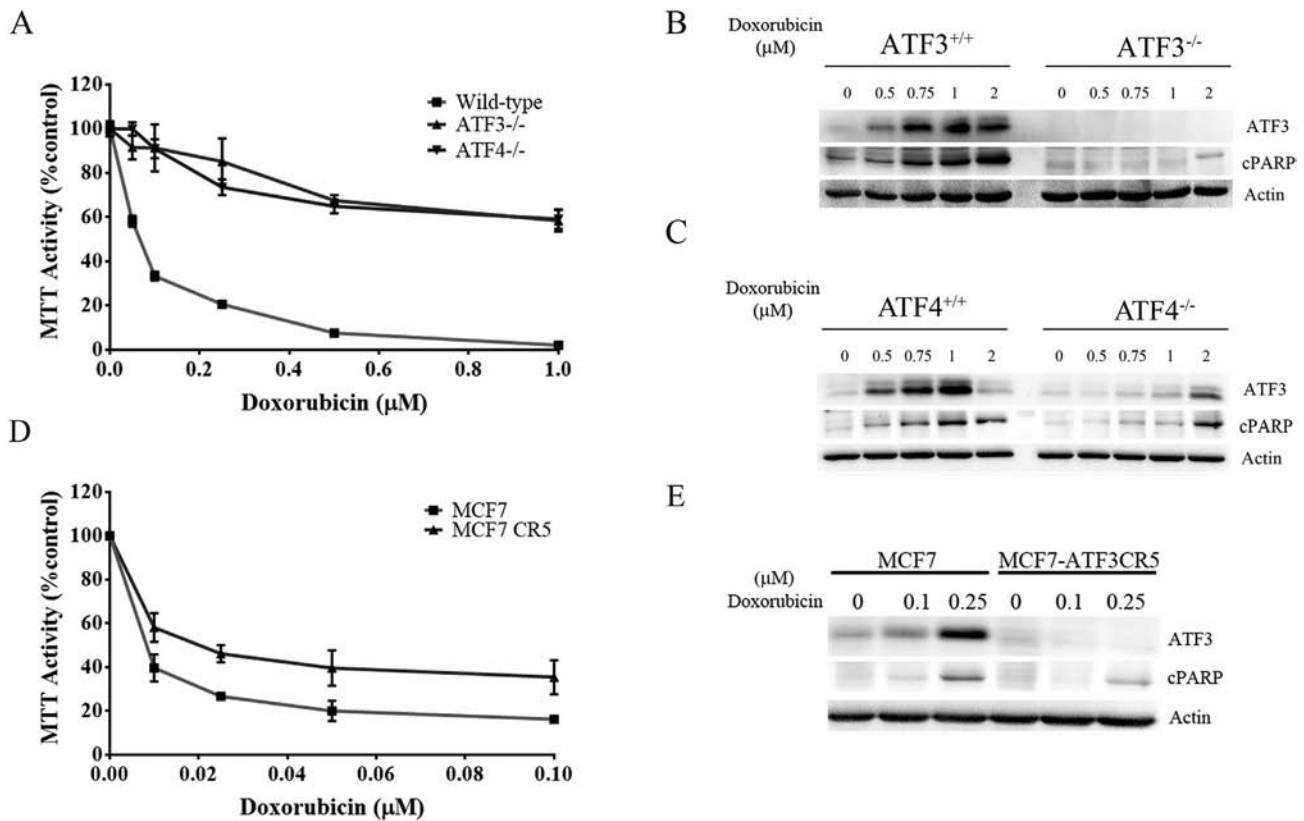
Figure 3. (A) MTT analysis in wild-type, ATF3$^{-/-}$, and ATF4$^{-/-}$ MEFs treated with doxorubicin for 48 hours. As observed, loss of ATF3 resulted in a significant reduction in sensitivity to doxorubicin treatment. Error bars, SD from the mean of three replicates ($n = 3$). (B) Western blot analysis for wild-type and ATF3$^{-/-}$ MEFs treated with various concentrations of doxorubicin for 24 hours. (C) Western blot analysis for wild-type and ATF4$^{-/-}$ MEFs treated with various concentrations of doxorubicin for 24 hours. (D) MTT analysis in MCF7 and MCF7 ATF3 CRISPR knockout cells treated with doxorubicin for 72 hours. Error bars, SD from the mean of three replicates ($n = 3$). (E) Western blot analysis for MCF7 and ATF3 CRISPR knockout cells treated with doxorubicin for 72 hours.

To further characterize the potential mechanisms involved in mediating doxorubicin-induced ATF3 expression, we investigated pathways involved in the cellular response to DNA damage. Previously, our laboratory demonstrated the role of MAPK signaling in regulating ATF3 expression following cisplatin treatment [18]. Treating the human breast cancer cell lines with chemical inhibitors for JNK (SP600125), ERK1/2 (U0126), and p38 (SB203580), we demonstrate that the inhibition of JNK, but not the others, results in an inhibition of doxorubicin-induced ATF3 expression, suggesting an important role for JNK in mediating doxorubicin-induced ATF3 expression (Figure 4, C-E). Since doxorubicin is a potent inducer of DNA double-strand breaks (DSBs), we lastly looked at the role of ATM in regulating ATF3 expression following doxorubicin treatment. Inhibition of ATM with the inhibitor KU55933 resulted in reduced ATF3 expression (Figure 4F). Together, these results identify three major signaling pathways that can regulate doxorubicin-induced ATF3 expression (Figure 4A). Each of these pathways presents potential targets that can be exploited to enhance ATF3 expression through potential combination therapies with doxorubicin.

Inducers of ATF3 Enhance Doxorubicin Cytotoxicity

The identification of compounds that can enhance the cytotoxicity of doxorubicin will have the potential to either enhance the efficacy of this agent or allow for its use in lower less toxic but equally effective doses. The combination of ATF3-inducing compounds with doxorubicin may provide a means to sustain and elevate ATF3 expression in order to induce tumor cell apoptosis and enhance treatment efficacy. To identify agents that are dependent on ATF3 for their cytotoxicity and can enhance doxorubicin cytotoxicity, we performed two independent high-throughput library screens of 1200 FDA-approved compounds [18]. This first screen was performed comparing the responses by MTT analysis of 5-μM treatments of the library in ATF3+/+ and ATF3−/− MEFs. Agents with at least a 25% difference in cytotoxicity in the ATF3+/+ MEFs compared to the ATF3−/− MEFs were characterized as ATF3 cytotoxicity dependent (Figure 5A). Of the 1200 FDA-approved agents tested, 86 showed significant cytotoxicity with 42 dependent on ATF3. Employing the same 1200 FDA-approved compound library, we further screened these compounds in combination with doxorubicin to identify agents that could enhance doxorubicin cytotoxicity in MCF7 cells (Figure 5B). In this screen, the MCF7 cells were pretreated with 1 mM of each library drug for 24 hours followed by 10-nM doxorubicin treatments for a further 48 hours. This screen identified 10 compounds as enhancers of doxorubicin cytotoxicity (>20% higher cytotoxicity than library or doxorubicin alone). Importantly, of the identified compounds, 4 of the 10 were also identified as ATF3 dependent in the previous screen and included vorinostat, trifluridine, 6-mercaptopurine, and monensin. Vorinostat and trifluridine were chosen from the lead compounds for
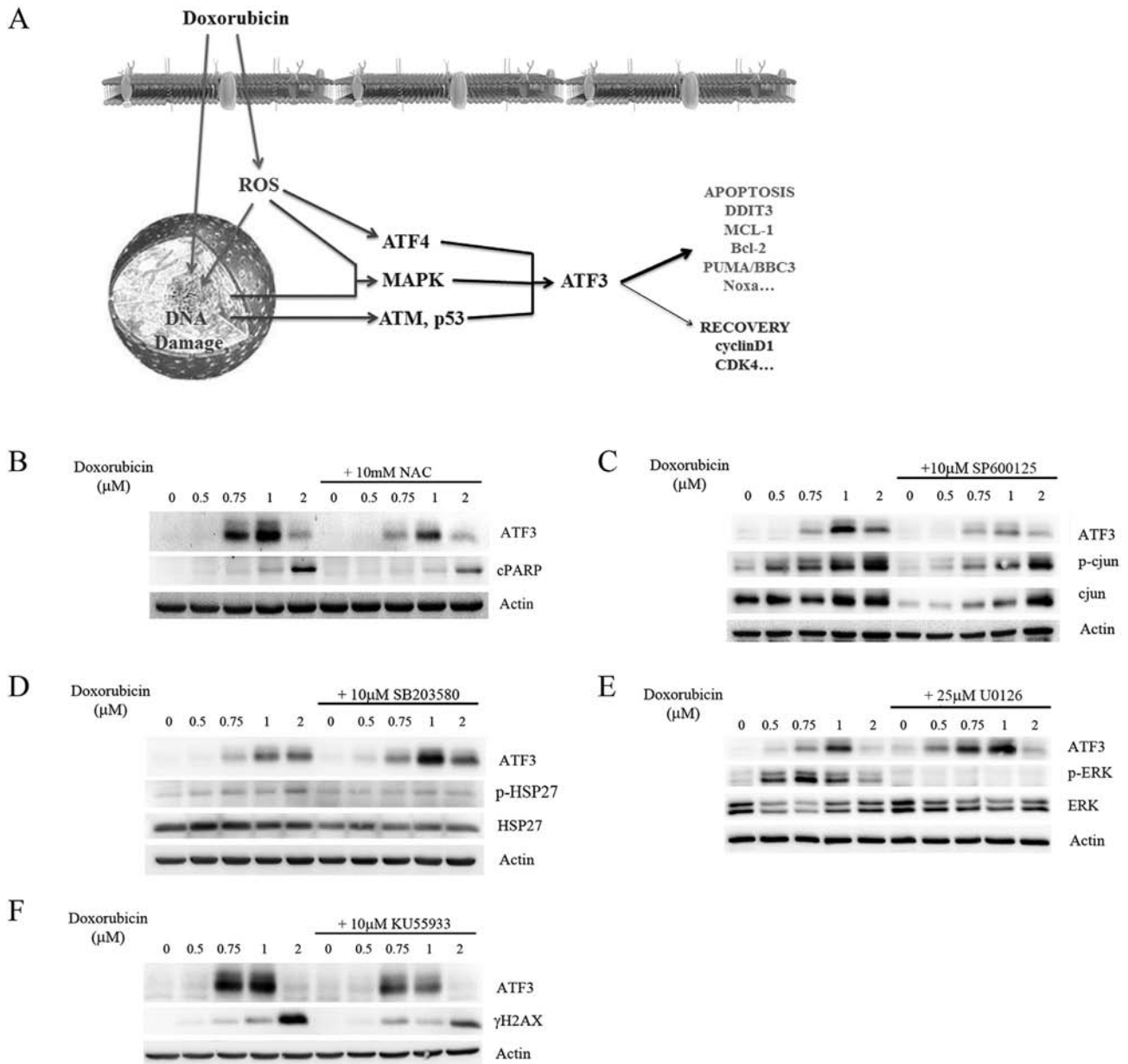
Figure 4. (A) Schematic depicting the potential mechanisms involved in regulating doxorubicin induced ATF3 expression. DNA damage and ROS produced by doxorubicin treatment can initiate various signaling pathways, such as the ISR through ATF4, the MAPK signaling pathways, and DDR pathways. Upregulation of ATF3 by these signaling pathways can lead to both cell recovery and apoptosis. Cell death occurs when ATF3 expression is elevated and sustained. (B-F) Western blot analysis for ATF3 expression following 24-hour doxorubicin treatment with or without n-acetylcysteine or chemical inhibitors for JNK (SP600125), ERK1/2 (U0126), p38 (SB203580), and ATM (KU55933) in the MCF7 cells.

further evaluation as they represent clinically relevant agents. The combination of vorinostat with doxorubicin has demonstrated clinical benefit in a phase 1 clinical trial [30], and trifluridine has recently been FDA-approved for the treatment of metastatic colon cancer and has potential for the treatment of other solid tumors [31,32].

We specifically validated the data from the library screens by MTT analysis using the ATF3$^{-/-}$ MEFs, which demonstrated reduced sensitivity to a series of treatments with vorinostat or trifluridine (Figure 5C). Western blot analysis confirmed that both drugs induce ATF3 expression in the wild-type MEFs and the human breast cancer cell lines tested (Figure 5, D-F). We next determined whether vorinostat and/or trifluridine could enhance doxorubicin cytotoxicity in these breast cancer

cell lines. MTT analysis on the combination treatment of vorinostat or trifluridine with doxorubicin compared to doxorubicin alone demonstrated enhanced cytotoxicity in the MCF7 cells but not the MDA-MB-231 cell lines (Figure 6, A and B). Both drug combinations were synergistic in the MCF7 cell line (Figure 6, D and E). The combination treatment with vorinostat or trifluridine also resulted in an enhancement of ATF3 expression in the MCF7 cells where synergistic cytotoxicity was demonstrated but not in the MDA-MB-231 cells where these combinations failed to induce synergistic cytotoxicity (Figure 6C). To address the role of ATF3 in breast cancer response to adjuvant therapy in a more robust manner, we directly assessed basal ATF3 expression and patient response to chemotherapy using publically
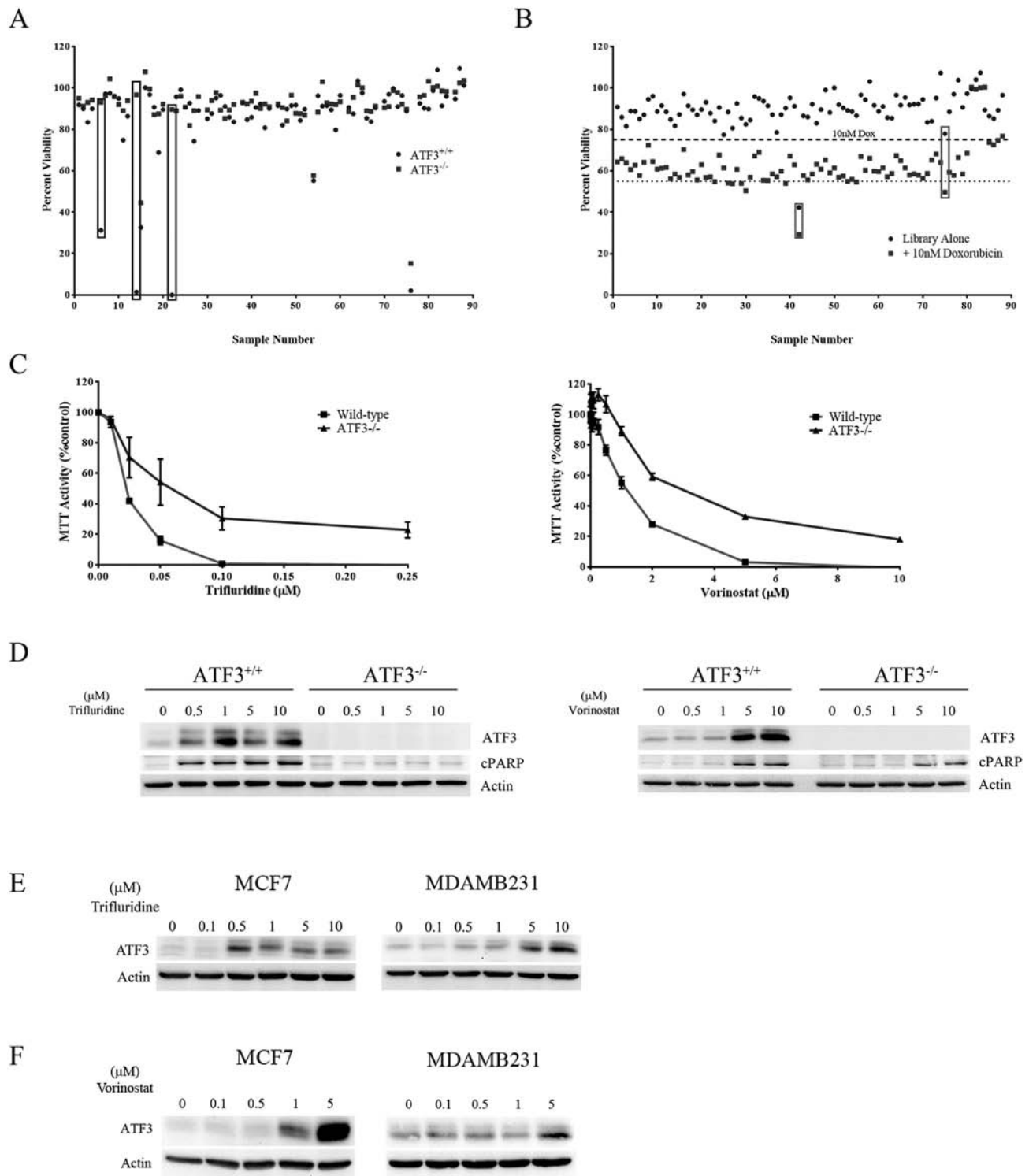
Figure 5. A 1200 FDA-approved library screen was performed to identify inducers of ATF3. (A) Representative plot of the data obtained from the screen performed in the wild-type and ATF3$^{-/-}$ MEFs. While there was no difference in cytotoxicity for many of the compounds between the two groups, there was a significant reduction in sensitivity to some drugs (highlighted in green). (B) Representative plot of the data obtained from the library screen performed in the MCF7 cell lines to identify enhancers of doxorubicin cytotoxicity. (C) MTT analysis demonstrating that the loss of ATF3 in MEFs results in a reduction in sensitivity to trifluridine and vorinostat. Error bars, SD from the mean of three replicates ($n = 3$). (D-F) Western blots analysis demonstrating the ability of trifluridine and vorinostat to induce ATF3 expression in the wild-type MEFs and the human breast cancer cell lines following 24-hour treatment.

available gene expression data with patient survival data (www. kmplot.com) [33]. Patient outcomes were assessed following adjuvant chemotherapy in breast cancer patients with high or low ATF3 expression. Kaplan-Meier survival curves demonstrate improved survival outcomes for breast cancer patients with elevated ATF3 expression (Figure 6F). These data suggest that patients with
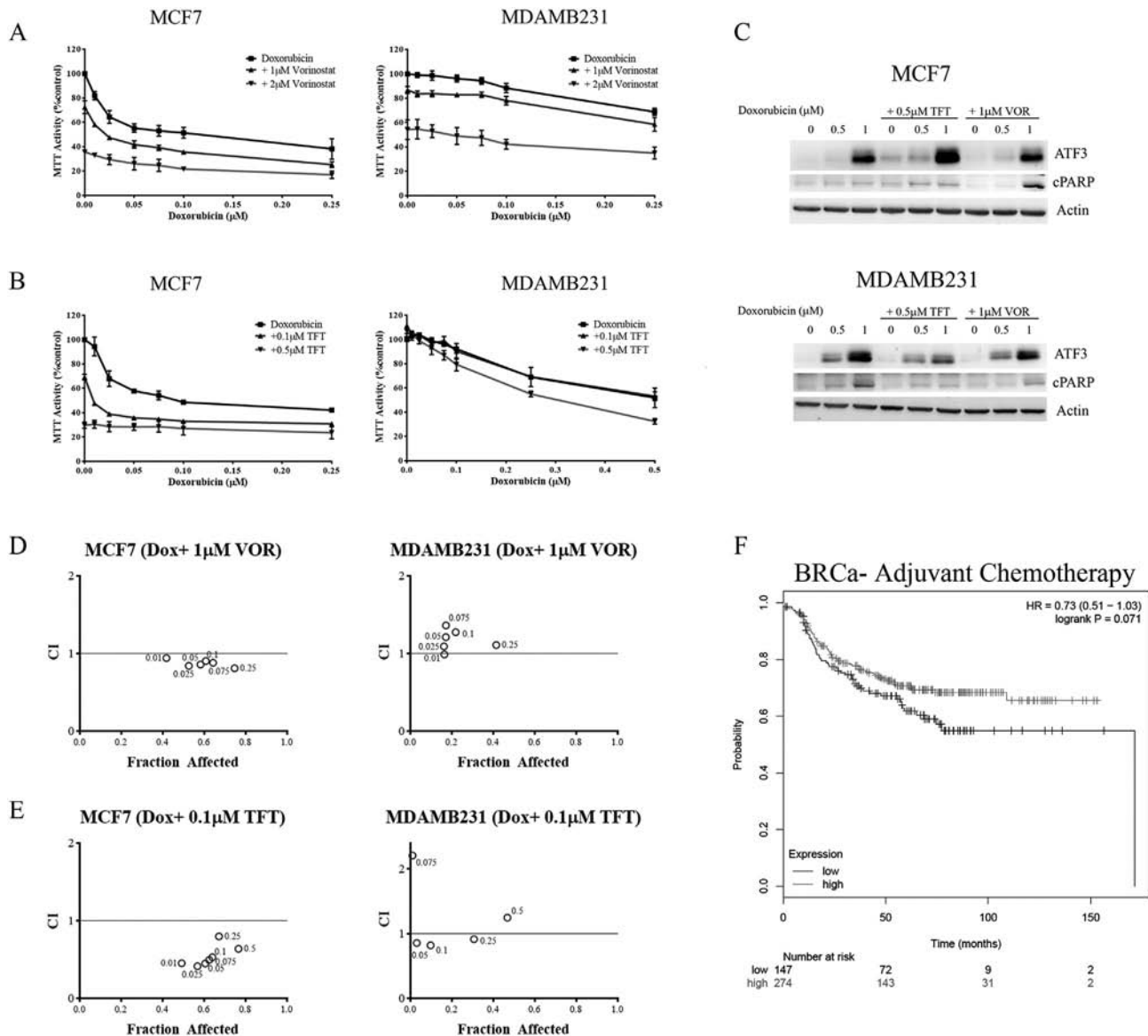
Figure 6. MTT analysis for the combination of vorinostat (A) and trifluridine (B) with doxorubicin in the human breast cancer cell lines. Cells were pretreated with vorinostat or trifluridine for 24 hours followed by 48-hour treatment with doxorubicin. Error bars, SD from the mean of three replicates ($n = 3$). (C) Western blot analysis for the expression of ATF3 following the combination treatment of vorinostat or trifluridine with doxorubicin in the breast cancer cell lines. (D and E) CI was determined for the combination of vorinostat and trifluridine with doxorubicin. CI < 1 is synergistic, CI = 1is additive, and CI > 1 is antagonistic. (F) Kaplan-Meier survival curves for breast cancer patients with high and low expression of ATF3 that received adjuvant chemotherapy. Data obtained from kmplot.com [30].

elevated ATF3 expression show enhanced response and greater overall survival with adjuvant chemotherapy treatment.

Taken together, these results demonstrate that combining inducers of ATF3 with doxorubicin can be a potential rationale approach to enhance doxorubicin-induced tumor cell cytotoxicity. The combination of ATF3 inducers with doxorubicin represents a potential novel combination therapeutic strategy for enhancing doxorubicin efficacy.

Discussion

Anthracyclines, such as doxorubicin, are a consistently employed class of chemotherapeutics for the treatment of advanced breast cancer [4]. While it remains to be effective in the treatment of advanced breast cancer, there are many unfavorable toxicities associated with doxorubicin use, with the most limiting being cardiotoxicity [34]. These

associated toxicities result in having to limit the dose patients receive, which ultimately results in decreased effectiveness [34]. In this study, we have demonstrated that ATF3 is a key regulator of doxorubicin-induced cytotoxicity and that combining ATF3-inducing agents, such as trifluridine and vorinostat, with doxorubicin can enhance tumor cell cytotoxicity in breast cancer cell lines. This represents a novel rationale combination-based therapeutic strategy where inducing sustained and elevated ATF3 expression can drive tumor cell apoptosis and enhance the activity compared to the use of each agent alone.

Doxorubicin is primarily considered to be a DNA-damaging agent through its ability to inhibit topoisomerase IIa [5]. It targets topoisomerases in two ways: 1) it intercalates DNA which prevents topoisomerase binding and results in stalled replication forks, and 2) it stabilizes topoisomerase IIα following the formation of DSBs [5]. Both of

these mechanisms result in the formation of double-strand DNA breaks that elicit a DDR. Doxorubicin treatment also results in other off-target toxicities that result in tumor cell cytotoxicity which include the formation of DNA adducts and free radicals, and the overproduction of ceramides [35]. Combination therapeutic approaches are one strategy to try and overcome some of the clinical limitations of doxorubicin use. The identification of compounds that can enhance doxorubicin efficacy may allow for lower doses of doxorubicin to be used while maintaining therapeutic efficacy. Understanding the molecular mechanisms involved in mediating the effect of the combinations also allows for the identification of more targeted combination therapies.

Trifluridine, a fluoropyrimidine, has demonstrated potent anticancer activity, but due to poor bioavailability, its use has been limited. Trifluridine is rapidly phosphorylated to produce its active monophosphate form which is incorporated into DNA, resulting in cell cycle arrest which is attributed to be the primary mechanism of trifluridine cytotoxicity [36]. Recent advances have demonstrated that the combination of trifluridine with a thymidine phosphorylase inhibitor (tipiracil) can greatly enhance its bioavailability and efficacy [37–39]. Trifluridine /tipiracil (TAS-102) treatment has recently been FDA approved for the treatment of metastatic colorectal cancer. Vorinostat is an HDAC inhibitor that has been approved for the treatment of cutaneous T-cell lymphoma and is being assessed in the treatment of other cancers. HDAC inhibitors have demonstrated preclinical and clinical activity as single agents and in combination with other chemotherapies, such as platins [40–42]. A Phase 1 clinical trial assessed the combination of vorinostat and doxorubicin and demonstrated that the combination displays some clinical benefit for the treatment of solid tumors [30]. But due to lack of specificity and off-target toxicities associated with treatment, the use of HDAC inhibitors is limited [43]. In our study, the combination of trifluridine or vorinostat with doxorubicin enhanced tumor cell cytotoxicity, demonstrating the potential of these drug combinations for therapeutic use. In the case of trifluridine, in order for it to be effectively used *in vivo*, the combination of TAS-102 with doxorubicin needs to be evaluated.

Previously, we demonstrated that ATF3 plays an important role in mediating platin cytotoxicity in NSCLC and that the dysregulation of ATF3 may result in resistance to platin therapy [18]. In breast cancer, other studies have demonstrated a role for ATF3 in mediating the proapoptotic effect of drugs such as digitoxin and indole-3-carbonol [19, 20]. Doxorubicin was demonstrated by our group and others to be an inducer of ATF3 expression [18, 24]. We have also demonstrated that the loss of ATF3 results in a significant reduction in sensitivity to doxorubicin treatment in MEFs. It has been well characterized that doxorubicin cytotoxicity can occur through various mechanisms and cell death pathways, which may explain why the loss of ATF3 expression results in only a modest reduction in sensitivity to doxorubicin treatment in the human cell lines. In our study, we observed the activation of the ISR, DDR, and MAPK signaling pathways following doxorubicin treatment, all of which have been demonstrated to initiate cellular apoptosis and can upregulate ATF3 expression [15–17].

The synergistic enhancement of doxorubicin cytotoxicity observed in the combination treatments with trifluridine or vorinostat may therefore be attributed to the upregulation of ATF3 or the activation of upstream pathways regulating ATF3 expression. Previous reports have demonstrated the role of JNK and ATM as mediators of doxorubicin cytotoxicity [44–46], and in our study, the inhibition of these pathways attenuated doxorubicin-induced ATF3 expression in the breast cancer cell lines. JNK has also been demonstrated to be involved in mediating the enhancement of doxorubicin cytotoxicity in combination with gamitrinib [47]. In our study, the overexpression of an activated JNK construct in the breast cancer cell lines resulted in enhanced ATF3 expression following doxorubicin treatment (data not shown). Our laboratory has also previously demonstrated that JNK is an important regulator of ATF3 expression in NSCLC [18]. Dysregulation of JNK resulted in an attenuation of ATF3 induction following cisplatin treatment and contributed to the resistance of the cell lines to cisplatin. Vorinostat has previously been demonstrated to upregulate JNK activity [48], and trifluridine treatment was able to activate JNK signaling in the breast cancer cell lines (data not shown). Therefore, vorinostat and trifluridine may enhance doxorubicin cytotoxicity through the activation of JNK signaling and its downstream targets, including ATF3. These data suggest that high basal levels of ATF3 expression in breast cancer patients may lead to enhanced response to chemotherapy. In adjuvant treatment regimens of breast cancer, doxorubicin and 5-fluorouracil (similar mechanism to trifluridine) are the most commonly employed agents. In an evaluation of breast cancer patients treated with adjuvant chemotherapy, patient tumors with elevated basal ATF3 levels showed enhanced overall survival.

In conclusion, combination approaches with compounds that upregulate the expression of ATF3 may provide novel strategies for next-generation doxorubicin combination therapies. The combination of trifluridine and vorinostat with doxorubicin represents a potential novel combination therapeutic approach in breast cancer that requires further investigation.

Acknowledgements

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