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NADPH quinone oxidoreductase 1 mediates breast cancer cell resistance to thymoquinone-induced apoptosis

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ABSTRACT

Thymoquinone (TQ), a bioactive component of black caraway seed (*Nigella sativa*) oil, is reported to have antineoplastic properties. In this study we investigated the effect of TQ on a panel of human breast cancer cell lines. Cell viability assays showed that TQ killed T-47D, MDA-MB-231, and MDA-MB-468 cells via p53-independent induction of apoptosis; however, MCF-7 cells were refractory to the cytotoxic action of TQ. Western Blot analysis showed that MCF-7 cells expressed high levels of cytoprotective NADPH quinone oxidoreductase 1 (NQO1), which was responsible for TQ-resistance since inhibition of NQO1 with dicoumarol rendered MCF-7 cells TQ-sensitive. These findings may be clinically important when considering TQ as a possible adjunct treatment for breast cancer since a high percentage of breast tumors express NQO1.

1. Introduction

Thymoquinone (TQ) is the major bioactive component of oil obtained from black caraway seeds (*Nigella sativa*), also referred to as black cumin [1]. Black caraway seed has a history of use in traditional Arabic herbal medicine for the treatment of such diverse ailments as gastrointestinal problems, rheumatism, diabetes and hypertension [2]. Moreover, recent laboratory findings indicate that TQ is a potent antioxidant, modulates inflammatory responses, and inhibits the growth of many types of cancer cells [3,4]. Effects of TQ on cancer cells include DNA damage, cell cycle arrest, and apoptosis in human glioblastoma cells [5], reactive oxygen species-dependent apoptosis in human colon carcinoma cells [6], and reduced growth of human prostate tumor xenografts in nude mice [7]. TQ also kills multi-drug resistant human tumor cells [8], including doxorubicin-resistant breast cancer cells [9], and chemosensitizes pancreatic cancer cells to gemcitabine and oxaliplatin by preventing NFκB activation [10]. In addition, TQ may have antimetastatic activities since it inhibits *in vitro* migration, adhesion and invasion by human glioblastoma cells [11]. Importantly, TQ does not substantially affect the viability of normal human lung fibroblasts, intestinal cells, and noncancerous prostate epithelial cells at concentrations that are cytotoxic for cancer cells [5–7]. The potent anticancer activities of TQ in cell culture and animal models support its possible use in cancer treatment.

Breast cancer is a leading cause of cancer death in North American and European women, who have a one in eight lifetime risk of the disease [12]. Advanced breast cancer that cannot be cured by surgery alone is typically treated with adjunct chemotherapy [13]; however, chemotherapeutic drugs can cause secondary malignancies [14] and have additional detrimental side effects due to their inability to discriminate between rapidly proliferating cancer cells and healthy dividing cells [15]. Moreover, breast cancer cells frequently develop chemoresistance due to the emergence of multidrug resistance protein-expressing variants [16]. The need for new treatments for breast cancer that have fewer adverse side effects has generated interest in exploiting the anticancer properties of dietary phytochemicals and other natural products that are generally well tolerated [17].

In this investigation we determined the effect of TQ on four different breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468, T-47D) with differing estrogen receptor (ERα) and progesterone receptor expression [18–20], as well as p53 status [21]. None of the breast cancer cell lines in our panel overexpress human epidermal growth factor receptor-2 (HER2) [22]. Information gained from this study will help to determine whether TQ

Abbreviations: Ab, antibody; DMSO, dimethyl sulfoxide; ERα, estrogen receptor; HER2, human epidermal growth factor receptor-2; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO1, NADPH quinone oxidoreductase 1; PBS, phosphate-buffered saline; PI, propidium iodide; TQ, thymoquinone.

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might be broadly useful as an adjuvant treatment for breast cancer since the panel of breast cancer cells that was investigated is representative of several commonly observed breast cancer phenotypes [23].

2. Materials and methods

2.1. Cell lines

MDA-MB-468 breast carcinoma cells were obtained from Dr. P. Lee (Dalhousie University, NS) while MCF-7 and T-47D breast cancer cells were provided by Dr. J. Blay (Dalhousie University, NS). MDA-MB-231 breast carcinoma cells were a kind gift from Dr. S. Drover (Memorial University of Newfoundland, NL). Breast cancer cell lines were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Canada Ltd., Oakville, ON), supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (7.4 pH), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Corp., Burlington, ON). Breast cancer cells were passaged every 72 h or at 70–90% confluency by treatment with trypsin plus 0.25% EDTA for 1 min at room temperature or TrypLE for 3 min at 37 °C (both from Invitrogen).

2.2. Reagents

TQ (99% pure), phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dicoumarol, pifithrin-α, and phosphatase substrate were from Sigma–Aldrich Canada. A 100 mM stock solution of TQ was prepared in dimethyl sulfoxide (DMSO) and stored in aliquots at −20 °C. Goat anti-human actin polyclonal antibody (Ab), horseradish peroxidase (HRP)-conjugated bovine anti-goat IgG Ab, and goat anti-mouse IgG-HRP Ab were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-human NADPH quinone oxidoreductase 1 (NQO1) monoclonal Ab was from Cell Signaling Technology, Inc. (Beverly, MA).

2.3. Cell viability assays

Breast cancer cell viability was determined using colorimetric MTT and acid phosphatase assays. Cells were plated in quadruplicate wells of a 96-well flat-bottom tissue culture plates at a density of 5×10^3 cells/well and incubated overnight at 37 °C in a 10% CO_2 humidified atmosphere. Cells were then treated with TQ or its vehicle (DMSO) and cultured for an additional 24 h, 48 h, or 72 h. For MTT assays, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well 2 h before the end of culture, at which time plates were centrifuged at 1400*g* for 5 min, the supernatant was discarded, and DMSO (0.1 ml/well) was added to solubilize the formazan crystals. The plate was shaken for 1 min on a plate shaker and the absorbance was measured at 490 nm using an ELx800 UV Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). For acid phosphatase assays, at the end of the culture period cell monolayers were washed 3 times with PBS followed by the addition of 0.2 ml of a 1:2 dilution of assay buffer (0.1 M sodium acetate, pH 5.5, 0.1% Triton X-100, and 4 mg/ml phosphatase substrate) in PBS to each well. After incubation for 90 min at 37 °C in a 10% CO_2 humidified atmosphere, 10 μl of 1 N NaOH was added to each well to stop the reaction and absorbance was measured at 405 nm. For both MTT and acid phosphatase assays, the % reduction in cell number was calculated using the equation $[1 - E/C] \times 100$, where E is the absorbance of samples from TQ-treated cells and C is the absorbance of samples from control cells.

2.4. Apoptosis/necrosis assay

Annexin-V-FLUOS/propidium iodide (PI) staining was used to assess the amount of apoptosis and/or necrosis in cultures of breast cancer cells treated with vehicle (DMSO) or TQ. MDA-MB-468 and MCF-7 cells were seeded at 5×10^4 cells/well in 6-well flat-bottom plates and incubated overnight at 37 °C in a 10% CO_2 humidified atmosphere. Cells were then treated with TQ or its vehicle (DMSO) and cultured for an additional 24 h. Culture supernatant was collected and combined with adherent cells that were harvested using TrypLE. Cells were washed with PBS, then stained with Annexin-V-FLUOS diluted according to the manufacturer's instructions and PI (1 μg/ml) in staining buffer (10 mM HEPES, 10 mM NaCl, and 5 mM $CaCl_2$) for 15 min at room temperature. Cell fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON).

2.5. Western Blot analysis

MDA-MB-468 and MCF-7 cells were harvested with TrypLE, washed with PBS, and resuspended in ice-cold RIPA lysis buffer solution (0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 20 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml apotinin, 1 mM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, 1 mM dithiothreitol, 10 mM NaF, and 10 μM phenylarsine oxide). Cells were incubated on ice for 25 min and then centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant containing cell proteins was collected and stored at −80 °C. The protein content of the cell lysates was measured by Bradford assay (Bio-Rad Laboratories Ltd., Mississauga, ON). Proteins were resolved on sodium dodecyl sulfate–polyacryl-

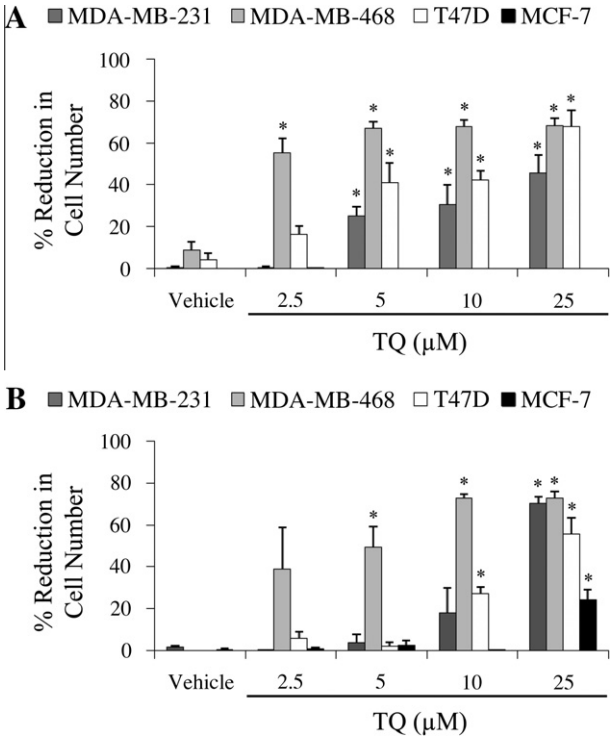

Fig. 1. TQ causes a dose-dependent reduction in breast carcinoma cell number. MDA-MB-468, MDA-MB-231, MCF-7, and T-47D human breast carcinoma cells were exposed to the indicated concentrations of TQ or its vehicle (DMSO) and the reduction in cell number was quantified by (A) MTT assay or (B) acid phosphatase assay after 24 h of culture. Data are an average of 3 experiments, shown as % reduction in cell number ± SEM. Statistical significance in comparison to the vehicle control was determined by the Tukey–Kramer multiple comparisons test; *denotes $p < 0.05$.

amide gels and then transferred to a nitrocellulose membrane using the iBlot transfer system (Invitrogen). Membranes were blocked overnight at 4 °C using 5% (w/v) skim milk powder in Tris-buffered saline (200 mM Tris, 1.5 M NaCl at pH 7.6) containing 0.05% Tween-20, then washed for 30 min and incubated with anti-NQO1 Ab (1:1000) overnight at 4 °C. After extensive washing, membranes were probed for 1 h at room temperature with HRP-conjugated goat anti-mouse IgG-HRP Ab (1:1000). Protein bands were visualized on X-ray film using an enhanced chemiluminescence Western Blotting detecting reagent (Bio-Rad Laboratories Inc., Hercules, CA). To control for equal protein loading, membranes were re-probed with anti-actin Ab (1:1000) and HRP-conjugated bovine anti-goat IgG Ab (1:1000). Protein bands were visualized as described above.

2.6. Statistical analysis

Data were analyzed using the Instat statistics program (GraphPad Software Inc., San Diego, CA) for one-way analysis of variance and the Tukey–Kramer multiple comparisons test; $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Differential sensitivity of breast cancer cell lines to TQ

A panel of four HER2-negative human breast carcinoma cell lines with differing estrogen receptor, progesterone receptor, and

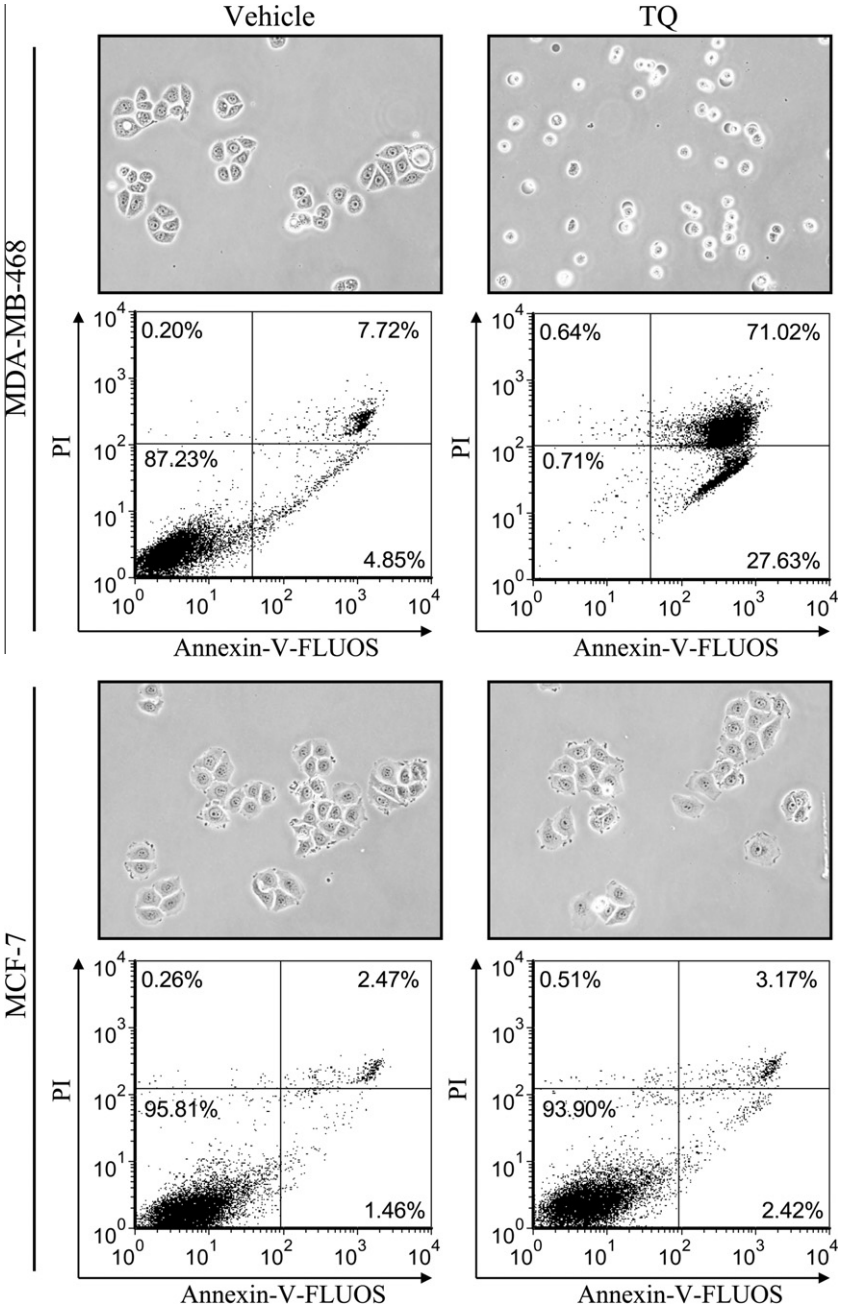

Fig. 2. TQ induces apoptosis in MDA-MB-468 but not MCF-7 breast cancer cells. MDA-MB-468 or MCF-7 breast carcinoma cells were cultured for 24 h in the presence of the drug vehicle (DMSO) or 10 μM TQ, harvested, and stained with annexin-V-FLUOS and PI. Flow cytometry was then used to determine the relative staining of annexin-V-FLUOS and PI to establish the percentage of viable cells (lower left quadrant) in comparison to those in early apoptosis (lower right quadrant) versus late apoptosis/necrosis (upper right quadrant). Cell cultures were also photographed under phase contrast illumination at 100× magnification. Data are representative of at least 3 independent experiments.

p53 status were cultured for 24 h in the absence or presence of different concentrations of TQ (2.5–25 μM). MTT and acid phosphatase assays were used to determine the impact of TQ treatment on breast cancer cell number. Both colorimetric assays showed a dose-dependent reduction in the number of MDA-MB-231, MDA-MB-468, and T-47D breast cancer cells following TQ treatment (Fig. 1). MDA-MB-468 breast cancer cells showed the greatest sensitivity to TQ. In contrast, MCF-7 breast cancer cells were resistant to concentrations of TQ that were cytotoxic for the other breast cancer cell lines. MCF-7 resistance to 2.5–25 μM TQ was maintained at 48 h and 72 h time points (data not shown); however, 24 h exposure to 100 μM TQ did result in a substantial reduction in MCF-7 cell number (54 ± 5%), indicating that TQ resistance was not absolute.

MCF-7 and T-47D cells express ERα whereas MDA-MB-231 and MDA-MB-468 cells are negative for ERα [18]. Progesterone receptor is expressed by T-47D cells but not by MCF-7, MDA-MB-231 or MDA-MB-468 cells [19,20]. Although MCF-7 cells were least affected by TQ, ERα expression is unlikely to be a factor since T-47D cells that also express ERα, as well as progesterone receptor, were sensitive to killing by TQ. MDA-MB-231 and MDA-MB-468 cells are negative for HER2 [22], as well as ERα and progesterone receptor [18–20], and are therefore examples of triple-negative breast cancer cells. Approximately 15–20% of patients present with triple-negative breast cancer, which is associated with unusually high morbidity and mortality due to the aggressive nature of these tumors, their tendency to acquire chemoresistance, and failure to respond to hormonal therapy or HER2-targeted treatment [24]. Killing of triple-negative MDA-MB-231 and MDA-MB-468 cells by TQ suggests that this phytochemical should be further investigated for possible use in the treatment of triple-negative breast cancers.

P53 is a tumor suppressor protein that is activated in response to genotoxic stress, typically resulting in cell cycle arrest and apoptosis [25]. TQ-resistant MCF-7 cells express wild-type p53 whereas TQ-sensitive T-47D, MDA-MB-231, and MDA-MB-468 cells have single-point mutations that render p53 non-functional [21]. The capacity of TQ to kill breast cancer cells that lack functional p53 is an important finding since p53 mutations that lead to chemoresistance are common in breast cancer [26]. Although activation of wild-type p53 can in some cases protect normal and cancer cells from drug-induced cytotoxicity [27,28], wild-type p53 activity in MCF-7 cells was not involved in TQ resistance since the p53 inhibitor pifithrin-α [29] at concentrations as high as 10 μM did not render MCF-7 cells more sensitive to TQ (data not shown). The p53-independent action of TQ on breast cancer cells is consistent with a report that TQ decreases cell survival more potently in p53-deficient MG63 osteosarcoma cells than in their p53 wild-type counterparts [30].

3.2. Differential induction of apoptosis by TQ

Flow cytometric analysis of TQ-treated MDA-MB-468 and MCF-7 cells that were subsequently stained with annexin-V-FLUOS and PI revealed extensive apoptosis in MDA-MB-468 cell cultures but very little apoptotic cell death in MCF-7 cultures (Fig. 2), which was consistent with the results obtained from MTT and alkaline phosphatase assays. MDA-MB-468 and MCF-7 cells that were cultured for 24 h in the absence or presence of 10 μM TQ were also examined by phase-contrast microscopy. TQ-treated MDA-MB-468 cell cultures showed an increase in rounded cells with morphology characteristic of apoptosis whereas TQ treatment did not substantially alter the appearance of MCF-7 cells.

TQ-mediated induction of apoptosis in MDA-MB-468 breast cancer cells is in line with TQ-induced apoptosis previously reported in glioblastoma cells and colon carcinoma cells [5,6]. Depending on the cell type, TQ-induced apoptosis can occur via a p53-dependent or p53-independent pathway [30,31]. Our data indicate that TQ-induced apoptosis in MDA-MB-468 cells was p53-independent since p53 is mutated and not functional in these cells [21]. Although an examination of the precise mechanism of apoptosis caused by TQ was beyond the scope of the present investigation, TQ-induced apoptosis in breast cancer cells is reported to involve a peroxisome proliferators-activated receptor γ-dependent pathway and a subsequent reduction in protein levels of survivin [32], which is a multifunctional cell-protective protein that is expressed at high levels in most breast cancer cells [33].

3.3. NQO1 mediates MCF-7 resistance to TQ

The majority of breast tumors express NQO1 [34], which is a multifunctional cytoprotective flavoprotein with the capacity to

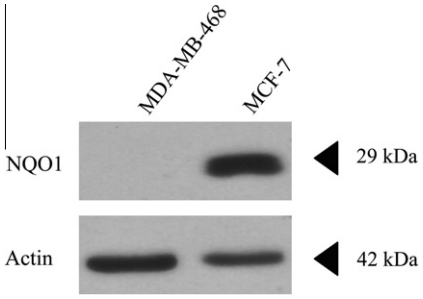

Fig. 3. Differential expression of NQO1 by MDA-MB-468 and MCF-7 breast cancer cells. Freshly harvested MDA-MB-468 and MCF-7 cells were lysed and cellular protein extracts were prepared for Western Blot analysis to detect NQO1 expression. Equal protein loading was verified by examining actin expression. Data are from one experiment representative of 3 independent experiments.

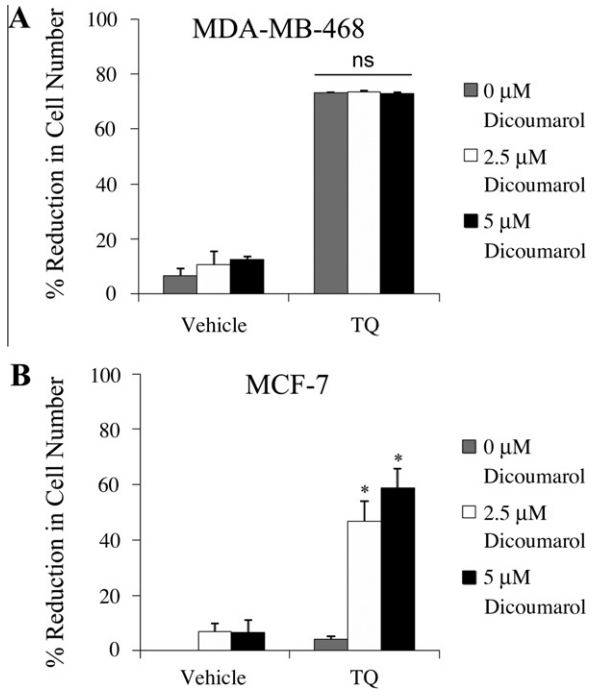

Fig. 4. NQO1 inhibition sensitizes MCF-7 breast carcinoma cells to killing by TQ. (A) MDA-MB-468 or (B) MCF-7 breast cancer cells were treated with the drug vehicle (DMSO) or indicated concentrations of dicoumarol for 1 h prior to culture in the presence of 25 μM TQ. The effect on cell number was measured by MTT assay after 24 h. The data are shown as % reduction in cell number ± SEM from 4 independent experiments. Statistical significance in comparison to the vehicle control was determined by the Tukey–Kramer multiple comparisons test; *denotes $p < 0.05$.

detoxify quinones [35]. We therefore asked whether the level of NQO1 expression might determine the sensitivity of breast cancer cells to TQ-induced cytotoxicity. Western Blot analysis showed that MDA-MB-468 cells, which exhibited the greatest sensitivity to killing by TQ, expressed little NQO1 whereas NQO1 was abundant in TQ-resistant MCF-7 cells (Fig. 3). To confirm that NQO1 was responsible for protecting MCF-7 cells from TQ-induced cytotoxicity, TQ-sensitive MDA-MB-468 or TQ-resistant MCF-7 breast cancer cells were pretreated for 1 h with the NQO1 inhibitor, dicoumarol [36], prior to 24 h culture in the absence or presence of TQ. Pretreatment with dicoumarol did not increase TQ-mediated killing of MDA-MB-468 cells (Fig. 4A) but clearly sensitized MCF-7 cells to killing by TQ (Fig. 4B). In fact, in the presence of 5 μM dicoumarol, TQ-mediated killing of MDA-MB-468 and MCF-7 cells was nearly equivalent. Dicoumarol by itself did not affect the viability of MDA-MB-468 or MCF-7 cells.

NQO1 (also called DT-diaphorase) catalyzes the two-electron reduction of quinones to hydroquinones, which prevents the one-electron reduction of quinones to semiquinones and the subsequent generation of toxic free radicals via redox cycling [37]. In addition, NQO1 functions as a superoxide scavenger [38]. Given that the generation of reactive oxygen species is reported to mediate TQ-induced apoptosis in cancer cells [6], it is not surprising that NQO1 was cytoprotective for TQ-treated MCF-7 cells. Elevated NQO1 expression by ERα-positive MCF-7 cells was consistent with a recent study showing a positive correlation between NQO1 and ERα expression in breast cancer patients [34]. Our findings are also in line with a report that high levels of NQO1 activity protect HT-29 colon carcinoma cells from the toxic effects of arylating naphthoquinones [39]. NQO1 expression by a high percentage of breast tumors [34] poses a potential obstacle to the application of TQ in breast cancer treatment; however, co-administration of oral dicourmarol, which is in common clinical use as an anticoagulant [40], should obviate this problem. We conclude that TQ should be further investigated as a possible adjunct agent for the treatment of a range of breast cancer phenotypes, including difficult-to-treat triple-negative breast cancers.

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