TRAIL-Induced Apoptosis in TRAIL-Resistant Breast Carcinoma Through Quercetin Cotreatment

Jasmine M Manouchehri1,2, Katherine A Turner1,2 and Michael Kalafatis1,2,3

1Department of Chemistry, Cleveland State University, Cleveland, OH, USA. 2Center for Gene Regulation in Health and Disease (GRHD), Cleveland State University, Cleveland, OH, USA. 3Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA.

ABSTRACT: Breast cancer is the most commonly diagnosed cancer in women. There is a continued interest for the development of more efficacious treatment regimens for breast carcinoma. Recombinant human tumor necrosis factor–related apoptosis-inducing ligand (rhTRAIL) shows potential as a potent anticancer therapeutic for the treatment of breast cancer, whereas displaying minimal toxicity to normal cells. However, the promise of rhTRAIL for the treatment of breast cancer is dismissed by the resistance to rhTRAIL-induced apoptosis exhibited by many breast cancers. Thus, a cotreatment strategy was examined by applying the natural compound quercetin (Q) as a sensitizing agent for rhTRAIL-resistant breast cancer BT-20 and MCF-7 cells. Quercetin was able to sensitize rhTRAIL-resistant breast cancers to rhTRAIL-induced apoptosis as detected by Western blotting through the proteasome-mediated degradation of c-FLIP, and through the upregulation of DR5 expression transcriptionally. Overall, these in vitro findings establish that Q is an effective sensitizing agent for rhTRAIL-resistant breast cancers.

KEYWORDS: Apoptosis, breast cancer, c-FLIP, DR5, quercetin, rhTRAIL

Introduction

Breast cancer is the most commonly diagnosed cancer in women.1–4 The development of more effective treatment regimens against the different forms of breast carcinoma is being explored.2,4,5 Patients with hormone-dependent and human epidermal growth factor receptor 2 (HER2) overexpression breast cancers often have a better prognosis because of the availability of hormone-targeted therapies.2,5,6 The triple-negative breast cancers (TNBCs) are more challenging to treat because there is no specific hormone to target; hence, TNBC is the deadliest form of breast cancer.2,4

One promising anticancer therapeutic of interest is recombinant human tumor necrosis factor–related apoptosis-inducing ligand (rhTRAIL)—the optimized form of the endogenous death ligand TRAIL (tumor necrosis factor–related apoptosis-inducing ligand). Recombinant human tumor necrosis factor–related apoptosis-inducing ligand consists of the extracellular death ligand TRAIL amino acids 114-281 lacking exogenous sequence tags.7–11 Recombinant human tumor necrosis factor–related apoptosis-inducing ligand has shown great potential as an effective anticancer therapeutic due to its ability to induce apoptosis in cancer cells, whereas exhibiting minimal toxicity to normal, nontransformed cells.7–11 Recombinant human tumor necrosis factor–related apoptosis-inducing ligand initiates the extrinsic pathway of apoptosis by binding to the extracellular death receptors (DRs) DR4 and DR5 leading to trimerization of the receptors followed by the activation of caspase 8; the subsequent activation of the executioner caspases 3, 6, and 7; and the eventual cleavage of poly (adenosine diphosphate-ribose) polymerase or PARP (hallmark of apoptosis).12–16 In addition, rhTRAIL can activate the intrinsic pathway of apoptosis independently of p53 through the caspase 8–mediated cleavage of Bid to truncated Bid (tBid) facilitating the release of cytochrome c from the mitochondria followed by the activation of caspase 9 and the subsequent activation of the executioner caspases.13–15,17–19 Despite this, most of the breast cancer cells exhibit resistance to TRAIL treatment due to the upregulation of antiapoptotic proteins such as cellular FLICE-like inhibitory protein (c-FLIP) and the downregulation of DR5. Clinical trials have been completed with TRAIL, but further trials have since been terminated due to a limited therapeutic efficacy.20–22 Consequently, many studies have focused on determining sensitizing agents that have the capability to overcome rhTRAIL resistance.

One potential sensitizing agent is Quercetin (Q); it is a naturally occurring flavonol found in different vegetables, fruits, tea, red wine, and coffee.23–25 Quercetin has been shown to produce antiproliferative and proapoptotic effects in cancer cells such as prostate, cervical, lung, breast, and colon.23,24,26–30 Quercetin can induce apoptosis in some cancer cell lines through the downregulation of antiapoptotic proteins, survivin, Bcl-xL, and Bcl-2, and through the upregulation of proapoptotic proteins, Bad and Bax.29,31–33 Investigations involving human hepatoma and prostate cancer cells have demonstrated that Q can enhance TRAIL-induced apoptosis through the...
upregulation of DR5. Furthermore, no major cytotoxic effects have been observed in different in vivo studies, and clinical trials have administered Q with no major cytotoxic effects cited. Therefore, these findings suggest that Q has the potential to be an effective sensitizing agent.

The intention of this study was to investigate the capability of Q to sensitize rhTRAIL-resistant TNBC BT-20 cells and hormone-dependent breast cancer MCF-7 cells and to elucidate the underlying mechanism for Q’s sensitization. Our study demonstrates that Q has the ability to induce the proteasome-mediated degradation of c-FLIPL and to induce the upregulation of DR5 facilitating the execution of the extrinsic pathway and thereby sensitizing breast cancers to rhTRAIL-induced apoptosis. Thus, the presented evidence reveals that Q is a good sensitizing agent for rhTRAIL-resistant breast cancers.

Methods

Drugs and chemicals

Recombinant human tumor necrosis factor–related apoptosis-inducing ligand was produced according to well-defined and previously detailed protocols. Recombinant human tumor necrosis factor–related apoptosis-inducing ligand was aliquoted and stored at −80°C. Quercetin dihydrate (lot no. D00166146, molecular weight of 338.3 g/mol; Calbiochem, San Diego, CA, USA) was dissolved in 7.5 mg/mL of polyethylene glycol (molecular weight of 400 g/mol; Fisher Scientific, Hampton, NH, USA) and then filtered, aliquoted, and stored at −20°C. MG132 proteasome inhibitor (molecular weight of 457.6 g/mol; Calbiochem) was dissolved in dimethyl sulfoxide to produce a 10 mM stock that was filtered, aliquoted, and stored at −20°C.

Cell culture

Human breast cancer MCF-7 (ATCC HTB-22) and BT-20 (ATCC HTB-19) cells were cultured in Dulbecco's Modified Eagle Medium (Cleveland Clinic Cell Services Media Core, Cleveland, OH, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), 1% antibiotics-antimycotics (Gibco), 1% l-glutamine (Gibco), 1% nonessential amino acids (Gibco), and 1% sodium pyruvate (Gibco). Human nontumorigenic breast epithelial MCF-10A (ATCC CRL-10317) cells were cultured in HuMEC Ready Medium (Gibco). Cells were maintained in a humidified atmosphere with 5% CO2 at 37°C. Cells were treated with drugs 24 hours after plating, incubated with drugs for an additional 72 hours, and collected for the different assays described below.

Annexin V/propidium iodide assays-flow cytometry

Cells were trypsinized, spun at 1000 rpm for 3 minutes, and washed with phosphate-buffered saline (PBS). Cells were incubated with Annexin V-FITC and propidium iodide (PI) solution (Annexin V: FITC Apoptosis Detection Kit I; BD Life Sciences, Sparks, MD, USA) for 15 minutes at room temperature in the dark. Apoptosis was detected using the BD FACS Canto II and applying FACS Diva software and Flowing Software 2. Each experiment was performed in triplicate, and 3 independent experiments were conducted for each cell line to obtain the mean percent of apoptotic cells ± SEM.

Determination of apoptotic-associated protein levels by Western blotting

Cells were collected and washed with PBS. Total cell lysates were prepared by lysing with radioimmunoprecipitation assay (RIPA) buffer (AMRESCO, Dallas, TX, USA) and a protease inhibitor cocktail (Calbiochem). The lysates were placed on ice for 30 minutes, were spun at 10000 rpm for 10 minutes, and were quantified by applying the Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of 35 μg of protein were prepared, denatured with 4× Laemmli sample buffer (250 mM Tris–HCl, 8% sodium dodecyl sulfate [SDS], 40% glycerol, 8% β-mercaptoethanol, and 0.02% bromophenol blue), and separated on 12% SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA, USA) employing the semidry transfer method (Bio-Rad, Richmond, CA, USA). Each membrane was blocked with 5% nonfat milk at room temperature for 1 hour and incubated overnight at 4°C with a diluted primary antibody: anti-PARP, anti-caspase 3, anticleaved caspase 3, anti-caspase 7, anti-caspase 8, anticleaved caspase 8, anti-Bid (Cell Signaling Technology, Danvers, MA, USA), or anti-FLIP (Enzo Life Sciences, Farmingdale, NY, USA). The membranes were washed in Tris-buffered saline (TBS)-TWEEN (0.15 M NaCl, 0.02 M Tris, and 0.3% Tween 20 with a pH of 7.4), incubated with a horse radish peroxidase–conjugated secondary antibody either goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad) diluted in 5% nonfat milk at 1 hour at room temperature, and washed in TBS-TWEEN. The membranes were then exposed on HyBlot CL Autoradiography Film (Denville Scientific, Holliston, MA, USA); the protein bands were detected using chemiluminescence via Pierce ECL2 Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of 35 μg of protein were prepared, denatured, and separated on 15% SDS-PAGE gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA, USA) employing the semidry transfer method (Bio-Rad, Richmond, CA, USA). Each membrane was blocked with 5% nonfat milk at room temperature for 1 hour and incubated overnight at 4°C with a diluted primary antibody: anti-PARP, anti-caspase 3, anticleaved caspase 3, anti-caspase 7, anti-caspase 8, anticleaved caspase 8, anti-Bid (Cell Signaling Technology, Danvers, MA, USA), or anti-FLIP (Enzo Life Sciences, Farmingdale, NY, USA). The membranes were washed in Tris-buffered saline (TBS)-TWEEN (0.15 M NaCl, 0.02 M Tris, and 0.3% Tween 20 with a pH of 7.4), incubated with a horse radish peroxidase–conjugated secondary antibody either goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad) diluted in 5% nonfat milk for 1 hour at room temperature, and washed in TBS-TWEEN. The membranes were then exposed on HyBlot CL Autoradiography Film (Denville Scientific, Holliston, MA, USA); the protein bands were detected using chemiluminescence via Pierce ECL2 Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) as the internal loading control.

Determination of cytochrome c release

Cells were collected, washed, and resuspended in permeabilization buffer (400 μg/mL digitonin, 75 mM KCl, 1 mM Na2HPO4, 8 mM NaH2PO4, 250 mM sucrose) with a protease inhibitor cocktail. All of the samples were kept on ice for 10 minutes and spun at 16000 g for 5 minutes at 4°C; the supernatants were kept as the cytosolic fractions. Protein quantification was executed as described above, and aliquots of 60 μg of protein were prepared, denatured, and separated on 15%
Complementary DNA synthesis was performed at 60°C for 30 minutes using the Applied Biosystems GeneAmp PCR System 9700. The PCR cycling conditions (40 cycles) were chosen as follows: denature for 2 minutes at 94°C, anneal for 30 seconds at 55°C for c-FLIP, and 65°C for DR5 and β-actin, extend for 1 minute and 30 seconds at 68°C, and execute a final extension for 10 minutes at 68°C. Reaction products were analyzed on 1.2% agarose gels. The bands were visualized by ethidium bromide (Invitrogen, Carlsbad, CA, USA) and a UV illuminator (UVP, Upland, CA, USA).

Flow cytometry analysis of DR4 and DR5 expression

Cells were collected with cell dissociation buffer (Gibco) and lysed with RIPA buffer as described above. After the membranes were blocked, the membranes were incubated overnight at 4°C with anti-DR4 (Imgenex, Littleton, CO) in 5% nonfat milk or anti-DR5 (Cell Signaling Technology) in 5% bovine serum albumin (Fisher Scientific). The membranes were washed and developed as described above. Densitometry was calculated from ImageJ software.

Western blot analysis of DR4 and DR5 expression

Cells were collected with cell dissociation buffer and spun at 1000 rpm for 3 minutes. Cells were resuspended in staining buffer (2% FBS, 0.02% sodium azide, and PBS) and incubated with anti-DR4-PE or anti-DR5-PE (eBioscience, San Diego, CA, USA) for 1 hour in the dark at 4°C; a mouse IgG1 K isotype control (eBioscience) was used to compensate for any nonspecific binding. Cells were washed with staining buffer and resuspended in staining buffer for analysis. DR4 and DR5 membrane expressions were analyzed on a BD FACSCanto II flow cytometer using FACSDiva software. Histograms were prepared employing Flowing Software 2. Each experiment was performed in triplicate, and 3 independent experiments were conducted for each cell line to obtain the fold increase in DR4 or DR5 cell surface expression relative to the vehicle-treated control ± SEM.

Reverse transcription–polymerase chain reaction analysis for DR5 and c-FLIP

Total RNA was extracted from cells using TRIzol Reagent (Ambion, Pittsburgh, PA, USA). Reverse transcription–polymerase chain reaction (RT–PCR) was performed following the manufacturer's protocol (Invitrogen SuperScript III One-Step RT–PCR System with Platinum Taq DNA Polymerase; Thermo Fisher Scientific). Human DR5 messenger RNA (mRNA) was amplified using the forward primer 5′-GGGAGCCGCT-CATGAGGAAGTTGG-3′ and the reverse primer 5′-GGCAAGTCTCTCCTGCCAGCGTCTC-3′ (182-bp [base pairs] product). For c-FLIP, forward primer 5′-GGGAGCCGCT-CATGAGGAAGTTGG-3′ and the reverse primer 5′-CCCATGAACATCCTCCTGCCAGCGTCTC-3′ (182-bp [base pairs] product).

Results

Fluorescence–activated cell sorting analysis of rhTRAIL–induced apoptosis

Fluorescence–activated cell sorting (FACS) analysis was completed on breast cancer cells treated with increasing concentrations of Q (12.5, 25, and 50 μM) in the presence or absence of 100 ng/mL rhTRAIL to ascertain Q's sensitizing effects on rhTRAIL–induced apoptosis (Figure 1). Quercetin enhanced rhTRAIL–induced apoptosis in both breast cancer cell lines. However, for breast cancer MCF-7 cells, Q did not have a considerable impact on promoting rhTRAIL–induced apoptosis when compared with Q–mediated rhTRAIL–induced apoptosis in breast cancer BT-20 cells. For example, breast cancer MCF-7 and BT-20 cells treated with 50 μM Q produced on average about 15% and 20% apoptotic cells, respectively (P < .05), whereas the cotreatment of 50 μM Q and 100 ng/mL
rhTRAIL on MCF-7 and BT-20 cells produced on average about 25% and 45% apoptotic cells, respectively (P < .05). It should be noted that 100 ng/mL rhTRAIL alone did not produce a significant amount of apoptotic breast cancer cells when compared with the vehicle-treated control.

**Detection of the pathway of apoptosis**

Breast cancer cells were treated the same as the FACS analysis. The protein levels of caspase 8, cytosolic cytochrome c, caspase 3 (only for BT-20 cells because MCF-7 cells lack procaspase 3 expression), caspase 7, and cleaved PARP were all upregulated with the cotreatment of Q and rhTRAIL, whereas Bid expression was downregulated when compared with the vehicle-treated controls and single-agent treatments in both breast cancer cell lines (Figure 2). Densitometry results are illustrated in Figure 3. These results indicate that the extrinsic pathway was induced as marked by caspase 8 activation, executioner caspase 3 and/or caspase 7 activation, and PARP cleavage. In addition, Q alone did exhibit minimal proapoptotic effects on breast cancer cells as illustrated by PARP cleavage. Furthermore, cytochrome c was released from the mitochondria in both breast cancer cells treated with 50 μM Q demonstrating the initiation of the intrinsic pathway of apoptosis. Also, it should be noted that PARP fragmentation was not observed with single-agent rhTRAIL treatment at 100 ng/mL for both breast cancer cell lines supporting data gathered from other researchers that breast cancer BT-20 and MCF-7 cells are TRAIL resistant. In addition, TRAIL did not induce apoptosis in MCF-10A cells. These data together with the data from FACS shown in Figure 1 strongly suggest that Q sensitized breast cancer cells to rhTRAIL-induced apoptosis through the induction of the extrinsic pathway of apoptosis.

**Synergism of Q and rhTRAIL**

The Chou-Talalay method was applied to calculate the combination index (CI) for the cotreatment of Q and rhTRAIL in both breast cancer cell lines (Figure 4). The CIs for both breast cancer cell lines were less than 1 indicating that a synergic effect was observed and not an additive effect (CI = 1) or an antagonistic effect (CI > 1).

**Q induces the proteasome-mediated degradation of c-FLIP_1.**

Considering that Q was shown to sensitize breast cancer to rhTRAIL-induced apoptosis through the extrinsic pathway by enhanced activation of caspase 8, Q’s impact on the expression of
the caspase 8 inhibitor c-FLIP was assessed. Results gathered from Western blot analysis demonstrate that Q downregulated the expression of the long form of c-FLIP (c-FLIPL) in breast cancer in a dose-dependent manner after 72 hours (Figure 5A). Preliminary assays were performed with 24-, 48-, and 72-hour Q-treated breast cancer cells, and Q's impact on c-FLIPL was not displayed until after 72 hours. At the highest concentration of Q used in our study (50 μM), expression of c-FLIP L was downregulated by about 9-fold and 3-fold in comparison with the vehicle-treated control for breast cancer BT-20 and MCF-7 cells, respectively. To observe whether Q-induced downregulation occurred through the proteasome-mediated degradation of c-FLIPL, a proteasome inhibitor MG132 was used. MG132 was used at a low concentration of 0.25 μM in presence and absence of 50 μM Q along with a vehicle-treated control. Inhibition of the proteasome resulted in the prevention of c-FLIPL downregulation through Q treatment. In addition, c-FLIPL expression did not differ between the vehicle-treated control and the breast cancer cells treated with only the proteasome inhibitor MG132 (Figure 5B). Furthermore, Co-IP was performed probing for ubiquitin demonstrating that Q promoted the ubiquitination of c-FLIPL in breast cancer (Figure 5C and D). Hence, for breast cancer, Q enhances rhTRAIL-induced apoptosis through the proteasome-mediated degradation of c-FLIPL via increased ubiquitination.

**Q's impact on DR4 and DR5 expression**

Western blot and flow cytometry analyses were performed to discover Q's effects on DR4 and DR5 protein and cell surface expression in breast cancer. DR5 protein expression was upregulated in BT-20 cells with Q treatment dose dependently only after 72 hours, but Q did not influence DR4 protein expression in BT-20 cells (Figure 6A). DR5 and DR4 protein expressions did not change significantly in Q-treated MCF-7 cells (Figure 7A). Moreover, FACS analysis revealed that BT-20 cells express both receptors on the cell surface (Figure 6B to D), and DR4 expression was not upregulated significantly with Q (P > .05 for all treatments when compared with control), whereas Q did induce the upregulation of DR5 membrane expression. The FACS analysis also showed that MCF-7 cells express both receptors on the cell surface (Figure 7B to D). DR4 cell surface expression was not upregulated significantly with single-agent Q treatment (P > .05 for all treatments when compared with control), but 50 μM Q slightly upregulated DR5 membrane expression when compared with the vehicle-treated control. The results obtained from FACS analysis agree with the data derived from Western blot analysis for both cell lines. Therefore, Q-induced DR5 upregulation is an additional factor for the heightened rhTRAIL sensitivity observed in TNBC cells.

**Comparing DR4 and DR5 expression in breast cells**

Western blot and flow cytometry analyses were performed to compare the expression of DR4 and DR5 in breast cancer BT-20 and MCF-7 cell lines to DR4 and DR5 expression in nontumorigenic breast epithelial MCF-10A cells. MCF-10A had the highest DR4 and DR5 membrane and protein expression, and BT-20 cells had the lowest DR4 and DR5 protein and membrane expression levels (Figure 8). In addition, Q did not affect the expression of DR4 and DR5 in MCF-10A cells. Overall, the data demonstrate that Q specifically upregulates DRs in malignant breast cancer cell lines only (Figure 9).
Figure 3. Densitometry results for apoptotic-associated proteins. Densitometry results for immunoblots were calculated for breast cancer (A) BT-20 and (B) MCF-7 cells using ImageJ software and graphs were generated applying GraphPad Prism. PARP indicates poly (adenosine diphosphate-ribose) polymerase.
**Q's effects on DR5 and c-FLIP**

Quercetin was shown to increase the expression of DR5 and decrease the expression of c-FLIP in breast cancer, but it was unknown whether Q acts at the transcriptional level to affect DR5 and c-FLIP. Therefore, RT-PCR was executed applying β-actin as a positive control (Figure 10). Through RT-PCR analysis, we show that Q did not induce any change in c-FLIP mRNA expression in breast cancer cells. The combined data suggest that Q-induced c-FLIP downregulation must occur at the posttranslational level. Finally, Q did increase DR5 mRNA levels dose dependently in TNBC BT-20 cells correlating with the increase in the protein expression observed through Western blot and FACS analyses. Therefore, Q-induced DR5 upregulation in TNBC occurs transcriptionally.

**Figure 4.** Combination indexes (CIs) for the cotreatment of Q and rhTRAIL indicate synergism. Combination indexes were calculated and the cotreatment of Q and rhTRAIL displayed synergistic effect (all CIs < 1.0) in both breast cancer cell lines (A) BT-20 and (B) MCF-7. Q indicates quercetin; rhTRAIL, recombinant human tumor necrosis factor–related apoptosis-inducing ligand.

**Discussion**

Breast cancer affects women worldwide. Traditional chemotherapy and radiation treatments for breast cancer rely on p53 to induce the intrinsic pathway of apoptosis, but many cancers possess a nonfunctional p53 gene resulting in necrosis rather than apoptosis after chemotherapy and radiation producing adverse side effects in patients. Recombinant human tumor necrosis factor–related apoptosis-inducing ligand possesses the ability to induce apoptosis through the induction of the extrinsic pathway of apoptosis in cancer cells and induce apoptosis through the induction of the intrinsic pathway of apoptosis independent of p53.7–10,43,44 Recombinant human tumor necrosis factor–related apoptosis-inducing ligand has been proposed to be used as an anticancer therapeutic. However, clinical trials using rhTRAIL as a potential anticancer therapeutic were terminated due to a lack of clinical efficacy, and in vitro studies have found that rhTRAIL treatment had limitations due to many cancer cell lines being resistant. This study focused on evaluating the potential of Q as a potent sensitizing agent for rhTRAIL-induced apoptosis in rhTRAIL-resistant TNBC BT-20 and hormone-dependent breast cancer MCF-7 cells; it should be noted that the combinatorial treatment of Q and rhTRAIL has not been examined before in the breast cancer cell lines of interest.28,34,35,45 Through in vitro analysis, we show that Q possesses the capability to act as a sensitizing agent for rhTRAIL-resistant breast carcinoma.

To assess the interaction of Q and rhTRAIL, Western blot analysis and Annexin V/PI assays were performed after 72 hours of treatment. The time course of 72 hours was chosen after preliminary experiments showed that Q's impact on c-FLIP and DR5 did not occur until after 72 hours. Other investigations have treated cells with Q at greater concentrations (100, 150, 175, and 200 μM) for 24 and 48 hours, whereas our investigation applies at most 50 μM of Q.23,34,40,46 Both assays confirmed that Q augments rhTRAIL-induced apoptosis in breast cancer BT-20 and MCF-7 cells via the execution of the extrinsic pathway and the activation of caspase 8, the activation of executioner caspases 3 and 7, and the cleavage of PARP; furthermore, the results demonstrated that Q had the ability to promote apoptosis as a single agent but rhTRAIL did not. In addition, the cotreatment of Q and rhTRAIL exhibited a synergistic effect in breast BT-20 and MCF-7 cells. Therefore, Q's mechanism of sensitization needed to be elucidated.

Previous studies have proposed that one reason cancer cell lines are resistant to rhTRAIL-induced apoptosis is through the upregulation of the c-FLIP, a significant inhibitor of the extrinsic pathway.47 The c-FLIP is structurally similar to caspase 8, and at high-expression levels, it has the ability to prevent caspase 8 activation when bound to the death-inducing signaling complex and thus suppressing the DR signaling pathway.47–49 Consequently, c-FLIP expression was evaluated and found to be significantly downregulated in Q-treated breast cancer cells.

Considering Q's induced downregulation of c-FLIP in breast cancer, the underlying mechanism for Q's sensitization needed
to be elucidated. Through RT-PCR analysis, Q was found to not affect the mRNA expression of c-FLIPL in either breast cancer cell line. Analysis was then performed at the posttranslational level through the treatment of both breast cancer cell lines with the 50 μM Q alone and in combination with Q for 72 hours, and the cotreatment of MG132 and Q recovered c-FLIPL protein expression in breast cancer. Co-IP was performed on (C) BT-20 and (D) MCF-7 cells treated in the presence and absence of 50 μM Q for 72 hours. Q enhanced the ubiquitination of c-FLIPL in breast cancer. Blots were also probed for c-FLIPL to confirm that the Co-IP was properly executed. Co-IP indicates co-immunoprecipitation; Q, quercetin.

Figure 5. Q promotes the proteasome-mediated degradation of c-FLIPL. Western blotting revealed that Q decreases c-FLIPL expression in (A) BT-20 and MCF-7 cells in a dose-dependent manner. (B) BT-20 and MCF-7 cells were treated with a proteasome inhibitor MG132 alone and in combination with Q for 72 hours, and the cotreatment of MG132 and Q recovered c-FLIPL protein expression in breast cancer. Co-IP was performed on (C) BT-20 and (D) MCF-7 cells treated in the presence and absence of 50 μM Q for 72 hours. Q enhanced the ubiquitination of c-FLIPL in breast cancer. Blots were also probed for c-FLIPL to confirm that the Co-IP was properly executed. Co-IP indicates co-immunoprecipitation; Q, quercetin.

Figure 6. DR4 and DR5 expression in Q-treated breast cancer BT-20 cells. (A) DR4 and DR5 (mature form) protein levels were assessed after 72 hours of treatment; Q upregulated DR5 expression in a dose-dependent manner. (B) DR5 and (C) DR4 cell surface expression levels for Q-treated BT-20 cells were analyzed by flow cytometry. For the representative histograms (B) and (C), vehicle-treated control = green, 12.5 μM Q = yellow, 25 μM Q = black, and 50 μM Q = blue. (D) The bar graphs represent the average fold increase in DR4 or DR5 cell surface expressions relative to the vehicle-treated control ± SEM from 3 independent experiments performed in triplicate (n = 9). P > .05 except * Q indicates quercetin.

findings we can conclude that Q sensitizes TNBC and hormone-dependent breast cancer cells to rhTRAIL-induced apoptosis through the proteasome-mediated degradation of c-FLIPL because of enhanced ubiquitination.

Previous investigations have proposed that some rhTRAIL-resistant cancer cell lines express low levels DR4 and DR5 and thereby making them less sensitive to rhTRAIL’s proapoptotic effects.²⁸,³⁴ Quercetin has been shown to upregulate DR5 expression level in prostate cancer and hepatoma but not in breast cancer.²⁸,³⁴ Hence, DR4 and DR5 protein expressions were examined by Western blotting, and DR4 and DR5 cell surface expressions were examined by FACS. The data proved that Q had the ability to
significantly upregulate DR5 expression in TNBC BT-20 cells, but Q had less of an effect on DR5 expression in hormone-dependent breast cancer MCF-7 cells. When comparing the expression levels of DRs, nontumorigenic breast epithelial MCF-10A cells had the highest expression of DRs, whereas TNBC BT-20 cells had the lowest expression of DRs. These results match another investigation comparing DR4 and DR5 protein expression in breast cancer MCF-7 cells and nontumorigenic breast epithelial MCF-10A cells where MCF-10A cells expressed more DR4 and DR5 in comparison with MCF-7 cells. In addition, Q did not affect DR4 and DR5 protein expression in nontumorigenic breast epithelial MCF-10A cells.

Since Q-induced DR5 upregulation in breast cancer, the underlying mechanism for this augmentation of rhTRAIL sensitivity, needed to be elucidated. The RT-PCR analysis demonstrated that Q enhanced DR5 mRNA expression in a dose-dependent manner most significantly in TNBC BT-20 cells. Thus, Q increases rhTRAIL sensitivity in TNBC cells through the upregulation of DR5 transcriptionally.
Figure 9. Densitometry results for death receptors and c-FLIP. Densitometry results for Western blots for DR4, DR5, and c-FLIP were calculated using ImageJ software and graphs were generated applying GraphPad Prism. (A) BT-20; (B) MCF-7; (C) BT-20, MCF-7, and MCF-10A; and (D) MCF-10A.
Conclusions
Overall, the data presented here demonstrate that Q is an effective sensitizing agent for rhTRAIL-resistant breast carcinoma. Through in vitro analysis, the cotreatment of Q and rhTRAIL proved efficacious for hormone-dependent breast cancer and TNBC. Our results suggest that the enhanced ubiquitination of c-FLIP<sub>L</sub> by Q could be a novel mechanism underlying the downregulation of c-FLIP<sub>L</sub> facilitating the enhanced rhTRAIL sensitivity. Therefore, this cotreatment should be explored further in vivo to determine its clinical efficacy as a potential breast carcinoma therapeutic regimen especially for the more fatal TNBCs.

Author Contributions
JMK wrote the first draft of the manuscript. JMK, KAT, and MK conceived and designed the experiments; analyzed the data; contributed to the writing of the manuscript; agree with manuscript results and conclusions; jointly developed the structure and arguments for the paper; made critical revisions and approved final version; and reviewed and approved the final manuscript.

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