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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

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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