IJC
International Journal of Cancer

Novel targets for sensitizing breast cancer cells to TRAIL-induced apoptosis with siRNA delivery

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in variety of cancer cells without affecting most normal cells, which makes it a promising agent for cancer therapy. However, TRAIL therapy is clinically not effective due to resistance induction. To identify novel regulators of TRAIL that can aid in therapy, protein targets whose silencing sensitized breast cancer cells against TRAIL were screened with an siRNA library against 446 human apoptosis-related proteins in MDA-231 cells. Using a cationic lipopolymer (PEI-αLA) for delivery of library members, 16 siRNAs were identified that sensitized the TRAIL-induced death in MDA-231 cells. The siRNAs targeting BCL2L12 and SOD1 were further evaluated based on the novelty and their ability to sensitize TRAIL induced cell death. Silencing both targets sensitized TRAIL-mediated cell death in MDA-231 cells as well as TRAIL resistant breast cancer cells, MCF-7. Combination of TRAIL and siRNA silencing BCL2L12 had no effect in normal human umbilical vein cells and human bone marrow stromal cell. The silencing of BCL2L12 and SOD1 enhanced TRAIL-mediated apoptosis in MDA-231 cells via synergistically activating capsase-3 activity. Hence, here we report siRNAs targeting BCL2L12 and SOD1 as a novel regulator of TRAIL-induced cell death in breast cancer cells, providing a new approach for enhancing TRAIL therapy for breast cancer. The combination of siRNA targeting BCL2L12 and TRAIL can be a highly effective synergistic pair in breast cancer cells with minimal effect on the non-transformed cells.

Molecular Cancer Biology

Resistance to apoptosis is one of the hallmarks of cancer.[1] Malignant cells develop resistance to apoptosis mainly by upregulating antiapoptotic proteins and/or diminishing proapoptosis signals. Facilitating apoptosis during therapy has a strong potential to eradicate cancer cells. Most conventional chemotherapy and radiotherapy regimens induce apoptosis via the intrinsic pathway that is p53-dependent.[2] However, p53 is functionally inactivated in some malignant cells as a result of mutation(s) or loss of expression, which makes these malignant cells display resistance to conventional therapies.[3] Alternatively, binding of death ligands to death receptors (DRs) triggers the extrinsic apoptosis pathway where p53 appears to be dispensable in most cases.[4] Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), in particular, have the potential to induce the extrinsic apoptosis pathway after binding to TRAIL-R1 (DR4) or TRAIL-R2 (DR5) on the surface of malignant cells.[5]

TRAIL has a unique capacity to induce apoptosis in a variety of tumor cell lines, but not in most normal cells, providing a highly promising avenue for therapy in cancer.[6–8] Several clinical trials (Phase I and II) demonstrated that TRAIL and TRAIL receptor agonists are safe,[9–11] but unlike the preclinical results, TRAIL therapy tested so far failed to exert a robust anticancer activity in patients. Resistance to TRAIL has been shown to occur through defects at every level of the TRAIL signaling pathways, from ligand binding to cleavage of the effector caspases.[5,12] Several inhibitory proteins such as cellular FLICE-inhibitory protein (cFLICE), antiapoptotic Bcl-2 family members and XIAPs were overexpressed in malignant cells and have been associated with TRAIL resistance.[10,13] Hence, TRAIL treatment along with inhibiting antiapoptotic proteins could augment proapoptotic signaling. Several independent studies found that chemotherapeutics and inhibitors of antiapoptotic proteins sensitized

Key words: apoptosis, breast cancer, tumor necrosis factor-related apoptosis-inducing ligand, siRNA library
Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Canadian Institute of Health Research (CIHR); **Grant sponsor:** A Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC); **Grant sponsor:** An Innovation Grant from the Canadian Breast Cancer Foundation (CBCF); **Grant sponsor:** NCPRM (NSERC CREATE Program for Regenerative Medicine PI: G. Laroche, University of Laval, Quebec City, WC, Canada)
DOI: 10.1002/ijc.31079
History: Received 15 Apr 2017; Accepted 19 Sep 2017; Online 27 Sep 2017
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What's new?

Triggering cell death via apoptosis is a key therapeutic strategy in cancer, making tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) an appealing agent for development as an anticancer therapy. Resistance to TRAIL in clinical studies, however, has limited its potential. In this study, two novel regulators, BCL2L12 and SOD1, were identified via siRNA library screening as targets capable of enhancing TRAIL effectiveness in breast cancer cells. Silencing of BCL2L12 and SOD1 in the presence of TRAIL resulted in synergistic activation of caspase-3 and reversed TRAIL resistance, with little effect on normal cells.

cells to TRAIL-induced apoptosis.[5,10,13] However, systemic studies to determine key inhibitors of TRAIL in breast cancer cells have not been clearly established.

In this study, we took advantage of RNAi screening technology to identify key targets that regulate TRAIL-induced apoptosis in breast cancer cells. Using synthetic siRNA-mediated RNAi screening against 446 human apoptosis-related proteins, complementary protein targets to TRAIL-induced apoptosis were identified and silenced to sensitize breast cancer cells to TRAIL treatment. For the delivery of siRNA, we employed nonviral cationic lipopolymers after grafting aliphatic lipids onto the low molecular weight (MW) polyethyleneimines (PEIs). Low MW PEIs are more biocompatible than conventional cationic biomolecules and could be cleared easier in body due to smaller size. To improve the stability of complexation with nucleic acids, we have been grafting aliphatic lipids onto PEI and such modifications enhanced the delivery efficacy via increased membrane interaction and intracellular uptake.[14–16] These lipopolymers effectively undertook siRNA delivery to breast cancer cells, including xenograft models in mouse.[16] Hence, this study pursued simultaneous induction of apoptosis by TRAIL and lipopolymer-mediated siRNA delivery against TRAIL-sensitizing targets for a superior anticancer activity in breast cancer cells.

METHODS

Polymer synthesis and characterization

Chemical modification of low MW (0.6, 1.2 and 2 kDa) PEIs was performed via *N*-acylation using α-linoleoyl chloride (αLA) as a hydrophobic moiety as described earlier[17] (Fig. 1). Structural composition of PEI-αLAs was elucidated through ^{1}H-NMR spectroscopy (Bruker 300 MHz, Billerica, MA).

Cell culture

Human breast cancer cells MDA-231 and MCF-7 and normal breast cells MCF-10A were obtained from Dr. Judith Hugh (Department of Oncology, University of Alberta). MDA-231 and MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS 100 unit/mL Penicillin, and 100 μg/mL Streptomycin. MCF-10A cells were maintained in DMEM: F12 (1:1) supplemented with 5% Horse Serum 100 unit/mL Penicillin, 100 μg/mL Streptomycin, Hydrocortisone (500 ng/mL), hEGF (20 ng/mL), Human Insulin (0.01 mg/mL) (Sigma-Aldrich, St. Louis, MO) and Cholera Toxin (100 ng/mL) (List Biological Laboratories, Campbell, CA). Human umbilical vein cells (HUVEC) were obtained from Dr. Janet Elliott (Department of Chemical and Material Engineering, University of Alberta) and cultured in rat tail type I collagen coated flasks with endothelial growth medium-2 with manufacturer's growth factor BulletKit™ (Lonza), 10% FBS, 100 unit/mL Penicillin and 100 μg/mL Streptomycin. Human bone marrow stromal cells (hBMSC) were obtained and maintained as described previously.[18]

Uptake of PEI-αLA/siRNA complexes in breast cancer cells

The cellular uptake of PEI-αLA/siRNA complexes was assessed in MDA-231 through flow cytometry and confocal microscopy using FAM-labelled siRNA. For flow cytometry studies, cells were seeded (10^5 cells/mL) in 24-well plate and grown overnight. The polymer/FAM-siRNA complexes were prepared at room temperature by incubating polymer and siRNA (ratio 6, w/w) in the medium and then directly added to the cells in triplicate. After 24 hr, cells were analyzed by flowcytometer as described earlier.[19] For confocal microscopy study, MDA-231 cells were seeded (10^5 cells/mL) on cover slips (15 mm diameter) inserted into 24-well plates and grown overnight. Cells were treated with the complexes as described above. After 24 hr cells were processed and observed under confocal microscope as described earlier.[19]

siRNA library screening

siRNA library screenings were conducted using siRNAs against 446 apoptosis-related genes (siGENOME Human Apoptosis siRNA Library; G-003905, GE Dharmacon). The desired cells (MDA-231 and MCF-10A) were seeded in 96-well cell culture plates using a Perkin Elmer Janus Automated Workstation. After 24 hr, 1.0 μM dilution plate sets were prepared from the 96-well 5.0 μM plates of the siRNA Library. Polymer solution was then added to the siRNA solutions (ratio 6, w/w) and allowed to complex for 30 min at room temperature. Then 10 μL of complexes was added to the cells (30 nM final siRNA concentration) in triplicate. When indicated, paired screens were conducted, where one screen received 20 μL of complete medium after 24 hr of incubation, whereas the other screen received 20 μL of recombinant human TRAIL (Life Technologies, Grand Island, NY) solution to make a final concentration of 5 ng/mL TRAIL. Treated cells were then incubated for another 48 hr, before the final evaluation of cell growth by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich,

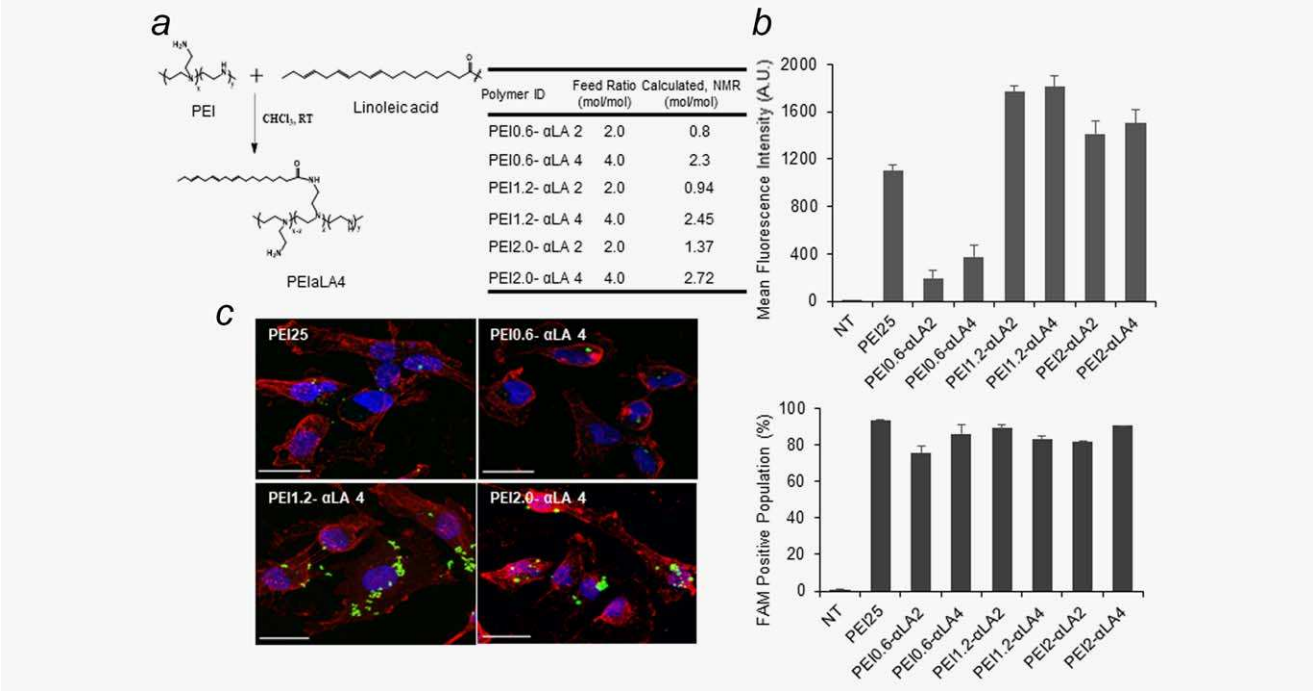

Figure 1. Scheme for synthesis of αLA-substituted PEIs and the obtained αLA substitutions for individual polymers (a), flowcytometer analysis of polymer/FAM-siRNA uptake using in MDA-231 cells (b) and confocal micrographs showing polymer/FAM-siRNA uptake using select αLA-substituted PEIs and PEI25 (c; scale bar is 20 μm). Complexes were prepared at the ratio of 6 (w/w) and MDA-231 cells were analyzed by flowcytometry or confocal microscopy after 24 hr of treatment. FAM-siRNA delivery efficiency of αLA substituted 1.2 kDa PEI was higher than PEI25 as revealed from confocal microscopy and flow cytometry. [Color figure can be viewed at wileyonlinelibrary.com]

St. Louis, MO) assay. To ensure the efficiency of siRNA silencing using the selected delivery system, CDC20 silencing siRNA (Cat # HSC.RNAI.N001255.12.1, IDT) was used as positive control, which was the most powerful siRNA we identified from a siRNA library against cell cycle proteins.[20] In addition, we used 2 scrambled siRNAs as negative controls in our screens. The siRNAs from the library are 21 mer siRNAs, so that one negative control was a scrambled 21-mer siRNA (CsiRNA; Ambion, Cat# AM4635). The positive control, CDC20 siRNA was a dicer-substrate 27-mer siRNA, so that the negative control siRNA for this reagent was a dicer-substrate scrambled 27 mer siRNA (Cat # DS NC1, IDT).

Validation of identified siRNA targets for cell growth inhibition

Efficacy of identified targets was validated in MDA-231 and MCF-7 cells by monitoring cell growth inhibition using the MTT assay. Cells were seeded (10^5 cells/mL) in 48-well plates and incubated for 24 hr. The polymer/siRNA complexes were prepared as described earlier and directly added to the cells. The final siRNA concentration was 30 nM. A custom synthesized two sets of siRNAs targeting BCL2L12 (cat#HSS.RNAI.N001040668.12.2 and HSS.RNAI.N001040 668.12.1, IDT), two sets siRNAs targeting SOD1 (cat# HSC.RNAI.N000454.12.1 and HSC.RNAI.N000454.12.2, IDT) and negative control scrambled siRNA were used. To determine synergistic effect with TRAIL, 20 μL of TRAIL solution was added to cells to give 5 ng/mL or 50 ng/mL TRAIL after 24 hr of siRNA transfection. Treatment groups without TRAIL received 20 μL of complete medium. After 48 hr of further incubation, MTT assay was proceed as described above.

Gene silencing by real-time PCR

After treatment with polymer/siRNA complexes, gene knockdown at the mRNA level was assessed by real-time PCR (qPCR). Briefly, MDA-231 and MCF-7 cells were seeded (10^5 cells/mL) in 6-well plates and allowed to grow overnight. Cells were then treated with polymer/siRNA complexes (ratio 6, w/w) at final siRNA concentration of 30 nM. Total RNA was isolated after 48 hr of transfection using TRIzol (Invitrogen). Then, RNA was converted into cDNA using M-MLV reverse transcriptase (Invitrogen) according to manufacturer's instruction. The qPCR was performed using 15 ng of each cDNA sample on StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA) with SYBR Green Mastermix containing ROX (MAF Center, University of Alberta, Edmonton, Canada). Two endogenous housekeeping gene, human GAPDH (Forward: 5′-TCA CTG TTC TCT CCC TCC GC-3′and Reverse: 5′-TAC GAC CAA ATC CGT TGA CTC C-3′) and human β-actin (Forward: 5′-GCG AGA AGA TGA CCC AGA T-3′ and Reverse: 5′- CCA GTG GTA CGG CCA GA-3′), specific BCL2L12 primers (Forward: 5′-CCC GCC CCT ATG CCC TTT TT-3′ and Reverse: 5′-ATA

ACC GGC CCA GCG TAG AA-3′) and SOD1 primers (Forward: 5′-GCA CAC TGG TGG TCC ATG AAA-3′ and Reverse: 5′-TGG GCG ATC CCA ATT ACA CC-3′) were used for amplification (all primers were obtained from IDT). Levels of mRNA for each gene were measured using the comparative threshold cycle method and presented as fold-change of the target relative to individual GAPDH and β-actin. In order to see effect of TRAIL on silencing of the targets, MDA-231 cells were treated with polymer/siRNA complexes (ratio, 6 w/w) at final siRNA concentration 30 nM in paired treatment. After 24 hr of transfection, one set received 50 μL of TRAIL to give a final concentration of 5 ng/mL and another set received 50 μL of complete medium. Total RNAs were extracted after 48 hr of TRAIL treatment and qPCR was performed as described above. In addition, kinetics of siRNA silencing was observed in MDA-231 cells by analyzing the mRNA levels of BCL2L12 and SOD1 by qPCR at different time points after siRNA treatment.

Analysis of apoptotic cell population

Percentage of cells undergoing apoptosis was determined by using FITC-Annexin V and Propidium Iodide staining (BD Biosciences). MDA-231 cells were seeded in the 12-well plate and treated with Polymer/siRNA complexes with or without TRAIL as described above. After 48 hr of TRAIL treatment apoptosis was assessed by the FITC-Annexin V and Propidium Iodine staining kit following the manufacturer's protocol. Briefly, cells were collected using Accutase® digestion and washed with HBSS. Then, cells were washed with apoptosis binding buffer (1×) and, aliquots of about 1×10^5 cells diluted in 100 μL of 1× binding buffer were incubated with 2.5 μL of FITC-Annexin V and 2.5 μL of Propidium Iodide in dark for 15 min at room temperature. Then, cells were analyzed with flow cytometer within 30 min.

Caspase activation assays

Cells were seeded in 24-well plate and treated with polymer/siRNA complexes TRAIL as described above. After 24 hr of TRAIL treatment, cells were lysed and cells extracts were collected. Total protein concentration in each extract was determined using the BCA Protein Assay (ThermoScientific, Waltham, MA) according to manufacturer's instruction. Caspase-3 and caspase-8 activity were determined using fluorogenic substrates, *N*-acetylaspartyl-glutamylvalinylaspartyl-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) and *N*-acetylleusylglutamylthreonylaspartyl-7-amino-4-triflurome-thylcoumarin (Ac-LETD-AFC), respectively, according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). Caspase activity was expressed as increase of relative fluorescent units per hour and normalized with respect of total protein content.

Statistical analysis

The data were presented as mean ± standard deviation of three different replicates and analyzed for statistical significance by Student's two-tailed t test (assuming equal variance). A value of $p < 0.05$ was considered significant. For siRNA library screening, relative cell growth was calculated as a percentage of cell growth in nontreatment control group and statistical significance was calculated by student's two-tailed t test and z scores. The outliers were singled out by selecting the responses with $-1.96 < z < 1.96$. z values were calculated by following equation,

$$Z = \frac{xi - \mu}{s},$$

where xi is the percentage of the cell growth compared to nontreatment cells for each well, μ is the average and s is the standard deviation of all xi in the whole plate.

RESULTS

Polymer synthesis, characterization and siRNA delivery

For effective delivery of siRNAs into breast cancer cells, we prepared a series of polymers. In previous studies,[14,20] linoleic acid (C18) substituted PEIs were identified as the most promising carrier. To further optimize the siRNA carriers, we synthesized a small cationic lipopolymer library where 0.6, 1.2 and 2.0 kDa PEIs were substituted with αLA (Fig. 1*a*). Modification of PEI with αLA was confirmed by the ^{1}H-NMR (Fig. S1, Supporting Information). A higher level of αLA was evident at higher lipid/polymer feed ratio during synthesis, but a similar level of substitution was obtained irrespective of the MW of the PEI (2–3 lipids/PEI). These polymers were screened for uptake of polymer/siRNA complexes in MDA-231 cells using flow cytometry (Fig. 1*b*). Hydrophobic modification significantly improved the siRNA uptake efficacy of the polymers to MDA-231 cells that was most evident with PEI1.2 and PEI2.0 irrespective of the level of αLA substitution. Among the polymers, PEI1.2-αLA4 showed highest uptake as revealed by the mean fluorescence intensity from the flow cytometer analysis. The siRNA uptake efficacy of αLA derivatives of PEI1.2 and PEI2.0 was significantly higher than the PEI25, the broadly effective nonviral gene delivery carrier and commonly used as a transfecting agent. Despite the differences in mean fluorescence intensity, percentage of FAM-siRNA positive population was similar for all groups, indicating uniform uptake of siRNA complexes among the cell population. Confocal micrograph images (Fig. 1*c*) showed that FAM-siRNA complexes with PEI25 were relatively small, uniformly distributed in all cells, but with low fluorescence intensity in each cell. On the other hand, FAM-siRNA complexes with PEI1.2-αLA4 and PEI2-αLA4 were distributed to all cells but in clusters resulting higher mean fluorescence intensity at distinct spots. From this screening, PEI1.2-αLA4 was selected as the most effective siRNA carrier for further experiment.

Identifying effective targets by siRNA library screening

In order to identify targets whose silencing could enhance TRAIL-induced apoptosis, a library of 446 apoptosis-related

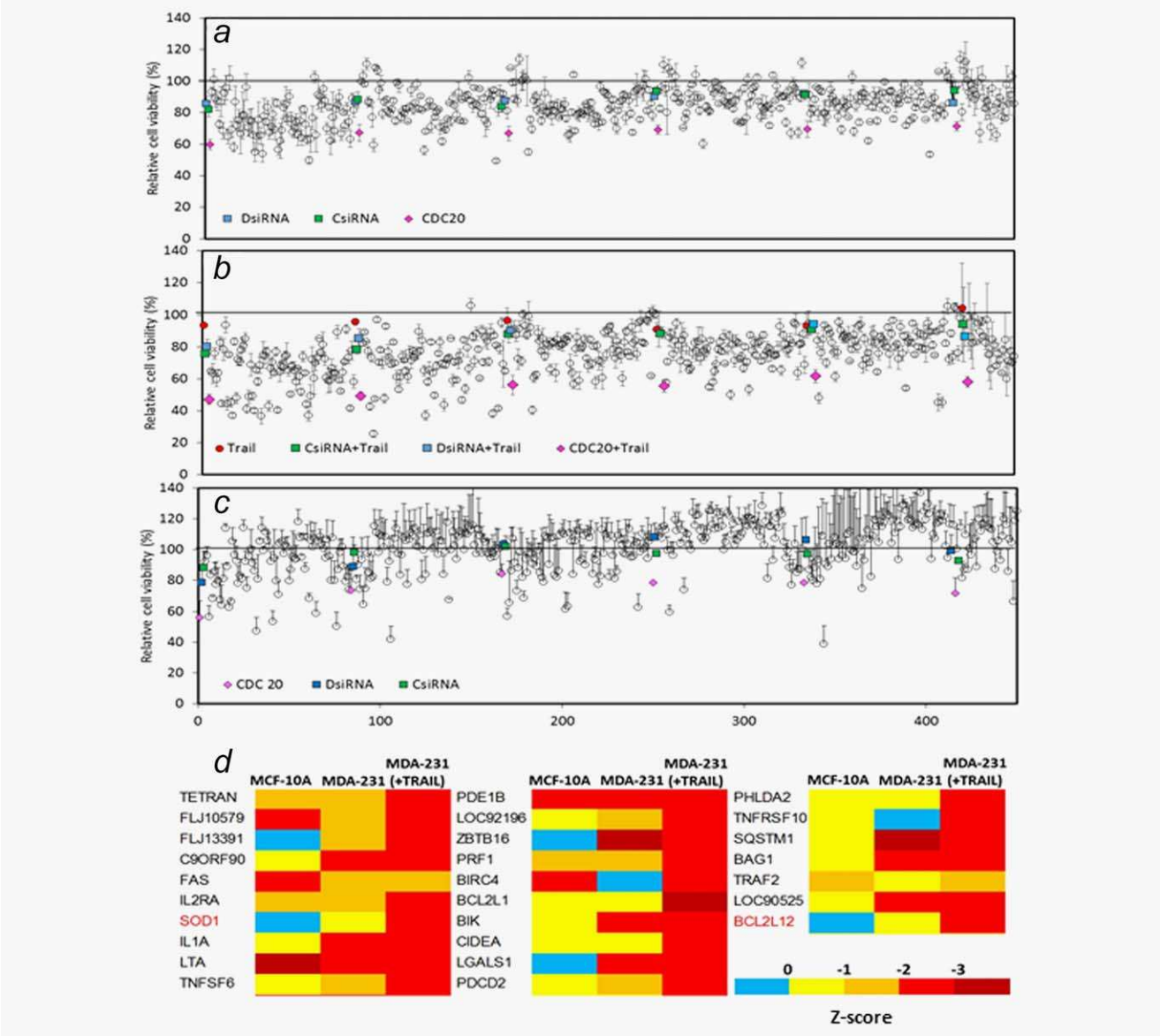

Figure 2. siRNA library screen in MDA-231 cells with (a) or without (b) TRAIL (5 ng/mL) and in MCF-10A cells (c). The relative cell growth for treated cells was calculated as a percentage of cell growth of non-treated group. Final concentration of siRNA used for cell treatment was 30 nM. CDC20 siRNA was used as positive control and DsiRNA and CsiRNA were two scrambled control siRNAs. (d) Heat map for the siRNAs that induced significant cell death ($z < -1.96$) in MDA-231 cells (with and without TRAIL) and MCF-10A cells. Many siRNAs including BCL2L12, SOD1, BCL2L2, FLJ13391 and LGALS1, showed significant cell death in the presence of TRAIL in MDA-231 cells without showing significant effects in MCF-10A cells (without TRAIL). [Color figure can be viewed at wileyonlinelibrary.com]

siRNAs were screened for inhibition of MDA-231 growth in the presence and absence of TRAIL. A siRNA against CDC20, a cell cycle protein whose silencing was most effective previously in inhibiting growth of MDA-231 cells,[20] served as a positive control. This siRNA provided more effective inhibition of cell growth than ~95% of the screened siRNAs (Fig. 2*a, b and c*), still confirming its potency among the screened siRNAs. As compared to siRNAs' effects on MDA-231 cells, most of the siRNAs in the library were unable to inhibit the growth of normal MCF-10A cells, although a few siRNAs also showed significant inhibition of growth in the latter cells (Fig. 2*c*).

Using the relative cell growth <70% and the *z* scores <–1.96 as a cut-off, 23 siRNAs were found to inhibit the growth of MCF-10A cells on their own. Among them, only 3 siRNAs in the presence of TRAIL and 2 siRNAs in the presence/absence of TRAIL significantly inhibited growth of MDA-231 cells, indicating a unique set of 18 siRNAs that are only effective in normal cells (and should be avoided). With the same cut-off criteria, only 14 siRNAs were found to inhibit growth of MDA-231 cells. Importantly, only 2 of them were effective on MCF-10A cells, leaving 12 siRNA affecting only MDA-231 cells, while 11 of them also showed significant growth inhibition in the presence of TRAIL in MDA-231

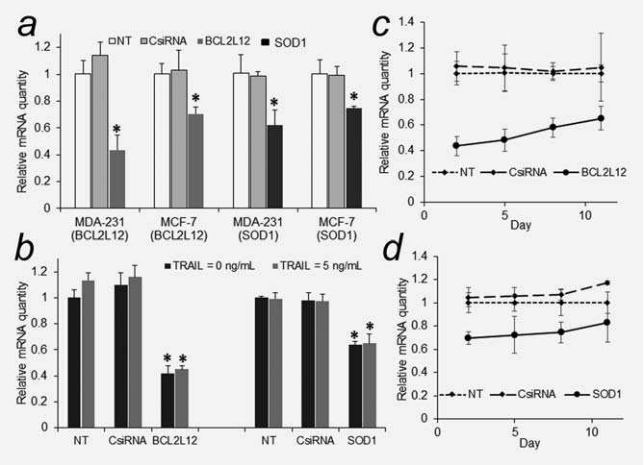

Figure 3. Analysis of mRNA by qPCR in MDA-231 and MCF-7 cells after 48 hrs of treatment with the indicated siRNAs (30 nM) (a). qPCR analysis of silencing effect of BCL2L12 and SOD1 siRNAs in MDA-231 in absence/presence of TRAIL (b) and time course analysis of BCL2L12 and SOD1 mRNAs after specific siRNA (30 nM) treatments (c and d) in MDA-231 cells. The relative quantity of mRNA transcripts was calculated relative to untreated cells using housekeeping genes GAPDH and β-actin as reference. After normalization, the results from the two reference genes were pooled together. *$p < 0.05$ compared with nontreated group.

cells. The critical targets which showed growth inhibition in the presence of TRAIL in MDA-231 cells ($z < -1.96$) were shown in Figure 2*d*. A total 27 siRNAs were found to inhibit growth of MDA-231 cells in the presence of TRAIL. Among them 5 siRNAs significantly inhibited MCF-10A cell growth (not desirable) and 11 of which retarded growth of MDA-231 cells without TRAIL. Importantly, 16 of the 27 siRNAs sensitized the TRAIL induced cell death in MDA-231 cells. Among the 16 siRNAs, some targets (e.g., TNFRSF10D, BCL2L1, TRAF2 and BIRC4) have been reported to be involved in TRAIL induced apoptosis. Functions of some of the identified targets (e.g., FLJ13391, PHLDA2) are not completely understood based on our literature search. Among these siRNAs, we selected relatively more potent BCL2L12 and SOD1 as "leads," which sensitized the TRAIL induced cell death in MDA-231 cells but did not affect growth of MCF-10A cells. We further validated the effects of these two targets on TRAIL-induced apoptosis in MDA-231 cells.

Validation of targets to enhance TRAIL induced cell death

In order to validate the involvement of BCL2L12 and SOD1 in TRAIL activity, the levels of targeted mRNAs were evaluated by qPCR after specific siRNA treatments. An independent set of siRNAs were secured from a different vendor, and MCF-7 cells were additionally used to explore siRNA efficacy with a different breast cancer cell model (Fig. 3*a*). The BCL2L12 and SOD1 mRNA levels were significantly reduced after treatment with siRNAs for 48 hr in both MDA-231 and MCF-7 cells. The silencing appeared to be effective to the same extent in both MDA-231 and MCF-7 cells at the siRNA concentration used (30 nM). In addition, we explored the effects of two additional siRNAs, each targeting BCL2L12 and SOD1 in both cell lines. These siRNAs were also able to silence the target mRNA (Fig. S2, Supporting Information). Then, we investigated the changes in the mRNA levels of BCL2L12 and SOD1 after treatment with combination of siRNA complexes and TRAIL in MDA-231 cells. TRAIL was added after 24 hr of siRNA complex treatment and mRNA levels were investigated after 48 hr exposure to TRAIL. Silencing efficiency of TRAIL and siRNA complexes was equivalent to the single treatment with siRNA complexes, indicating no effect of TRAIL on silencing of the siRNA targets (Fig. 3*b*). We next analyzed the time-course of silencing of BCL2L12 and SOD1 after treatment with siRNA complexes (Fig. 3*c* and *d*); maximum silencing of BCL2L12 and SOD1 were observed after Day 2 of siRNA treatment and the levels of mRNA increased with time. BCL2L12 mRNA was significantly lower than the untreated group up to Day 11 of siRNA treatment. However, SOD1 mRNA level was significantly lower than nontreated group up to Day 8 of siRNA treatment.

The effect of the combination of siRNAs and TRAIL were evaluated on cell viabilities of MDA-231 and MCF-7 cells. To optimize the dose of siRNA and TRAIL for synergistic effects, two concentrations of siRNA (15 and 30 nM) were employed in the presence of TRAIL (0–100 ng/mL). TRAIL was added 24 hr after siRNA complexes in these experiments, since this approach gave higher cell death than TRAIL treatment immediately after siRNA treatment (Fig. S3, Supporting Information). The MDA-231 cells were responsive to TRAIL induced cell death where concentration-dependent cell death was observed at <20 ng/mL TRAIL (Fig. 4*a* and *b*). Both siRNAs against BCL2L12 and SOD1 gave no significant cell death when used alone, but were able to enhance the cell death in the presence of TRAIL. The TRAIL response was significantly higher even at low concentrations (e.g., 5 ng/mL) with these siRNA treatments. On the other hand, MCF-7 cells showed an attenuated response to TRAIL even at higher concentrations (Fig. 4*c* and *d*), indicating a certain TRAIL resistance. The TRAIL effect on MCF-7 was increased with BCL2L12 and SOD1 siRNA treatments; siRNA silencing BCL2L12 resulted in significant effects with TRAIL at lower concentration (15 nM) whereas siRNAs silencing SOD1 was effective with TRAIL at higher concentration (30 nM) in MCF-7 cells. The siRNA silencing BCL2L12 had the ability to inhibit growth of MCF-7 cells at 30 nM without TRAIL, and sensitized the TRAIL-mediated inhibition of growth (Fig. 4*d*).

Based on these results, 30 nM siRNA and 5 ng/mL TRAIL was considered optimal for MDA-231 cells. Since no significant cell death was observed at 5 ng/mL TRAIL in MCF-7 cells (Fig. S4, Supporting Information), combination of 30 nM siRNA and 50 ng/mL TRAIL were considered optimal for this cells. The effects of these combinations were further evaluated in HUVEC and hBMSC. Both combinations of the

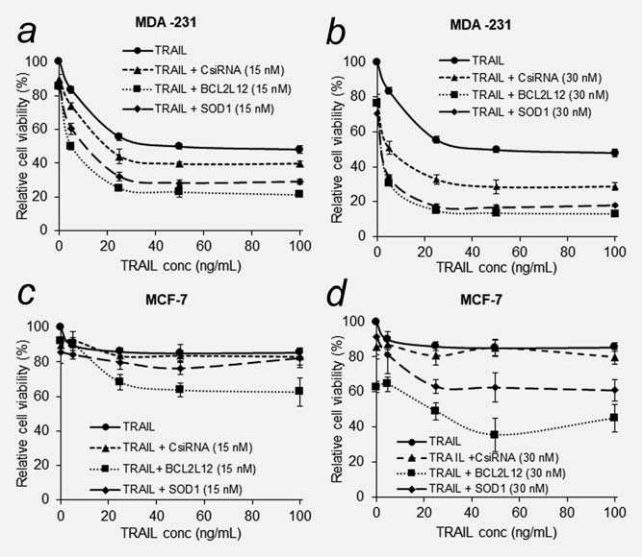

Figure 4. Inhibition of cell growth using siRNAs against BCL2L12 and SOD1 at 15 nM (a and c) and 30 nM (b and d) concentration with different concentration of TRAIL (0–100 ng/mL) in MDA-231 (a and b) and MCF-7 (c and d) cells. Cells were first treated with siRNA complexes (24 hr) and incubated with TRAIL for another 48 hr.

siRNA and TRAIL that previously inhibited the growth of MDA-231 and MCF-7 had no effects on HUVEC cells (Fig. 5*a* and *b*), and BCL2L12 and SOD1 siRNAs did not sensitize HUVEC cells to TRAIL induced cell death. In hBMSC cells, although 5 ng/mL of TRAIL did not retard growth, higher dose of TRAIL (50 ng/mL) inhibited cell growth to some extent (∼20%). Both siRNAs at 30 nM did not affect growth of hBMSC growth significantly, but their combination with TRAIL had mixed response. Silencing BCL2L12 did not show any synergistic effect with TRAIL (5 or 50 ng/mL); while silencing SOD1 enhanced TRAIL (5 or 50 ng/mL) induced cell death in hBMSC (Fig. 5*c* and *d*). The response of MDA-231 and MCF-7 cells were similar to the above response (See Fig. S5, Supporting Information). In addition, a different set of siRNAs targeting BCL2L12 and SOD1 also sensitized the TRAIL-induced cell death in both MDA-231 and MCF-7 cells (Fig. S6, Supporting Information). However, these siRNAs were less efficient than the previous set of siRNAs.

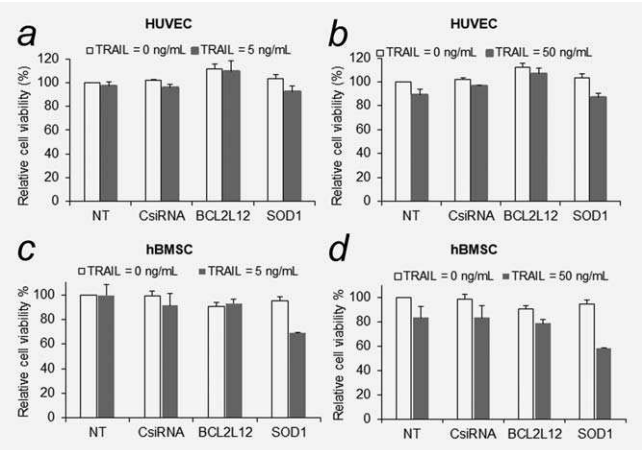

Figure 5. Effects of siRNA and TRAIL combinations in untransformed cells; HUVEC cells (a and b) and hBMSC (c and d). Concentration of siRNA used was 30 nM and TRAIL was 5 ng/mL (a and c) 50 ng/mL (b and d) which were optimal to retard growth of breast cancer cells; MDA-231 and MCF-7 cells, respectively.

Apoptosis induction and caspase-3 activation by TRAIL/siRNA combination

Since MDA-231 cells were more sensitive towards TRAIL-induced apoptosis, we further explored the ability of identified siRNAs to enhance TRAIL induced apoptosis in MDA-231 cells (Fig. 6). The percentage of early apoptotic population was small as compared to late apoptotic population in all treated groups (Fig. 6*b*). TRAIL (5 ng/mL) alone did not induce a clear increase in apoptosis, based on the percentage of late apoptotic cells in TRAIL-treated cells (Fig. 6*c*). Induction of apoptosis by siRNAs targeting BCL2L12 and SOD1 was not significantly higher than by control siRNA in the absence of TRAIL. On the other hand, combination of TRAIL and siRNA targeting BCL2L12 or SOD1 induced significant apoptosis; with the combination of TRAIL/BCL2L12 siRNAs, late apoptotic population was $71.1 \pm 2.5\%$ whereas the late apoptotic population was $61.3 \pm 3.1\%$ with the combination of TRAIL/SOD1 siRNAs.

In order to probe which caspases were activated to induce apoptosis, we investigated the level of caspase-3 and caspase-8 activation by the TRAIL and siRNAs in MDA-231 cells (Fig. 6*d* and *e*). As compared to nontreatment group, TRAIL (5 ng/mL) significantly induced the activation of caspase-3 and caspase-8, even though there was no apoptosis induction based on Annexin/PI staining. Silencing BCL2L12 and SOD1 siRNAs alone (in the absence of TRAIL) was unable to activate the caspase-3 and caspase-8. However, the combination of TRAIL and either BCL2L12 or SOD1 siRNA increased the caspase-3 activity by ∼3-fold (Fig. 6*d*). The caspase-8 activation by either of siRNA (BCL2L12 or SOD1), on the other hand, and the TRAIL combination was not significant than the control siRNA and TRAIL combination. Doing an assessment of caspase-8 activity at earlier time point (3 hr vs. 24 hr as in Fig. 6*e*) gave similar trends (Fig. S7, Supporting Information). Hence, siRNAs against BCL2L12 or SOD1 enhanced TRAIL induced apoptosis via synergistically activating caspase-3 in MDA-231 cells without any evidence of caspase-8 involvement.

DISCUSSION

The siRNA approach has become a powerful tool for its specificity and efficiency to knock-down therapeutic targets.[21–23] However, safe and efficient delivery systems for siRNA are paramount for a functional effect. Lipid moieties such as linoleic and caprylic acids were used in our previous studies to substitute PEI amines, which converts the ineffective polymers into effective siRNA delivery agents via increasing the interaction with anionic cell membrane, facilitating their

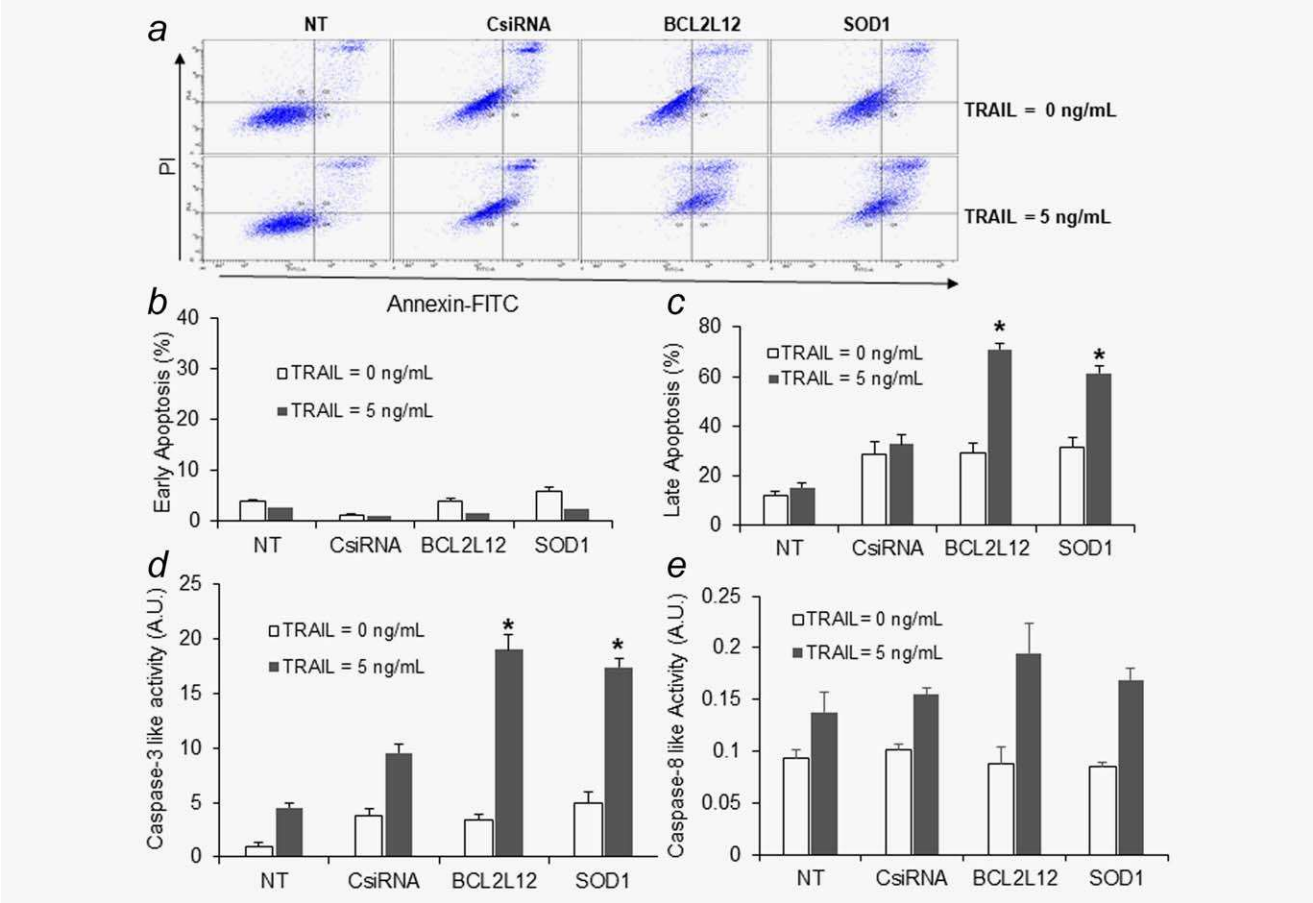

Figure 6. Annexin-FITC/PI apoptosis and caspases assay in MDA-231 cells. MDA-231 cells were treated with siRNAs (30 nM) targeting BCL2L12 and SOD1 followed by addition of 5 ng/mL TRAIL. After 48 hr of TRAIL treatment apoptosis assay was conducted (a). Early apoptotic population (b) was the percentage of Annexin$^+$/PI$^-$ population and late apoptotic population (c) was the percentage of Annexin$^+$/PI$^+$ population. Percentage of apoptotic cells after treatment with combination of TRAIL and either siRNA was significantly higher than TRAIL and siRNAs alone. Effects of siRNA, TRAIL and their combination on the caspase-3 (d) and caspase-8 activity (e) in MDA-231 cells. MDA-231 cells were treated with siRNA (30 nM) targeting SOD1 and BCL2L12 for 24 hr, after which TRAIL was added (0 or 5 ng/mL) and caspase activities were determined after 24 hr. Caspase-3 was significantly increased after siRNA and TRAIL combination treatment. *$p < 0.05$ compared with CsiRNA and TRAIL. [Color figure can be viewed at wileyonlinelibrary.com]

cellular entry.[16,20] Although we reported several polymers in the past for siRNA delivery to breast cancer cells,[14,16,20] αLA substituted PEIs used in this study appear to be the most effective system to-date and they have been used here to identify targets for enhancing TRAIL sensitivity in breast cancer cells.

TRAIL has been recognized as a potent agent to induce apoptosis in malignant cells, but the underlying determinants of TRAIL sensitivity are not clearly understood. Apoptosis-related proteins that regulate TRAIL-induced apoptosis could be important, as they could be used as predictive biomarkers of TRAIL sensitivity and/or provide additional targets for enhancing TRAIL-induced apoptosis. Similar to our siRNA library screenings in this study, an independent group also recently identified several positive and negative regulators of TRAIL action by using RNAi-based screen in MDA-231 cells.[24] The reported screen included many kinases and antiapoptotic proteins as negative regulator of TRAIL, but little overlap was evident between our results and the results in that study. Of the 16 genes selected as putative negative regulator of TRAIL in this independent study, only BCL2L1 was common with our results. Although the same type of cell was used for both studies, several significant differences were noted. First, we performed screening of human apoptosis-related siRNAs (446 genes), whereas the prior study was focused on human kinome (691 genes), phosphatome (320 genes) and 300 additional genes. Second, our selection of targets was based on the relative cell growth after 72 hr after treatment, whereas the other study measured caspase-3/7 activation 1 hr after addition of TRAIL. Another important difference was the delivery system; while we used a polymeric carrier for siRNA delivery, that study employed a liposomal carrier (RNAiMax^{TM}) and it is possible that physiological differences could arise due to carrier effects on the cells. Another siRNA library screening of 510 genes (380 kinases, 20 genes of interest, 100 genes of unknown function and 10

genes that play a role in apoptosis and TRAIL-mediated signaling) was also conducted to reveal regulators of TRAIL in HeLa cells.[25] Evaluation was based on cell viability after 48 hr of siRNA treatment followed by 20 hrs TRAIL treatment, which is a similar strategy to our study. Since the siRNA targets and the cell line were significantly different from this study, none of the top 20 negative regulator of TRAIL matched the outcome of our screening.

Several well-established antiapoptotic proteins including BCL2L1 (BCL-XL) and BIRC4 (XIAP) were identified in our hands as targets, whose silencing enhanced TRAIL-induced cell death, supporting the validity of the screening results. Another identified target, TNFRSF10D (tumor necrosis factor receptor superfamily member 10d) and TRAF2 were already known to play an inhibitory role in TRAIL-induced cell apoptosis.[10,26] In addition to these targets, we reported two new targets, BCL2L12 and SOD1, for the first time to sensitize TRAIL induced apoptosis. BCL2L12 (for BCL2-like-12) is a proline-rich and BH2 domain-containing protein, which is known to inhibit effector caspase-3 and −7.[27,28] Two independent studies in glioblastoma showed that BCL2L12 inhibits caspase-7 via physical interaction[27] and caspase-3 via induction of the oncoprotein αβ-crystallin, which then interacts and inhibits caspase-3.[28] Our study showed that BCL2L12 silencing did not increase the activation of caspase-3 by itself but synergistically activated caspase-3 in the presence of TRAIL. Although the role of BCL2L12 is well characterized in glioblastoma, its role in breast cancer is not completely understood and remains paradoxical. BCL2L12 was found highly expressed in low-stage breast cancer clinical samples.[29] Based on the gene expression analysis in breast cancer tissues, over-expression of BCL2L12 was speculated to lead to lower relapse or mortality rate[29] in contrast to its expected role as an anti-apoptotic protein. A shRNA mediated silencing of BCL2L12 resulted in acquired resistance to cisplatin in MDA-231 cells.[30] Contrary to this result, siRNA mediated silencing of BCL2L12 sensitized MDA-231 and MCF-7 cells to doxorubicin and cisplatin induced apoptosis in a separate study,[31] in line with its expected role as anti-apoptotic protein. In our study, silencing BCL2L12 sensitized MDA-231 and MCF-7 cells to TRAIL-induced apoptosis, which was in line with the latter study.

Another target, SOD1 is well known to catalyze the conversion of superoxide ion (O_2^-) into H_2O_2 and O_2 to maintain low levels of reactive oxygen species (ROS) in lung adenocarcinoma cells.[32] Transformed cells have persistently higher levels of ROS than the non-transformed cells because of increased metabolic activity and the dysregulation of redox balance. Excessive amount of ROS results in oxidative damage to lipids, proteins and cellular DNA, and induces apoptosis.[33] Hence, transformed cells including breast cancer cells have typically elevated levels of SOD1 to maintain cellular ROS under a critical threshold and protect cells from the ROS damage.[34] Inhibition of SOD1 with small molecules had led to cell death in various cancer,[35,36] including breast cancer models *in vitro*.[34] In addition, many apoptotic stimuli upregulate the SODs among the other prosurvival molecules which delays apoptosis.[37] Downregulation of SOD1 using a small molecule was attempted to enhance TRAIL induced apoptosis; embelin sensitized the inflammatory breast cancer rSUM149 cells to TRAIL-induced apoptosis,[38] but embelin is an inhibitor of XIAP as well so that its effect might have been mediated by both mechanisms. Although an siRNA against SOD1 were reported to inhibit growth of lung adenocarcinoma cells *in vitro,*[32] a synergistic effect of SOD1 silencing with TRAIL has not been reported before.

We have also shown that the mechanism of increased apoptosis is a result of enhanced caspase-3 activation in MDA-231 cells, which is a central event in apoptosis of malignant cells. However, MCF-7 cells are known to have lost the expression of capsase-3[39] and, accordingly, we were not able to detect caspase-3 activity in the MCF-7 cells. However, significant cell death was still observed in this cell type after treating with TRAIL and siRNA targeting BCL2L12 or SOD1. Indeed, TRAIL resistance in MCF-7 was reversed by silencing the BCL2L12 and SOD1. Several independent studies previously showed that despite the absence of caspase-3, MCF-7 can undergo apoptosis after induction of various stimuli.[40,41] One study showed apoptosis induction in MCF-7 cells via sequential activation of caspases-9, −7 and −6 without involvement of caspase-3.[40] In this study, we evaluated activation of caspase-3 using substrate DEVD, which is also a substrate for caspase-7. DEVDase activity was not significant in MCF-7 cells after treatment implying lack of involvement of caspase-7. However, another independent study showed apoptotic induction in MCF-7 cells after treatment with staurosporin via activation of caspase-6 independent of caspase-3 and −7.[42] Further studies are needed to find out the specific mechanism associated with MCF-7 response with the proposed combination therapy.

CONCLUSIONS

In this study, we identified and explored two new targets, namely BCL2L12 and SOD1 to enhance TRAIL-induced apoptosis in breast cancer cells by screening human siRNA library using lipid-substituted PEI polymer as a non-viral siRNA carrier. The siRNA-mediated silencing of these targets was able to sensitize breast cancer cells a to cell death with minimum effects on the normal cells. These siRNA targets BCL2L12 and SOD1 enhanced the TRAIL-induced apoptosis via synergistically activating caspase-3 in MDA-231 cells. Further studies to elucidate the mechanisms of these targets to regulate TRAIL-induced effects in MCF-7 are needed. Hence, the present study pointed out the importance of a combination therapy with the highly promising TRAIL protein and siRNAs targeting two specific apoptosis mediators to retard growth of breast cancer with minimal effect on normal cells.

ACKNOWLEDGEMENTS

The authors RBKC and HU are the founder and shareholders in RJH Biosciences Inc. that are developing the lipopolymers for medical applications.

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