Published in final edited form as:

J Cell Biochem. 2018 July ; 119(7): 6216–6230. doi:10.1002/jcb.26848.

Calmodulin antagonist enhances DR5-mediated apoptotic signaling in TRA-8 resistant triple negative breast cancer cells

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Abstract

Patients with triple negative breast cancer (TNBC) have no successful “targeted” treatment modality, which represents a priority for novel therapy strategies. Upregulated death receptor 5 (DR5) expression levels in breast cancer cells compared to normal cells enable TRA-8, a DR5 specific agonistic antibody, to specifically target malignant cells for apoptosis without inducing normal hepatocyte apoptosis. Drug resistance is a common obstacle in TRAIL-based therapy for TNBC. Calmodulin (CaM) is overexpressed in breast cancer. In this study, we characterized the novel function of CaM antagonist in enhancing TRA-8 induced cytotoxicity in TRA-8 resistant TNBC cells and its underlying molecular mechanisms. Results demonstrated that CaM antagonist(s) enhanced TRA-8 induced cytotoxicity in a concentration and time-dependent manner for TRA-8 resistant TNBC cells. CaM directly bound to DR5 in a Ca²⁺ dependent manner, and CaM siRNA promoted DR5 recruitment of FADD and caspase-8 for DISC formation and TRA-8 activated caspase cleavage for apoptosis in TRA-8 resistant TNBC cells. CaM antagonist, trifluoperazine, enhanced TRA-8 activated DR5 oligomerization, DR5-mediated DISC formation, and TRA-8 activated caspase cleavage for apoptosis, and decreased anti-apoptotic pERK, pAKT, XIAP, and cIAP-1 expression in TRA-8 resistant TNBC cells. These results suggest that CaM could be a key regulator to mediate DR5-mediated apoptotic signaling, and suggests a potential strategy for using CaM antagonists to overcome drug resistance of TRAIL-based therapy for TRA-8 resistant TNBC.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article except that Dr. Donald J. Buchsbaum has intellectual property rights in TRA-8.

Keywords

Calmodulin antagonist; death receptor 5; DISC; DR5 oligomerization; apoptosis; TRA-8 resistant TNBC cells

Breast cancer is the unregulated growth of cells from breast tissue, including carcinomas of breast epithelial tissue layers, adenocarcinoma of breast glandular tissue and sarcomas of breast connective tissue [1]. Breast cancer is one of the most common malignancies among American women and affects an estimated 1.3 million women annually worldwide [2, 3]. Patients with triple negative breast cancer (TNBC) [4, 5] that occupies about 15–20 percent of breast cancers, have no successful “targeted” treatment strategy as anti-estrogen therapy in ER-positive breast cancer or anti-HER2 therapy in HER2-amplified tumors, which represent a priority for novel therapy strategies [6]. Death receptor 5 (DR5) has been shown to be an important regulator in breast cancer pathogenesis [7–10]. DR5 is a member of the tumor necrosis factor (TNF) receptor superfamily and is one of five receptors bound by TNF receptor apoptosis inducing ligand (TRAIL) [11–13]. TRAIL has been reported with strong antitumor activity [11–13], however, TRAIL can induce apoptosis of normal human hepatocytes [14]. Upregulated death receptor 5 (DR5) expression levels in breast cancer cells compared to normal cells enable TRA-8, a DR5 specific agonistic antibody, to specifically target malignant cells for apoptosis without inducing normal hepatocyte apoptosis [8–10]. DR5 transduces its apoptotic signal via the death inducing signaling complex (DISC) formation and the activation of caspase signaling [15]. DISC formation is critical for signaling apoptosis [16, 17]. DR5 mutants could affect the proper assembly of DISC [18–20] and the degree of DR5 oligomerization is directly related to the TRA-8 driven cytotoxicity in breast cancer [21]. Drug resistance is a common obstacle in TRAIL-based therapy for breast cancer, including TNBC [10, 22–24]. Basal B subtype of TNBC cell lines have substantial *in vitro* sensitivity to TRAIL-mediated cytotoxicity in contrast to TRAIL resistance by basal A subtype of TNBC cell lines [25]. Half of the basal A subtype TNBC cell lines are resistant to TRA-8/TRAIL treatment, including HCC1143 and HCC1937 basal A TNBC cell lines [23]. Regulation of DR5-mediated apoptosis is a promising approach to prevent breast cancer progression and to overcome drug resistance [8–10, 23, 26].

Calmodulin (CaM), an intracellular mediator for Ca^{2+} signals, regulates various cellular processes [27, 28]. CaM is overexpressed in breast cancer [29, 30]. CaM antagonist treatment of breast cancer cells shows the inhibition of cell growth or the induction of apoptosis in a time-dependent manner [31–34]. Our recent studies have shown that CaM directly binds to DR5 in a Ca^{2+} dependent manner and CaM binding to DR5-mediated DISC to signal apoptosis in TRA-8 sensitive ER-positive and triple negative breast cancer cells [35, 36]. An earlier study show that tamoxifen, one of the CaM antagonists, induces apoptosis via $\text{ER}\beta$ in TRA-8 sensitive basal B TNBC, and combined tamoxifen and TRA-8 treatment for TRA-8 sensitive basal B TNBC showed an antagonistic effect for antitumor effect [37]. Understanding the role of CaM and CaM antagonist in regulating DR5-induced DISC formation for apoptosis in TRA-8 resistant TNBC could lead to the identification of novel strategies and drug targets, including more specific and potent agent development, for enhancing apoptosis to overcome drug resistance for TNBC treatment.

In this study, we characterized the novel function of CaM antagonists in enhancing TRA-8 induced cytotoxicity in TRA-8 resistant TNBC cells and its underlying molecular mechanisms. Results demonstrated that CaM antagonists enhanced TRA-8 induced cytotoxicity at the optimized concentration and treatment time. CaM bound to DR5 in a Ca^{2+} -dependent manner and CaM knockdown promoted DR5 recruitment of FADD and caspase-8 for DISC formation in TRA-8 resistant TNBC cells. CaM antagonist, trifluoperazine (TFP), enhanced TRA-8 activated DR5 oligomerization, DR5-mediated DISC formation and TRA-8 activated caspase cleavage for apoptosis, and decreased anti-apoptotic pERK, pAKT, XIAP, and cIAP-1 expression in TRA-8 resistant TNBC cells. These results suggest that CaM could be a key regulator to mediate DR5-mediated apoptotic signaling, and suggests a potential strategy for using CaM antagonists to overcome drug resistance of TRAIL-based therapy for TRA-8 resistant TNBC.

MATERIALS AND METHODS

Cell Culture and Reagents

TRA-8 resistant TNBC cell lines HCC1143 and HCC1937 [23] were kindly provided by Dr. Donald Buchsbaum at the University of Alabama at Birmingham (UAB) (Birmingham, AL). HCC1143 and HCC1937 breast cancer cells were cultured in RPMI-1640 media (Hyclone GE Healthcare Lifesciences, South Logan, UT) supplemented with 1 mM sodium pyruvate and 4500 mg/L glucose. HCC1143 and HCC1937 cells were maintained in 1% penicillin, 1% streptomycin, 1% amphotericin B, and 20% FBS at 37°C, 5% CO_2 and 95% relative humidity. Calmodulin antagonists trifluoperazine (TFP) and tamoxifen (TMX) were purchased from MP Biomedicals (Solon, OH). Calmodulin antagonist, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), was purchased from Tocris Bioscience (Bristol, United Kingdom).

Cell Viability Assay Using ATPLite

HCC1143 or HCC1937 cells (3000 cells /well) were seeded onto tissue-culture treated 96-well plates (Costar #3595, Corning, Inc., NY) in 100 μL of complete media, as described in the cell culture and reagents section. HCC1143 or HCC1937 cells were incubated overnight at 37°C before initiating drug treatments. CaM antagonists TFP, TMX, or W-7 were diluted in culture medium from stock solution immediately before use. Cell viability was assessed by measuring cellular ATP levels with the ATPLite luminescence-based assay (Perkin Elmer Waltham, MA), following the manufacturer's recommended protocol.

For cell viability experiments, HCC1143 or HCC1937 cells were treated with a serial dilution of 0, 0.156, 0.313, 1.25, 10, 20, or 25 μM TFP alone, or serial dilutions of 0, 15.6, 31.3, 62.5, 125, 500, or 1000 ng/mL TRA-8 alone, or the combination of each TFP and each TRA-8 concentration for 24 hours. For the time dependence of TFP on HCC1143 cell viability experiments, HCC1143 cells were treated with 500 ng/mL TRA-8 only, 20 μM TFP only, or the combination of 500 ng/mL TRA-8 with 20 μM TFP for the time points of 0, 2, 4, 8, 12, 16, or 24 hours. We also determined the effect of CaM antagonists TMX or W-7 at varied concentrations on TRA-8 induced cytotoxicity of TRA-8 resistant HCC1143 cells. HCC1143 cells were treated with 0, 10, 20, or 25 μM TMX or W-7 only or the combination

of 500 ng/mL TRA-8 and TMX or W-7 at the concentration of 10, 20, or 25 μ M for 24 hours.

For fluorescence microscope imaging and analysis experiments using Cy5.5 dye conjugated to TRA-8, as a control experiment, we tested the effect of Cy5.5 dye conjugation to TRA-8, on TRA-8 cytotoxicity with or without TFP in HCC1143 cells. HCC1143 cells were treated with 500 ng/mL Cy5.5-TRA-8 or TRA-8 alone, or in combination with 10 μ M TFP or 20 μ M TFP for 24 hours. Following drug treatment, HCC1143 cell viability was assessed using ATPLite assay.

Apoptosis and Caspase Signaling Analysis

The effect of TFP on DR5-mediated apoptosis and caspase cleavage in TRA-8 resistant TNBC HCC1143 or HCC1937 cells was characterized with Annexin V and propidium iodide (PI) apoptosis assay and western blot analysis. HCC1143 or HCC1937 cells (5×10^6) were seeded on 100 mm dishes and cultured in media overnight. The cells were untreated (control) or treated with 500 ng/mL TRA-8 only, treated with 20 μ M TFP only, or treated with 20 μ M TFP and 500 ng/mL TRA-8 for 16 hours. The apoptosis assay was performed using the Dead Cell Apoptosis Kit with Annexin V FITC and propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol. Following each treatment, cells were stained with annexin V conjugated to green-fluorescent FITC dye and the red-fluorescent propidium iodide (PI) nucleic acid binding dye. Apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show no fluorescence. Fluorescence images were taken using a Nikon eclipse TE2000-S microscope equipped with a Hamamatsu ORCA-flash 4.0 camera (Hamamatsu Corporation USA Headquarters, Bridgewater, NJ). The mean fluorescence intensity that is the average fluorescence intensity of the pixels in the image area measured, for Annexin V FITC fluorescence images and for PI fluorescence images for apoptosis assay, was determined using the ImageJ 1.50i software [38]. For western blot analysis, following each treatment, cells were washed with cold PBS and lysed with RIPA buffer with 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA). Samples were sonicated on ice for 10 seconds, and total protein concentration was determined using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA). Then 6 $\times$ laemmli buffer with 2-mercaptoethanol was added to samples, 50 μ g of total protein were loaded for western blot.

To characterize the effect of CaM knockdown on DR5-mediated caspase cleavage in TRA-8 resistant TNBC cells, DR5-mediated caspase cleavage in HCC1143 cells that were treated with control siRNA or CaM siRNA, was characterized with western blot analysis. HCC1143 cells were nucleofected using 5 μ g control siRNA or siRNAs generated against calmodulin 1, calmodulin 2, and calmodulin 3 together with siRNA Reagent System (sc-45064) (Santa Cruz biotechnology, Inc., Santa Barbra, CA) per the manufacturer's protocol. Following nucleofection, cells were transferred to complete media for HCC1143 cells and incubated for 12 hours. Then, the HCC1143 cells pretreated with siRNA control or CaM siRNA were treated with or without 500 ng/mL TRA-8 for 3 hours. The interference of CaM expression by CaM siRNAs and caspase-8, caspase-3, and PARP-1 cleavage was verified with western blot analysis. For western blot analysis of the DR5-mediated caspase cleavage in HCC1143

cells that were treated with control siRNA or CaM siRNA, following each treatment, cells were washed with cold PBS and lysed with RIPA buffer with 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA). Samples were sonicated on ice for 10 seconds, and total protein concentration was determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA). Then 6× laemmli buffer with 2-mercaptoethanol was added to samples, 50 µg of total protein were loaded for western blot.

Protein Complex Immunoprecipitation

To determine CaM-DR5 binding in TRA-8 resistant TNBC cells, DR5 was immunoprecipitated (IP) from HCC1143 cell lysates using TRA-8 conjugated sepharose beads [8], which were kindly provided by Dr. Tong Zhou at UAB. Iso-IgG conjugated sepharose beads (Cell Signaling Technologies Beverly, MA, specificity: none, source: mouse, Cat. 3420S, Lot # 3 Cat.: 3420S, Lot #: 3) were used for a control IP experiment. HCC1143 cells were lysed using a 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA) buffer at 4°C for 30 minutes. The total protein content was determined using BCA assay (Thermo Fisher Scientific, Waltham, MA), and 5 mg/mL of total protein cell lysates were incubated with TRA-8 conjugated sepharose beads or mouse Iso-IgG conjugated sepharose beads overnight at 4°C. Following overnight incubation, TRA-8 conjugated sepharose beads or mouse Iso-IgG conjugated sepharose beads were washed using a 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.5% Triton X-100 washing buffer five times. After washing, 40 µL of 2× laemmli sample buffer with 2-mercaptoethanol was added to the beads to elute the proteins for western blot analysis.

To characterize the effect of CaM knockdown on DR5-mediated DISC formation in TRA-8 resistant TNBC cells, DR5-mediated DISC in HCC1143 cells was immunoprecipitated with cells treated with control siRNA or CaM siRNA. HCC1143 cells were nucleofected using 5 µg control siRNA or siRNAs generated against calmodulin 1, calmodulin 2, and calmodulin 3 together with siRNA Reagent System (sc-45064) (Santa Cruz biotechnology, Inc., Santa Barbra, CA) per the manufacturer's protocol. Following nucleofection, cells were transferred to complete media for HCC1143 cells and incubated for 12 hours. The interference of CaM expression by CaM siRNAs was verified with western blot analysis. Then, the HCC1143 cells pretreated with siRNA control or CaM siRNA were treated with or without 500 ng/mL TRA-8 (Daiichi-Sankyo Company, Ltd, Tokyo, Japan), gift from Dr. John Mountz's lab at UAB, for 3 hours. To determine the effect of CaM antagonist, TFP, on DR5-mediated DISC in TRA-8 resistant TNBC cells, HCC1143 cells treated with different conditions: 1) untreated, 2) treated with 500 ng/mL TRA-8 only, 3) treated with 10 µM or 20 µM of TFP only, or 4) pretreated with 10 µM or 20 µM of TFP for 19 hours followed by 500 ng/mL of TRA-8 treatment for 3 hours.

Following each treatment for different experiments as described above, HCC1143 cells were lysed using 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA) buffer at 4°C for 30 minutes. The total protein concentration was determined with BCA assay (Thermo Fisher Scientific, Waltham, MA) and a total protein concentration of 5 mg/mL was used for co-IP

experiments. HCC1143 cell lysates were precleared with 40 μ L of 4B-sepharose beads (Sigma-Aldrich, St. Louis MO) at 4 °C for 1 hour. DR5-mediated DISC was co-IP from HCC1143 cell lysates using anti-DR5 (D-6) antibody (Santa Cruz Biotechnology, Inc., Santa Barbra, CA. Specificity: DR5 source: mouse, Cat. sc-166624, Lot# C2811) with protein A/G Plus-Agarose beads (Santa Cruz biotechnology, Inc., Santa Barbra, CA). Cell lysates were incubated with anti-DR5 (D-6) antibody overnight at 4°C, then incubated with 40 μ L of protein A/G PLUS-Agarose beads slurry for 2 hours the following morning. Following IP experiments, anti-DR5 (D-6) antibodies and A/G PLUS-Agarose beads were washed using 50 mM Tris-HCL pH 7.5, 150 mM NaCl, 0.5% Triton X-100 wash buffer five times. After washing, 40 μ L of 2 $\times$ laemmli sample buffer with 2-mercaptoethanol were added to IP samples. IP and lysate samples in laemmli sample buffer were heated at 100 °C for 5 minutes and then centrifuged for 1 minute at 3000 rpm. 40 μ L of IP sample and 40 μ g of total protein from lysate were loaded into a SDS-PAGE gel for western blot analysis.

As a control experiment, DR5 was immunoprecipitated from TRA-8 untreated and TRA-8 treated HCC1143 cell lysates using anti-DR5 (D-6) antibody to test CaM association with DR5 with or without TRA-8 treatment.

CaM Pull-Down Assay

To further characterize CaM and DR5 interaction in a Ca^{2+} -dependent manner, DR5 was pulled-down using recombinant CaM conjugated 4B-sepharose beads from HCC1143 lysates supplemented with 1 mM Ca^{2+} or 2 mM Ca^{2+} chelator, EGTA. HCC1143 breast cancer cells were lysed with phosphate buffered saline with 1% Triton X-100 (PBST), and 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA) supplemented with 1 mM CaCl_2 or 2 mM EGTA. Cell lysates were incubated with 100 μ L of recombinant CaM conjugated 4B-sepharose beads (GE Healthcare Bio-Science) or control 4B-sepharose beads (Sigma-Aldrich, St. Louis MO) for 4 hours at 4°C. After CaM pull-down, beads or control 4B-sepharose beads were washed 5 times with the PBST buffer with 1 mM Ca^{2+} or 2 mM EGTA, and then 100 μ L of 2 $\times$ laemmli buffer was added to elute proteins. 35 μ L of IP sample was used for western blot analysis.

Analysis of inhibitors of apoptosis (IAP) expression and AKT and ERK phosphorylation

To characterize the effect of combined TFP and TRA-8 on anti-apoptotic protein expression, the expression of DR5, pERK, pAKT, XIAP and cIAP-1 in TRA-8 resistant TNBC cells was characterized with western blot analysis. 5×10^6 HCC1143 cells were seeded on 100 mm dishes and cultured in media overnight. The cells were untreated (control) or treated with 500 ng/mL TRA-8 only, treated with 20 μ M TFP only, or treated with 20 μ M TFP and 500 ng/mL TRA-8 for 16 hours. Following each treatment, cells were washed with cold PBS and lysed with RIPA buffer with 1:100 Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA). Samples were sonicated on ice for 10 seconds, and total protein concentration was determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA). Then 6 $\times$ laemmli buffer with 2-mercaptoethanol was added to samples, and 40 μ g of total protein were loaded for western blot analysis.

Western Blot Analysis

Samples of immunoprecipitations (IPs) of CaM-DR5 complex and DR5-mediated DISC, CaM pull-down of DR5, cell lysate from the effect of TFP on DR5-mediated caspase cleavage and cell lysate of the effect of combined TFP and TRA-8 on anti-apoptotic proteins expression in TRA-8 resistant TNBC cells, were assessed by western blot analyses. Immunoprecipitation, pull-down, or cell lysate samples in laemmli buffer were heated at 100 °C for 5 minutes. Protein samples were resolved by SDS-PAGE, transferred onto a 0.45 µm nitrocellulose membrane (Bio-Rad Hercules, CA). For the immunoprecipitation samples to determine CaM-DR5 binding, membranes were incubated overnight at 4 °C with primary antibodies against DR5 (Abcam Cambridge, MA, specificity: DR5 isoform 1 and 2, source: rabbit, Cat. Ab47179, Lot# GR199335-1) and CaM (Milipore Billerica, MA, source mouse, Cat. 05173, Lot# 2123697). For the samples of CaM pulldown of DR5, membranes were incubated with primary antibodies against DR5 (Abcam, specificity: DR5 isoform 1 and 2, source: rabbit, Cat. Ab47179, Lot# GR199335-1). For the samples for co-IPs of DR-mediated DISC, membranes were incubated overnight at 4 °C with primary antibodies against DR5 (Abcam, specificity: DR5 isoform 1 and 2, source: rabbit, Cat. Ab47179, Lot# GR199335-1), FADD (Cell Signaling Technologies Beverly, MA, specificity: FADD, source: rabbit, Cat. 2782S, Lot# 4), Caspase-8 (Cell Signaling Technologies Beverly, MA, specificity: Caspase-8 (D3562) (57/10 kDa), source: rabbit, Cat. 2782S, Lot# 2), or CaM (Milipore Billerica, MA, source mouse, Cat. 05173, Lot# 2123697). For Caspase signaling analysis, membranes were incubated with primary antibodies against Caspase-8 (Cell Signaling Technologies, specificity: Caspase-8 (D3562) (57/10kDa), source: rabbit, Cat. 4790S, Lot# 2), Caspase-9 (Cell Signaling Technology, specificity: Caspase-9 (35/37/47kDa), source: rabbit, Cat. 9502S, Lot#8), Caspase-3 (Cell Signaling Technology, specificity: Caspase-3 (35/19/17kDa), source: rabbit, Cat. 9662S, Lot# 15), and PARP (Cell Signaling Technology, specificity: PARP1(116/89 kDa), source: rabbit, Cat. 9542S, Lot# 13). For IAP expression and phosphorylation of AKT and ERK analysis, membranes were incubated with primary antibodies against pERK (Cell Signaling Technologies, specificity: pERK (44/42kDa), source: rabbit, Cat.9102S, Lot#26), pAKT (Cell Signaling Technologies, specificity: pAKT(S473) (60 kDa), source: rabbit, Cat.4060S, Lot#19), XIAP (Cell Signaling Technologies, specificity: XIAP (3B6) (53 kDa), source: rabbit, Cat.2045S, Lot#3), and cIAP-1 (Cell Signaling Technologies specificity: cIAP-1(62 kDa), source: rabbit, Cat.4952S, Lot#5).

Following overnight incubation, membranes were washed 3 times with TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies (Southern Biotechnologies Birmingham, Alabama) at room temperature for 1–2 hour. Blots were developed using enhanced chemiluminescence reagents (Thermo Fisher Scientific Waltham, MA) per manufacturer's instructions. Quantitative analysis of western blot images for the effect of TFP or CaM siRNA on DR5-mediated DISC formation and for the effect of combined TFP and TRA-8 on IAP expression and phosphorylation of AKT and ERK analysis was performed using the ImageJ 1.50i software [38].

Fluorescence Microscope Imaging and Analysis

To investigate the effect of TFP on TRA-8 activated DR5 oligomerization in TRA-8 resistant TNBC cells, fluorescence microscope imaging was conducted and the distributions of Cy5.5-labeled TRA-8 was analyzed. 1×10^5 HCC1143 cells were seeded onto 35-mm glass bottom culture dishes (MatTek Corp, Ashland, MA) and incubated overnight in complete medium as described in the cell culture section. The monofunctional dye Cy5.5 was conjugated to TRA-8 at a 3:1 molar ratio according to manufacturer's protocol. HCC1143 cells were washed with PBS and then treated with 5 $\mu\text{g/mL}$ Cy5.5 labeled TRA-8 with or without 10 μM TFP for 24 hours. Following treatment, HCC1143 cells were washed with PBS and then placed in complete medium without phenol red. Nine images at 100 $\times$ magnification from randomly selected areas were taken per each treatment group using a Leica DMIRE2 inverted microscope equipped with a Nuance camera (CRI Inc., Woburn, MA). The distribution of Cy5.5 labeled TRA-8 was analyzed using a fully automated computer software package as described in our previous study [21]. In brief, the computer software found the best fitting 2-dimensional Gaussian curve to the distribution of Cy5.5-TRA-8 in each region of oligomer, and the peak amplitude and the full-width at half maximum (FWHM) of the Gaussian curve were extracted. About 100 oligomers were detected in each image (about 900 oligomers per group). The computer software package was developed by Dr. Harrison Kim at UAB using Labview, version 2014 (National Instrument, Austin, TX). As a control, we tested the effect of Cy5.5 dye conjugation to TRA-8, on TRA-8 cytotoxicity with or without TFP in HCC1143 cells. We chose the 500 ng/mL TRA-8 concentration used in the Cell Viability Assay Using ATPLite section with HCC1143 cells. HCC1143 cells were treated with 500 ng/mL Cy5.5-TRA-8 or TRA-8 alone, or in combination with 10 μM TFP or 20 μM TFP for 24 hours. Following treatment HCC1143 cell viability was assessed using ATPLite assay.

Statistical Analysis

One-way ANOVA was used to compare the amplitudes of best fitting Gaussian curves for the signal intensities of Cy5.5 labeled TRA-8 on HCC1143 cells with or without TFP treatment. Pairwise t-tests were performed on cell viability data. *P*-values less than 0.05 were considered significant. Data are presented as the mean $\pm$ standard error where indicated. Nonlinear regression analysis was performed on cell viability data using SAS 9.3 [39] to obtain the drug concentration that provided a yield of 50 % inhibition for either single drug treatment or combined drug treatment [40–42]. Combination index (CI) for dose–effect relationships of TRA-8 and TFP in combination (equation 1) was calculated on the basis of the multiple drug–effect equation of Chou–Talalay [43, 44]. Using the CI theorem offered by Chou and Talalay, CI enabled a quantitative prediction of drug interaction by characterizing CI = 1 as an additive effect, CI > 1 as antagonism, and CI < 1 as synergism [44]. By definition, synergism is more than an additive effect, while antagonism is less than an additive effect [44].

$$CI = \frac{D_{TFP}}{IC_{50, TFP}} + \frac{D_{TRA-8}}{IC_{50, TRA-8}} \quad (1)$$

where D_{TFP} and D_{TRA-8} represent concentrations of TFP and TRA-8 that yield a 50 % inhibition when both drugs are given to the cells in combination and where $IC_{50, TFP}$ and $IC_{50, TRA-8}$ represent concentrations of TFP and TRA-8 that yield a 50 % inhibition when each drug is present alone [40, 43].

RESULTS

CaM antagonists enhanced TRA-8 induced cytotoxicity in TRA-8 resistant TNBC cells

We determined the effect the CaM antagonist, TFP, on TRA-8 cytotoxicity in TRA-8 resistant TNBC HCC1143 and HCC1937 cells using ATPLite cell viability assay. Figure 1A shows the cell viability results for HCC1143 cells treated with TFP alone, TRA-8 alone, or the combinations of TFP and TRA-8 at varied concentrations for 24 hours. For HCC1143 cells, TRA-8 treatment alone decreased HCC1143 cell viability (5%–25%) with increasing TRA-8 concentration (15.6–1000 ng/mL) and TFP treatment alone decreased HCC1143 cell viability (6–38%) with increasing TFP concentration (0.156–25 μ M). For the HCC1143 cells with combined TRA-8 and TFP treatment at varied concentrations, cell viability was significantly decreased with the increased TRA-8 or TFP concentrations (Fig. 1A). At low concentrations of TFP (0.156–1.25 μ M), TFP combined with relatively high concentrations of TRA-8 (125–1000 ng/mL) resulted in significantly decreased cell viability of HCC1143 cells compared to TFP only, or TRA-8 only treatment (Fig. 1A). At the relatively high concentrations of TFP (10–25 μ M), combined TRA-8 (15.6–1000 ng/mL) and TFP treatment resulted in significantly decreased cell viability of HCC1143 cells compared to either TFP only or TRA-8 only ($p < 0.05$) (Fig. 1A). Figure 1B shows the cell viability results for HCC1937 cells treated with TFP alone, TRA-8 alone, or the combinations of TFP and TRA-8 at varied concentrations for 24 hours. For HCC1937 cells, TRA-8 treatment alone decreased HCC1937 cell viability (2%–26%) with increasing TRA-8 concentration (15.6–1000 ng/mL) and TFP treatment alone decreased HCC1937 cell viability (8–54%) with increasing TFP concentration (0.156–25 μ M). For the combination of TFP and TRA-8 treatment at varied concentrations, cell viability was significantly decreased with the increased TRA-8 or TFP concentrations (Fig. 1B). For HCC1937 at low concentrations of TFP (0.156–1.25 μ M), TFP combined with relatively high concentrations of TRA-8 (125–1000 ng/mL) resulted in significantly decreased cell viability of HCC1937 cells compared to TFP only, or TRA-8 only treatment (Fig. 1B). At the relatively high concentrations of TFP (10–25 μ M), combined TRA-8 (15.6–1000 ng/mL) and TFP treatment resulted in significantly decreased cell viability of HCC1937 cells compared to either TFP only or TRA-8 only ($p < 0.05$) (Fig. 1B). Cell viability data demonstrated that at the optimized concentration of TFP and TRA-8, TFP enhanced TRA-8 induced cytotoxicity in TRA-8 resistant TNBC cells.

Cell viability data was further analyzed using SAS 9.3[39] to obtain the drug concentration that provided a yield of 50 % inhibition for either single drug treatment ($IC_{50, TFP}$ and $IC_{50, TRA-8}$) or combined drug treatment (D_{TFP} and D_{TRA-8}) and combination indexes (CIs) for different concentration combinations of each drug were calculated. CIs were used to quantify the combination drug effect and then characterize it as additive ($CI = 1$), antagonistic ($CI > 1$), or synergistic ($CI < 1$) (Table S1). Table S1A shows the TFP

concentration conversions from μM units used in cell viability assay (Fig. 1) to ng/mL units used for CI analysis (Table S1 B and C). For both TRA-8 resistant TNBC HCC1143 and HCC1937 cell lines, CI analyses of cell viability data predicts that combination of TFP and TRA-8 has synergistic effect ($\text{CI} < 1$) to result in 50 % inhibition of cell viability at low concentrations of TRA-8 (15.6–62.5 ng/mL) together with low concentration of TFP (0.14–0.19 ng/mL) (Table S1B), or at low concentrations of TFP (0.156–1.25 μM) together with low concentration of TRA-8 (0.11–0.18 ng/mL) (Table S1C).

Figure S1 in the Supplemental Materials shows a time-dependent effect of TFP on enhancing TRA-8 induced cytotoxicity in HCC1143 cells. The significant enhancement effect of TFP (20 μM) on TRA-8 (500 ng/mL) induced cytotoxicity in HCC1143 cells was observed starting at the 12-hour incubation time point ($p < 0.05$) (Fig. S1).

We also investigated the effect of CaM antagonists TMX and W-7 on TRA-8 induced cytotoxicity in TRA-8 resistant HCC1143 cells (Fig. S2). TMX or W-7 treatment alone decreased HCC1143 cell viability by 20–50% or 20–30% respectively in a concentration dependent manner (Figs. S2A and B) and 500 ng/mL TRA-8 treatment alone reduced about 20% cell viability during 24-hour period (Figs. S2A and B). The combined TRA-8 with TMX or W-7 treatment significantly reduced cell viability of HCC1143 cells compared to TMX or W-7 treatment alone (Figs. S2A and B) ($p < 0.05$).

CaM antagonist TFP promoted DR5-mediated apoptosis and apoptotic caspase signaling in TRA-8 resistant TNBC cells

We characterized the effect of TFP on TRA-8 induced apoptosis using Dead Cell Apoptosis Kit with Annexin V FITC and propidium iodide (PI) in HCC1143 or HCC1937 cells (Fig. 2). Apoptotic cells were recognized under fluorescence microscopy by their positive labeling with annexin V, which binds the phosphatidylserine residues on the outer phospholipid leaflet of the plasma membrane showing green staining in early apoptosis. In late stage of apoptosis, cell membranes become permeable to the red-fluorescent propidium iodide nucleic acid binding dye and the cells stain red in addition to the green staining (FITC). Figures 2 A–D are the merged Annexin V fluorescence and PI fluorescence images for HCC1143 or HCC1937 cells. Figure 2 E is mean fluorescence intensity for Annexin V FITC fluorescence images or for PI fluorescence images from HCC1143 or HCC1937 cells. Apoptosis results show that no treatment, TFP or TRA-8 alone induce very little or no apoptosis in HCC1143 or HCC1937 cells (Figs. 2 B, C and E). However, the combination of TFP and TRA-8 treatment induced significant amount of apoptosis in TRA-8 resistant HCC1143 or HCC1937 TNBC cells (Figs. 2 D and E). TRA-8 induced DR5-mediated DISC to activate caspase-8, which then can activate effector caspases-3 and PARP leading to cellular disassembly [45, 46] and induction of cell apoptosis [47]. We characterized the effect of combined TFP and TRA-8 on the cleavage of caspase-8, caspase-3, and PARP in HCC1143 or HCC1937 cells. Results showed that TRA-8 or TFP alone only caused very little or no caspase-8, caspase-3, or PARP cleavage, however, combined TFP and TRA-8 treatment enhanced TRA-8-induced activation of caspase-8 and downstream caspase-3, and PARP cleavage in HCC1143 (Fig. 3A) or HCC1937 (Fig. 3B) cells. Results demonstrated that TFP enhances TRA-8-induced cytotoxicity and apoptosis in HCC1143 or HCC1937

cells as observed in Fig. 1 and Fig. 2 via promotion of TRA-8 induced DR5-mediated caspase signaling for apoptosis.

CaM bound to DR5 in calcium dependent manner in TRA-8 resistant TNBC cells

CaM was co-immunoprecipitated with DR5 from HCC1143 cell lysate (IP: TRA-8 lane) and CaM or DR5 was not found in the control Co-IP using the mouse Iso-IgG conjugated sepharose beads (IP: IsoIgG lane) (Fig. 4A). Co-IP results showed that CaM or DR5 did not interact with the sepharose bead of mouse IgG portion of the TRA-8 antibody and demonstrated that CaM interacted with DR5 in TRA-8 resistant TNBC cells. CaM may interact with target proteins in calcium (Ca^{2+}) dependent or independent manner [48, 49]. The calcium dependence of CaM and DR5 interaction was investigated by CaM pull-down of DR5 from HCC1143 cell lysates supplemented with 1 mM Ca^{2+} or 2 mM Ca^{2+} chelator, EGTA. Western blot of CaM pull-down samples showed DR5 bound to CaM but not to the control sepharose beads in the presence of calcium (Fig. 4B). However, CaM did not bind to DR5 from cell lysates supplemented with EGTA (Fig. 4B). Results demonstrated that CaM bound to DR5 in a calcium dependent manner in TRA-8 resistant TNBC HCC 1143 cells.

CaM knockdown promotes DR5-mediated apoptotic signaling in TRA-8 resistant TNBC cells

To determine CaM involvement in DR5-mediated apoptotic signaling in TRA-8 resistant TNBC cells, we investigated the effect of CaM knock-down using CaM siRNA on DR5-mediated DISC formation and caspase cleavage. HCC1143 cells were nucleofected with CaM 1, 2, and 3 siRNAs together or a control siRNA, followed by TRA-8 activation. DR5-mediated DISC was co-IP using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads. Co-IP results demonstrated that knock-down of CaM protein expression increased FADD and Caspase-8 recruitment to DR5, thus increasing DR5-mediated DISC formation (Fig. 5A). Quantitative analysis of the co-IP results using ImageJ software [38] showed that CaM knock-down increased FADD and caspase-8 recruitment into DR5-mediated DISC (Fig. 5B). Western blot analysis of siRNA nucleofected HCC1143 cell lysates showed that CaM protein expression was knocked-down (Figs. 5 A–C). Pretreatment of cells with control siRNA, TRA-8 alone only caused very little or no caspase-8, caspase-3, or PARP cleavage, however, CaM 1, 2, and 3 siRNAs together for pretreatment enhanced TRA-8-induced activation of caspase-8 and downstream caspase-3, and PARP cleavage in HCC1143 cells (Fig. 5C). These results confirmed CaM involvement in DR5-mediated DISC formation and caspase cleavage for apoptosis in TRA-8 resistant TNBC cells.

Control experiment that DR5 was immunoprecipitated from TRA-8 untreated or TRA-8 treated HCC1143 cell lysates using anti-DR5 (D-6) antibody, showed that CaM bound with DR5 upon TRA-8 activation in HCC1143 cells and CaM did not bind to DR5 in the absence of TRA-8 (Fig. S3).

TFP enhanced DR5-mediated DISC formation in TRA-8 resistant TNBC cells

To understand the enhancement effect of TFP on TRA-8 activated cytotoxicity and apoptosis in TRA-8 resistant TNBC cells as observed in Figs. 1 and 2, we investigated the effect of TFP on TRA-8 activated DISC formation and on the recruitment of CaM into the DISC.

CaM, DR5, FADD and caspase-8 expressions in the cell lysate before co-IP were not affected with or without TFP in HCC1143 cells (Fig. 6A). DR5-mediated DISC was co-IP using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose following TRA-8 activation of DR5. Co-IP results show that TFP increased FADD and caspase-8 recruitment into TRA-8 activated DISC (Fig. 6A). Further, with the increase of TFP concentration from 10 to 20 μ M, there was a noticeable increase in FADD and caspase-8 recruitment into TRA-8 activated DISC (Fig. 6A). However, TFP inhibited CaM recruitment into DR5-mediated DISC (Fig. 6A). These results supported that TFP enhanced TRA-8 activated cytotoxicity by promoting DR5-mediated DISC formation for apoptosis. Quantitative analysis of the western blot results for DR5-mediated DISC formation using ImageJ software [38] confirmed that TFP significantly increased FADD and Caspase-8 recruitment into TRA-8 activated DISC and the increase was also significantly different when TFP concentration increased from 10 μ M to 20 μ M ($p < 0.05$) (Fig. 6B).

TFP increased DR5 oligomerization in TRA-8 resistant TNBC cells

TRA-8 activated DR5 oligomerization is critical for DISC formation to signal apoptosis [50, 51]. To further understand the effect of CaM antagonist on DR5-mediated apoptotic signaling in TRA-8 resistant TNBC cells, we performed fluorescence microscopic imaging to analyze the cellular distributions of Cy5.5-labeled TRA-8 that activates DR5 oligomerization in TRA-8 resistant HCC1143 TNBC cells without and with TFP treatment. Figure S4 shows Cy5.5 conjugation to TRA-8 did not hinder TRA-8 induced cytotoxicity in HCC1143 cells. Figure 7A shows the representative fluorescence microscopic images of Cy5.5 labeled TRA-8 on HCC1143 cells with or without TFP treatment, in which the same color scale was applied for both images. The fluorescence images were overlapped with bright field images, showing cell morphology. Cy5.5 labeled TRA-8 activated DR5 oligomerization was observed in HCC1143 cells (Fig. 7A). The distribution of Cy5.5-TRA-8 on the representative cells indicated with white arrows in Fig. 7A followed a Gaussian curve, and TFP increased the peak signal intensity of Cy5.5-TRA-8 in HCC1143 cells (Fig. 7B). Peak amplitude of TRA-8-activated DR5 oligomers treated with TFP were significantly higher than that without TFP treatment ($p < 0.05$) in HCC1143 cells (Fig. 7C). These results demonstrated that TFP increased DR5 aggregation in HCC1143 cells, which directly enhanced DR5-mediated DISC formation (Fig. 6) and apoptotic signaling (Fig. 3).

Inhibition of AKT, ERK phosphorylation and IAP proteins sensitize TRA-8 resistant TNBC cells to TRA-8-induced cytotoxicity

IAP family of proteins are involved in the regulation of TRAIL-mediated apoptosis, and can block caspase activation [52, 53]. Down regulation of anti-apoptotic proteins such as cIAP-1 and XIAP (X-linked IAP), has been shown to increase DR5-mediated apoptosis in cancer cells [9, 54]. Akt and ERK have been reported to be involved in negatively regulating TRAIL-mediated apoptosis [55]. We characterized the effect of TFP on DR5, pAKT, pERK, XIAP, and cIAP-1 expression in TRA-8 resistant TNBC HCC1143 cells. Figure 8 A shows the western blot analysis of the lysates of HCC1143 cell treated with TRA-8 only, TFP only or combined TFP and TRA-8. Quantitative analysis was performed on the western blot image using ImageJ software [38] (Figure 8B). Results showed that TFP or TRA-8 alone or in combination did not significantly change DR5 expression in HCC1143 cells. TRA-8 only

treatment did not significantly change pERK, pAKT, XIAP, and cIAP1 expression compared to no drug treatment (control). TFP alone treatment did not significantly change pERK, XIAP, and cIAP-1 expression compared to control case, although TFP significantly decreased pAKT expression (Fig. 8B). Combined TFP and TRA-8 treatment significantly decreased pERK, pAKT, XIAP, and cIAP-1 expression compared to TRA-8 only treatment. These results support that TFP enhanced DR5-mediated apoptosis in TRA-8 resistant TNBC cells.

DISCUSSION

Our study shows that the cytotoxicity of TRA-8 to human breast cancer cell lines with DR5 expression, including TNBC cell lines, varies from strongly to weakly positive [10, 23]. Drug resistant TNBC are prolific with poor clinical outcome [56]. In this study, we characterized the novel function of CaM antagonist in enhancing TRA-8 induced cytotoxicity in TRA-8 resistant TNBC cells and its underlying molecular mechanisms.

Our cell viability assay results showed that CaM antagonists enhanced TRA-8 induced cytotoxicity in TRA-8 resistant TNBC cells in a concentration and time-dependent manner (Fig. 1, Figs. S1 and S2). Enhanced cytotoxicity has been observed in TRA-8 resistant breast cancer cell lines with the combined treatment of TRA-8 with other drugs, including doxorubicin or paclitaxel etc. [9, 10, 23], which involved in the increased TRA-8 induced DR5-mediated apoptotic signaling [9]. We characterized the effect of the CaM antagonist, TFP, on DR5-mediated apoptosis (Fig. 2) and apoptotic caspase signaling (Fig. 3) in TRA-8 resistant TNBC cells. TFP enhanced TRA-8-induced apoptosis and the activation of caspase-8 and downstream caspase-3 and PARP cleavage for apoptosis in TRA-8 resistant TNBC cells (Figs. 2 and 3).

We further investigated the molecular mechanism of the effect of CaM antagonist on TRA-8 induced apoptosis and cytotoxicity in TRA-8 resistant TNBC cells. CaM is overexpressed in breast cancer [29, 30]. CaM functions as an intracellular mediator of Ca^{2+} signals and regulates various cellular processes including cell growth and apoptosis [27, 28, 31–34]. DR5-mediated DISC formation that is composed of DR5, FADD, and caspase-8 is a critical step in DR5-mediated signaling of apoptosis [15, 57–59]. Our co-IP and CaM pull-down results showed that CaM bound with DR5 in a calcium dependent manner in TRA-8 resistant TNBC cells (Fig. 4). CaM knockdown increased FADD and caspase-8 recruitment into DR5-mediated DISC and the activation of caspase-8 and downstream caspase-3 and PARP cleavage for apoptosis (Fig. 5), which demonstrated that CaM was directly involved with DR5-mediated signaling for apoptosis. These results indicate that CaM could be a key regulator to mediate DR5-mediated apoptotic signaling to overcome drug resistance in TRA-8 resistant TNBC cells.

We characterized the effect of the CaM antagonist TFP on DR5-mediated DISC and DR5 oligomerization in TRA-8 resistant TNBC cells. TRA-8 resistance is correlated with the degree of TRA-8 induced DR5 oligomerization [60–62]. TRA-8 activated DR5 aggregation was essential to drive intracellular signals for death [50, 51]. Fluorescence microscopic imaging of TRA-8-activated DR5 oligomers has been able to predict TRA-8 therapeutic

efficacy in a breast cancer mouse model [21]. Results show that TFP decreased CaM recruitment into TRA-8 activated DISC, however, TFP increased FADD and caspase-8 recruitment into TRA-8 activated DISC in TRA-8 resistant TNBC cells (Fig. 6). Fluorescence microscopic image analyses of TRA-8 activated DR5 oligomerization showed that TFP promoted DR5 oligomerization in TRA-8 resistant TNBC cells (Fig. 7). CaM conformational changes by binding to TFP(s) [63] could directly affect CaM interaction with DR5, further affecting DR5 oligomerization and DR5 recruitment of FADD for DISC formation.

TFP can inhibit breast cancer cell growth, presumably by CaM regulation of pathways controlling the cell cycle [31–33]. Inhibition of CaM regulatory pathways for cell growth could enhance DR5-mediated caspase apoptotic signaling by down-regulation of caspase inhibitors bcl-2 [34, 64], c-IAP, Bcl-xL, and XIAP[64]. Our results show that TFP decreased anti-apoptotic proteins, pERK, pAKT, XIAP, and cIAP-1 expression in TRA-8 resistant TNBC cells (Fig. 8).

TFP enhancement of TRA-8-induced cytotoxicity and apoptosis in TRA-8 resistant TNBC cells as observed in Figs. 1 and 2 could be directly related to TFP promotion of DR5 oligomerization, DR5-mediated DISC formation and apoptotic caspase signaling as shown in Figs 3, 6 and 7, and the depression of anti-apoptotic proteins as observed in Fig 8. Taken together, the results from this study present the potential novel strategy of using the readily available CaM antagonists to promote TRA-8/TRAIL activated cytotoxicity for resistant TNBC cells.

Our recent studies demonstrated that CaM directly bound to DR5 in a Ca^{2+} dependent manner and CaM bound to DR5-mediated DISC for apoptosis in TRA-8 sensitive triple negative MDA-MB-231 and ER-positive ZR-75-1 breast cancer cells [36, 65]. We demonstrated that CaM antagonist, TFP, inhibited CaM recruitment into DISC, decreased TRA-8 activated DR5 oligomerization, attenuated DISC formation, and reduced TRA-8 activated caspase-dependent apoptotic signaling and TRA-8 induced cytotoxicity in TRA-8 sensitive MDA-MB-231 or ZR-75-1 breast cancer cells. However, in this study for TRA-8 resistant TNBC cell lines, we observed that CaM antagonists increased TRA-8 induced cytotoxicity in a concentration and time-dependent manner. Further, we observed that at the optimized concentration, TFP enhanced TRA-8 activated DR5 oligomerization, DR5-mediated DISC formation, TRA-8 activated caspase cleavage for apoptosis, and decreased anti-apoptotic pERK, pAKT, XIAP, and cIAP-1 expression in TRA-8 resistant TNBC cells. The phosphorylation of DR5 could decrease DR5 oligomerization and subsequently impede DR5-mediated DISC formation for apoptosis.

The phosphorylation of DISC components FADD and caspase-8 have been shown to impede DISC formation and death receptor induced apoptosis [66–68]. Studies have shown that TRA-8 sensitive MDA-MB-231 TNBC cells can become desensitized to TRA-8 after low dose exposure for an extended time [26, 69]. The developed resistance is due to the formation of an anti-apoptotic complex at the DR5 death domain, which includes glycogen synthase kinase-3 (GSK3), DDX3 and cellular inhibitor of apoptosis protein-1 (cIAP-1) prior to DR5 stimulation [23, 69]. The kinase Src and CaM can be recruited into death

receptor-mediated DISC [70, 71]. In TRA-8 sensitive breast cancer cells, TRA-8 induced DR5 stimulation can inactivate GSK3 associated with DR5, and induce proteolysis of DDX3 and cIAP-1 to disrupt the inhibitory anti-apoptotic complex [69]. However, for TRA-8 resistant cell lines, TRA-8 stimulation of DR5 does not inactivate GSK3, and DDX3 and cIAP-1 were not cleaved, which block DR5-mediated DISC formation for apoptosis [69]. An anti-apoptotic complex including GSK3, DDX, cIAP-1, and Src could impede DR5-mediated DISC formation for apoptosis in TRA-8 resistant TNBC cells. CaM could associate with GSK3 [72, 73] and the kinase Src is a CaM binding protein. CaM-mediated GSK3 and or Src recruitment to DR5 could cause DR5 phosphorylation at initial stages of activation, decreasing TRA-8 induced DR5 oligomerization to impede DR5-mediated DISC formation for apoptosis in TRA-8 resistant TNBC cells. CaM knockdown in TRA-8 resistant TNBC cells could reduce or prevent GSK3 or Src recruitment into DR5-mediated DISC, thus decreasing the phosphorylation of DR5 to promote DR5 oligomerization and FADD and caspase-8 recruitment to form DR5-mediated DISC as observed in our results (Fig. 5). AKT phosphorylation of GSK3 [74], regulates GSK3 activation. We have shown CaM antagonist, TFP, decreases cIAP-1 expression and phosphorylation of AKT in TRA-8 resistant TNBC cells (Fig. 8) and TFP can inhibit CaM-mediated Src recruitment to DR5 in cancer cells [75]. The decreased CaM recruitment to DR5 by TFP in TRA-8 resistant TNBC cells as observed in Fig. 6 could reduce GSK3 or Src recruitment to DR5, subsequently decreasing the phosphorylation of DR5 to promote DR5 oligomerization, and FADD and caspase-8 recruitment to form DR5-mediated DISC as observed in Figs. 6 and 7.

The increase in DR5-mediated DISC formation coupled with TFP inhibition of anti-apoptotic proteins (Fig. 8), would enhance TRA-8 activated caspase apoptotic signaling and cytotoxicity in TRA-8 resistant TNBC cells as observed in Figs. 1–3. The effect of CaM antagonist, TFP, on DR5 oligomerization, on DR5-mediated DISC formation and caspase apoptotic signaling, and cytotoxicity in TRA-8 resistant TNBC cells, could be directly related to TFP modulating the phosphorylation of DR5, FADD and caspase-8, the key components of DISC. Understanding the role of CaM in CaM-mediated GSK3 and or Src recruitment to DR5 to form an anti-apoptotic complex, in regulating DR5 or DISC component phosphorylation, in TRA-8 resistant breast cancer cells versus TRA-8 sensitive breast cancer cells, are important for expanding the knowledge about the role of CaM in DR5-mediated apoptosis in breast cancer cells with various sensitivity to TRA-8/TRAIL and could be the focus of future studies.

In summary, we demonstrated that CaM antagonists increased TRA-8 induced cytotoxicity in a concentration and time dependent manner in TRA-8 resistant TNBC cells. The molecular mechanisms underlying the effect of CaM antagonists on TRA-8 induced cytotoxicity in TRA-8 resistant TNBC cells are directly related to the role of CaM and CaM antagonist in DR5-mediated DISC formation and apoptotic caspase signaling, TRA-8 activated DR5 oligomerization and anti-apoptotic protein expression. Our results demonstrated that CaM knock-down and TFP enhanced FADD and caspase-8 recruitment into DISC formation and TRA-activated apoptotic caspase signaling, TFP promoted DR5 oligomerization, and TFP inhibition of anti-apoptotic proteins in TRA-8 resistant TNBC cells. Our data suggest that CaM could be a key regulator to mediate DR5-mediated apoptotic signaling to overcome drug resistance in TRA-8 resistant TNBC cells. These

results present the potential novel strategy of using CaM antagonists to promote TRA-8/TRAIL activated cytotoxicity for resistant TNBC cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by an NIH K25 award (NCI 5K25CA140791) to Yuhua Song and UAB Comprehensive Cancer Center support grant, NCI P30CA013148. We also acknowledge the UAB VP Office for Equity and Diversity and ALSAMP Bridge to the Doctorate Fellowship Program and the Comprehensive Minority Faculty and Student Development Program (CMFSDP) Fellowship to Romone Fancy.

ABBREVIATIONS

CaM	Calmodulin
DR5	Death Receptor 5
Cas-8	Caspase-8
Cas-3	Caspase-3
Cas-9	Caspase-9
PARP	poly ADP ribose polymerase
DISC	Death inducing signaling complex
FADD	Fas associated death domain
TFP	Trifluoperazine
TMX	Tamoxifen
W-7	N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride
TNF	Tumor necrosis factor

References

1. Fisher ER, Gregorio RM, Fisher B, Redmond C, Vellios F, Sommers SC. The pathology of invasive breast cancer. A syllabus derived from findings of the National Surgical Adjuvant Breast Project (protocol no. 4). *Cancer*. 1975; 36:1–85. [PubMed: 173455]
2. DeSantis CE, Fedewa SA, Goding Sauer A, Kramer JL, Smith RA, Jemal A. Breast cancer statistics, 2015: Convergence of incidence rates between black and white women. *CA Cancer J Clin*. 2016; 66:31–42. [PubMed: 26513636]
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians*. 2016; 66:7–30. [PubMed: 26742998]
4. Kohler BA, Sherman RL, Howlader N, Jemal A, Ryerson AB, Henry KA, Boscoe FP, Cronin KA, Lake A, Noone AM, Henley SJ, Ehemann CR, Anderson RN, Penberthy L. Annual Report to the Nation on the Status of Cancer, 1975–2011, Featuring Incidence of Breast Cancer Subtypes by Race/Ethnicity, Poverty, and State. *J Natl Cancer Inst*. 2015; 107:djv048. [PubMed: 25825511]

5. Howlader N, Altekruse SF, Li CI, Chen VW, Clarke CA, Ries LA, Cronin KA. US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. *J Natl Cancer Inst.* 2014; 106
6. Perez EA, Moreno-Aspitia A, Aubrey Thompson E, Andorfer CA. Adjuvant therapy of triple negative breast cancer. *Breast Cancer Res Treat.* 2010; 120:285–291. [PubMed: 20094772]
7. Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N, Timour MS, Gerhart MJ, Schooley KA, Smith CA, Goodwin RG, Rauch CT. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J.* 1997; 16:5386–5397. [PubMed: 9311998]
8. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, Zhang H, Mountz JD, Koopman WJ, Kimberly RP, Zhou T. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. *Nat Med.* 2001; 7:954–960. [PubMed: 11479629]
9. Amm HM, Zhou T, Steg AD, Kuo H, Li Y, Buchsbaum DJ. Mechanisms of drug sensitization to TRA-8, an agonistic death receptor 5 antibody, involve modulation of the intrinsic apoptotic pathway in human breast cancer cells. *Mol Cancer Res.* 2011; 9:403–417. [PubMed: 21357440]
10. Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Zhang S, Carpenter M, LoBuglio AF. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. *Clin Cancer Res.* 2003; 9:3731–3741. [PubMed: 14506165]
11. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem.* 1996; 271:12687–12690. [PubMed: 8663110]
12. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med.* 1999; 5:157–163. [PubMed: 9930862]
13. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokhi Z, Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest.* 1999; 104:155–162. [PubMed: 10411544]
14. Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, Strom SC. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med.* 2000; 6:564–567. [PubMed: 10802713]
15. Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, Krammer PH, Walczak H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity.* 2000; 12:599–609. [PubMed: 10894160]
16. Festjens N, Cornelis S, Lamkanfi M, Vandenabeele P. Caspase-containing complexes in the regulation of cell death and inflammation. *Biol Chem.* 2006; 387:1005–1016. [PubMed: 16895469]
17. Yang JK, Wang L, Zheng L, Wan F, Ahmed M, Lenardo MJ, Wu H. Crystal structure of MC159 reveals molecular mechanism of DISC assembly and FLIP inhibition. *Mol Cell.* 2005; 20:939–949. [PubMed: 16364918]
18. Bin L, Thorburn J, Thomas LR, Clark PE, Humphreys R, Thorburn A. Tumor-derived mutations in the TRAIL receptor DR5 inhibit TRAIL signaling through the DR4 receptor by competing for ligand binding. *The Journal of biological chemistry.* 2007; 282:28189–28194. [PubMed: 17666396]
19. McDonald ER 3rd, Chui PC, Martelli PF, Dicker DT, El-Deiry WS. Death domain mutagenesis of KILLER/DR5 reveals residues critical for apoptotic signaling. *The Journal of biological chemistry.* 2001; 276:14939–14945. [PubMed: 11279061]
20. Yang H, Song Y. Structural Insight for Roles of DR5 Death Domain Mutations on Oligomerization of DR5 Death Domain-FADD Complex in the Death-Inducing Signaling Complex Formation: A Computational Study. *J Mol Model.* 2016; 22:89. [PubMed: 26995783]
21. Kim H, Buchsbaum DJ, Zinn KR. A Novel Imaging Biomarker Extracted from Fluorescence Microscopic Imaging of TRA-8/DR5 Oligomers Predicts TRA-8 Therapeutic Efficacy in Breast

- and Pancreatic Cancer Mouse Models. *Molecular imaging and biology* : MIB : the official publication of the Academy of Molecular Imaging. 2016; 18:325–333. [PubMed: 26552657]
22. Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A, Ford HL. On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. *Oncogene*. 2013; 32:1341–1350. [PubMed: 22580613]
 23. Oliver P, LoBuglio A, Zhou T, Forero A, Kim H, Zinn K, Zhai G, Li Y, Lee C, Buchsbaum D. Effect of anti-DR5 and chemotherapy on basal-like breast cancer. *Breast Cancer Res Treat*. 2012; 133:417–426. [PubMed: 21901385]
 24. Chinnaiyan AM, Prasad U, Shankar S, Hamstra DA, Shanaiah M, Chenevert TL, Ross BD, Rehemtulla A. Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. *Proceedings of the National Academy of Sciences*. 2000; 97:1754–1759.
 25. Rahman M, Davis SR, Pumphrey JG, Bao J, Nau MM, Meltzer PS, Lipkowitz S. TRAIL induces apoptosis in triple-negative breast cancer cells with a mesenchymal phenotype. *Breast Cancer Res Treat*. 2009; 113:217–230. [PubMed: 18266105]
 26. Li Y, Wang H, Wang Z, Makhija S, Buchsbaum D, LoBuglio A, Kimberly R, Zhou T. Inducible resistance of tumor cells to tumor necrosis factor-related apoptosis-inducing ligand receptor 2-mediated apoptosis by generation of a blockade at the death domain function. *Cancer Res*. 2006; 66:8520–8528. [PubMed: 16951164]
 27. Cohen P. Calmodulin. 1988
 28. La, W., Van Eldik, D. Calmodulin and Signal Transduction. Academic Press; San Diego, CA: 1988.
 29. Hait WN, Lazo JS. Calmodulin: a potential target for cancer chemotherapeutic agents. *J Clin Oncol*. 1986; 4:994–1012. [PubMed: 2423656]
 30. Wei J, Morris H, Hickie R. Positive correlation between calmodulin content and hepatoma growth rates. *Cancer Res*. 1982; 42:2571–2574. [PubMed: 7083150]
 31. Strobl JS, Peterson VA. Tamoxifen-resistant human breast cancer cell growth: inhibition by thioridazine, pimozide and the calmodulin antagonist, W-13. *J Pharmacol Exp Ther*. 1992; 263:186–193. [PubMed: 1403784]
 32. Frankfurt OS, Sugarbaker EV, Robb JA, Villa L. Synergistic induction of apoptosis in breast cancer cells by tamoxifen and calmodulin inhibitors. *Cancer Letters*. 1995; 97:149–154. [PubMed: 7497456]
 33. Gulino A, Barrera G, Vacca A, Farina A, Ferretti C, Screpanti I, Dianzani MU, Frati L. Calmodulin Antagonism and Growth-inhibiting Activity of Triphenylethylene Antiestrogens in MCF-7 Human Breast Cancer Cells. *Cancer Research*. 1986; 46:6274–6278. [PubMed: 3022916]
 34. Zhang GJ, Kimijima I, Onda M, Kanno M, Sato H, Watanabe T, Tsuchiya A, Abe R, Takenoshita S. Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-X(L), without alteration of p53 protein levels. *Clin Cancer Res*. 1999; 5:2971–2977. [PubMed: 10537367]
 35. Fancy RM, Kim H, Zhou T, Zinn KR, Buchsbaum DJ, Song Y. Calmodulin Binding to Death Receptor 5-mediated Death-Inducing Signaling Complex in Breast Cancer Cells. *J Cell Biochem*. 2017; 118:2285–2294. [PubMed: 28092099]
 36. Fancy RM, Wang L, Schmid T, Zeng Q, Wang H, Zhou T, Buchsbaum DJ, Song Y. Characterization of the Interactions between Calmodulin and Death Receptor 5 in Triple-negative and Estrogen Receptor-positive Breast Cancer Cells: AN INTEGRATED EXPERIMENTAL AND COMPUTATIONAL STUDY. *The Journal of biological chemistry*. 2016; 291:12862–12870. [PubMed: 27129269]
 37. Kim H, Samuel SL, Zhai G, Rana S, Taylor M, Umphrey HR, Oelschlager DK, Buchsbaum DJ, Zinn KR. Combination therapy with anti-DR5 antibody and tamoxifen for triple negative breast cancer. *Cancer biology & therapy*. 2014; 15:1053–1060. [PubMed: 25084100]
 38. Schindelin J, Rueden CT, Hiner MC, Eliceiri KW. The ImageJ ecosystem: An open platform for biomedical image analysis. *Mol Reprod Dev*. 2015; 82:518–529. [PubMed: 26153368]
 39. Amm HM, Zhou T, Steg AD, Kuo H, Li Y, Buchsbaum DJ. Mechanisms of Drug Sensitization to TRA-8, an Agonistic Death Receptor 5 Antibody, Involve Modulation of the Intrinsic Apoptotic

Pathway in Human Breast Cancer Cells. *Molecular Cancer Research*. 2011; 9:403–417. [PubMed: 21357440]

40. Zhao L, Wientjes MG, Au JL-S. Evaluation of Combination Chemotherapy. *Clinical Cancer Research*. 2004; 10:7994–8004. [PubMed: 15585635]
41. Chou T-C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. *Pharmacological Reviews*. 2006; 58:621–681. [PubMed: 16968952]
42. Goutelle S, Maurin M, Rougier F, Barbaut X, Bourguignon L, Ducher M, Maire P. The Hill equation: a review of its capabilities in pharmacological modelling. *Fundamental & Clinical Pharmacology*. 2008; 22:633–648. [PubMed: 19049668]
43. Chou T-C, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Advances in Enzyme Regulation*. 1984; 22:27–55. [PubMed: 6382953]
44. Chou T-C. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. *Cancer Research*. 2010; 70:440–446. [PubMed: 20068163]
45. Kaufmann SH, Steensma DP. On the TRAIL of a new therapy for leukemia. *Leukemia*. 2005; 19:2195–2202. [PubMed: 16224489]
46. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*. 1998; 17:1675–1687. [PubMed: 9501089]
47. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 1997; 91:479–489. [PubMed: 9390557]
48. Rhoads AR, Friedberg F. Sequence motifs for calmodulin recognition. *FASEB J*. 1997; 11:331–340. [PubMed: 9141499]
49. Ahn EY, Lim ST, Cook WJ, McDonald JM. Calmodulin binding to the Fas death domain. Regulation by Fas activation. *J Biol Chem*. 2004; 279:5661–5666. [PubMed: 14594800]
50. Dumitru CA, Gulbins E. TRAIL activates acid sphingomyelinase via a redox mechanism and releases ceramide to trigger apoptosis. *Oncogene*. 2006; 25:5612–5625. [PubMed: 16636669]
51. Wagner KW, Punnoose EA, Januario T, Lawrence DA, Pitti RM, Lancaster K, Lee D, von Goetz M, Yee SF, Totpal K, Huw L, Katta V, Cavet G, Hymowitz SG, Amler L, Ashkenazi A. Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med*. 2007; 13:1070–1077. [PubMed: 17767167]
52. Schimmer AD, Welsh K, Pinilla C, Wang Z, Krajewska M, Bonneau MJ, Pedersen IM, Kitada S, Scott FL, Bailly-Maitre B, Glinsky G, Scudiero D, Sausville E, Salvesen G, Nefzi A, Ostresh JM, Houghten RA, Reed JC. Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell*. 2004; 5:25–35. [PubMed: 14749124]
53. Schimmer AD. Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Res*. 2004; 64:7183–7190. [PubMed: 15492230]
54. Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ, Borden EC. Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. *Cell Death Differ*. 2004; 11:915–923. [PubMed: 15118763]
55. Jin CY, Moon DO, Lee JD, Heo MS, Choi YH, Lee CM, Park YM, Kim GY. Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis through downregulation of ERK and Akt in lung adenocarcinoma A549 cells. *Carcinogenesis*. 2007; 28:1058–1066. [PubMed: 17183064]
56. Guarneri V, Conte PF. The curability of breast cancer and the treatment of advanced disease. *Eur J Nucl Med Mol Imaging*. 2004; 31(Suppl 1):S149–161. [PubMed: 15107948]
57. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity*. 2000; 12:611–620. [PubMed: 10894161]
58. Daniel PT, Wieder T, Sturm I, Schulze-Osthoff K. The kiss of death: promises and failures of death receptors and ligands in cancer therapy. 2001

59. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene*. 2003; 22:8628–8633. [PubMed: 14634624]
60. Delmas D, Rebe C, Lacour S, Filomenko R, Athias A, Gambert P, Cherkaoui-Malki M, Jannin B, Dubrez-Daloz L, Latruffe N, Solary E. Resveratrol-induced apoptosis is associated with Fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells. *J Biol Chem*. 2003; 278:41482–41490. [PubMed: 12902349]
61. Cremesti A, Paris F, Grassme H, Holler N, Tschopp J, Fuks Z, Gulbins E, Kolesnick R. Ceramide enables fas to cap and kill. *The Journal of biological chemistry*. 2001; 276:23954–23961. [PubMed: 11287428]
62. Gajate C, Mollinedo F. The antitumor ether lipid ET-18-OCH(3) induces apoptosis through translocation and capping of Fas/CD95 into membrane rafts in human leukemic cells. *Blood*. 2001; 98:3860–3863. [PubMed: 11739199]
63. Pan D, Yan Q, Chen Y, McDonald JM, Song Y. Trifluoperazine regulation of calmodulin binding to Fas: a computational study. *Proteins*. 2011; 79:2543–2556. [PubMed: 21656570]
64. Hwang MK, Min YK, Kim SH. Calmodulin inhibition contributes to sensitize TRAIL-induced apoptosis in human lung cancer H1299 cells. *Biochem Cell Biol*. 2009; 87:919–926. [PubMed: 19935877]
65. Fancy RM, Kim H, Zhou T, Zinn KR, Buchsbaum DJ, Song Y. Calmodulin Binding to Death Receptor 5-mediated Death-inducing Signaling Complex in Breast Cancer Cells. *J Cell Biochem*. 2017
66. Wang S, Chen Y, Wu Q, Hua ZC. Detection of Fas-associated death domain and its variants' self-association by fluorescence resonance energy transfer in living cells. *Mol Imaging*. 2013; 12:111–120. [PubMed: 23415399]
67. Cursi S, Rufini A, Stagni V, Condò I, Matafora V, Bachi A, Bonifazi AP, Coppola L, Superti-Furga G, Testi R, Barilà D. Src kinase phosphorylates Caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *The EMBO Journal*. 2006; 25:1895–1905. [PubMed: 16619028]
68. Izeradjene K, Douglas L, Delaney AB, Houghton JA. Casein Kinase I Attenuates Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis by Regulating the Recruitment of Fas-Associated Death Domain and Procaspase-8 to the Death-Inducing Signaling Complex. *Cancer Research*. 2004; 64:8036–8044. [PubMed: 15520213]
69. Sun M, Song L, Li Y, Zhou T, Jope RS. Identification of an antiapoptotic protein complex at death receptors. *Cell Death Differ*. 2008; 15:1887–1900. [PubMed: 18846110]
70. Yuan K, Jing G, Chen J, Liu H, Zhang K, Li Y, Wu H, McDonald JM, Chen Y. Calmodulin Mediates Fas-induced FADD-independent Survival Signaling in Pancreatic Cancer Cells via Activation of Src-Extracellular Signal-regulated Kinase (ERK). *The Journal of Biological Chemistry*. 2011; 286:24776–24784. [PubMed: 21613217]
71. Yuan K, Yong S, Xu F, Zhou T, McDonald JM, Chen Y. Calmodulin antagonists promote TRA-8 therapy of resistant pancreatic cancer. *Oncotarget*. 2015; 6:25308–25319. [PubMed: 26320171]
72. Woodgett JR, Davison MT, Cohen P. The calmodulin-dependent glycogen synthase kinase from rabbit skeletal muscle. Purification, subunit structure and substrate specificity. *Eur J Biochem*. 1983; 136:481–487. [PubMed: 6315430]
73. Woodgett JR, Tonks NK, Cohen P. Identification of a calmodulin-dependent glycogen synthase kinase in rabbit skeletal muscle, distinct from phosphorylase kinase. *FEBS Lett*. 1982; 148:5–11. [PubMed: 6293876]
74. Fang X, Yu SX, Lu Y, Bast RC, Woodgett JR, Mills GB. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:11960–11965. [PubMed: 11035810]
75. Yuan K, Yong S, Xu F, Zhou T, McDonald JM, Chen Y. Calmodulin antagonists promote TRA-8 therapy of resistant pancreatic cancer. *Oncotarget*. 2015; 6:25308–25319. [PubMed: 26320171]

FIGURE 1. CaM antagonist, TFP, enhanced TRA-8 induced cytotoxicity in TRA-8 resistant triple negative breast cancer (TNBC) cells

ATPLite cell viability assay results for TRA-8 resistant TNBC cells **(A)** HCC1143 and **(B)** HCC1937 with drug treatment of TFP alone, TRA-8 alone or the combined TRA-8 and TFP treatment at varied TFP or TRA-8 concentrations for 24 hours. For ATPLite cell viability assay, percent of cell viability represents the percent of ATP amount relative to the no treatment control. Results are averaged from 9 sets of tested samples and error bars show the standard error of the mean. * $p < 0.05$ represents the significant difference of HCC1143 or HCC1937 cell viability with combined TFP and TRA-8 treatment compared to that with either TFP only or TRA-8 only treatment.

FIGURE 2. TFP promoted DR5-mediated apoptosis in TRA-8 resistant TNBC cells

The apoptosis assay performed using Dead Cell Apoptosis Kit with Annexin V FITC and propidium iodide (PI) for HCC1143 or HCC1937 cells untreated (control) (A), treated with 20 μ M TFP only (B), treated with 500 ng/mL TRA-8 only (C), or treated with 20 μ M TFP with 500 ng/mL TRA-8 treatment (D) for 16 hours. (E) The mean fluorescence intensity for Annexin V FITC fluorescence images and for PI fluorescence images for apoptosis assay measured using the ImageJ software [38]. Apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. Representative results are from three independent experiments.

FIGURE 3. TFP promoted DR5-mediated apoptotic caspase signaling in TRA-8 resistant TNBC cells

Western blot analysis of TRA-8 resistant (A) HCC1143 cell or (B) HCC1937 cell lysates for caspase-8, caspase-3, and PARP-1 and their cleavage products. Cells untreated or treated with 500 ng/mL TRA-8 only, treated with 20 μ M TFP only, or pretreated with 20 μ M TFP with 500 ng/mL TRA-8 for 16 hours. Representative results are from two independent experiments.

FIGURE 4. CaM bound to DR5 in a calcium dependent manner in TRA-8 resistant TNBC cells

(A) Co-Immunoprecipitation (Co-IP) of DR5 and CaM from HCC1143 cell lysate.

Lysate lane: whole cell lysate of HCC1143 cells, **IP TRA-8 lane:** Co-IP using TRA-8

conjugated beads, **IP Iso-IgG lane:** Co-IP using a mouse Iso-IgG conjugated beads. **(B)**

CaM pull-down of DR5 from HCC1143 cell lysate supplemented with 1 mM Ca^{2+} or 2 mM

Ca^{2+} chelator, EGTA. **I:** input, **CaM-S:** pull-down using CaM conjugated sepharose beads,

S: pull-down control using sepharose beads. Representative results are from two

independent experiments.

FIGURE 5. CaM expression knock-down increased DR5-mediated DISC formation and caspase cleavage in TRA-8 resistant TNBC cells

(A) Co-IP of CaM, DR5, FADD and caspase-8 from HCC1143 cells with control siRNA or CaM 1, 2, and 3 siRNAs pretreatment, then followed with or without 500 ng/mL TRA-8 treatment for 3 hours. **Cell Lysate lane:** cell lysates before IP. **IP: DISC lane:** Co-IP of CaM with DR5-mediated DISC from cell lysates using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads. (B) Quantitative analysis of the western blot results of the IP results about the effect of CaM siRNA on DR5-mediated DISC using ImageJ software [38]. (C) Western blot analysis of lysates of HCC1143 cells with control siRNA or CaM 1, 2, and 3 siRNAs pretreatment, then followed with or without 500 ng/mL TRA-8 treatment

for 6 hours, for caspase-8, caspase-3, and PARP-1 and their cleavages. Western blot of lysate results are from two independent experiments. Co-IP results are from three independent experiments and error bars show the standard error of the mean. * $p < 0.05$ represents the significant difference.

FIGURE 6. TFP increased DR5-mediated DISC formation in TRA-8 resistant TNBC cells
(A) Co-IP of CaM, DR5, FADD and Caspase-8 from TRA-8 resistant HCC1143 breast cancer cells with 0 μ M, 10 μ M or 20 μ M TFP pretreatment for 19 hours, then treated with or without 500 ng/mL TRA-8 for 3 hours. **Cell Lysate lane:** cell lysates before IP. **IP: DISC lane:** Co-IP of CaM with DR5-mediated DISC from cell lysates using anti-DR5 (D-6) antibody with protein A/G Plus-Agarose beads. Representative results are from three independent experiments. **(B)** Quantitative analysis of the western blot results of DR5-mediate DISC IP results using ImageJ software [38]. Results are from three independent experiments and error bars show the standard error of the mean. * $p < 0.05$ represents the significant difference.

FIGURE 7. TFP enhanced DR5 oligomerization in TRA-8 resistant TNBC cells

(A) Representative fluorescence microscopic images of Cy5.5 labeled TRA-8 on HCC1143 cells with or without 10 μ M TFP treatment for 24 hours. The same color scale was applied for all images. The fluorescence images were overlapped with bright field images showing the cell morphology. (B) Gaussian curves fitting to the distribution of Cy5.5-TRA-8 on the representative HCC1143 cells indicated with white arrows as shown in (A). (C) Peak amplitudes of TRA-8 activated DR5 oligomers when cells were treated with Cy5.5-TRA-8 alone or TRA-8 in combination with TFP in HCC1143.

FIGURE 8. TFP decreased phosphorylation of Akt and ERK and promoted IAPs down-regulation in TRA-8 resistant TNBC cells
 Western blot analysis of HCC1143 cell lysates for DR5, pERK, pAkt, XIAP, and cIAP-1. Cells untreated or treated with 500 ng/mL TRA-8 only, treated with 20 μ M TFP only, or pretreated with 20 μ M TFP with 500 ng/mL TRA-8 for 16 hours. Results are from three independent experiments and error bars show the standard error of the mean. * $p < 0.05$ represents the significant difference.