XIAP Inhibition and Generation of Reactive Oxygen Species Enhances TRAIL Sensitivity in Inflammatory Breast Cancer Cells

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Abstract

We recently identified superoxide dismutase (SOD) overexpression and decreased induction of reactive oxygen species (ROS)-mediated apoptosis in models of inflammatory breast cancer (IBC) cells with acquired therapeutic resistance. This population of cells has high expression of X-linked inhibitor of apoptosis protein (XIAP), which inhibits both extrinsic and intrinsic apoptosis pathways. We therefore wanted to evaluate the effect of classical apoptosis inducing agent TRAIL, a proapoptotic receptor agonist that selectively triggers death receptor (DR)-mediated apoptosis in cancer cells, in the IBC acquired resistance model. XIAP levels and subsequent inhibition of caspase activity inversely correlated with TRAIL sensitivity our models of IBC. These include SUM149, a basal-type cell line isolated from primary IBC tumors, and isogenic SUM149-derived lines rSUM149 and SUM149 wtXIAP, models of acquired therapeutic resistance with endogenous and exogenous XIAP overexpression respectively. Inhibition of XIAP function using embelin, a plant-derived cell permeable small molecule, in combination with TRAIL caused a synergistic decrease in cell viability. Embelin treatment resulted in activation of ERK1/2 and ROS accumulation, which correlated with downregulation of antioxidant protein superoxide dismutase (SOD1) and consumption of redox modulator reduced glutathione in the XIAP overexpressing cells. Simultaneous treatment with an SOD mimic, which protects against ROS accumulation, reversed the decrease in cell viability caused by embelin+TRAIL treatment. Embelin primes IBC cells for TRAIL-mediated apoptosis by its direct action on XIAP’s anti-caspase activity and by shifting the cellular redox balance toward oxidative stress-mediated apoptosis. Thus, ROS modulators represent a novel approach to enhance efficacy of TRAIL-based treatment protocols in IBC.
Introduction

Inflammatory breast cancer (IBC) is an aggressive subtype of breast cancer that is highly invasive and often acquires resistance to chemo-, targeted- and radiotherapies, resulting in a relatively low disease-free survival rate compared to locally advanced breast cancer (LABC) (1, 2). Increase in reactive oxygen species (ROS) in response to many therapeutic agents has been identified to induce therapeutic apoptosis (3, 4). At the same time, drug resistance can develop with continuous treatment, which can reduce cellular ROS levels. We previously reported overexpression of a potent caspase inhibitor, X-linked inhibitor of apoptosis (XIAP) to be a key feature in acquired resistance to epidermal growth factor (ErbB1/EGFR and ErbB2/HER2) targeting agents in SUM149 (basal type with EGFR activation) and SUM190 (ErbB2 amplified) derived IBC cell models (5, 6). In addition to XIAP overexpression, we also observed that the clonal population of IBC cell lines with acquired therapeutic resistance gain antioxidant capacity (upregulation of superoxide dismutases-SOD1/2), making them cross-resistant to other oxidative stressors (7). Further, it has been shown that activation of AMP-activated protein kinase switched cell metabolism from an anabolic ATP-consuming to a catabolic ATP-generating state, which served to protect cells from apoptotic stimuli (7, 8). Therefore, it is clear that an imbalance between ROS and the cell’s antioxidant capacity and activation of cytoprotective stress responses can contribute to the development of autoresistance in IBC therapy. It is unknown how this altered profile of increased anti-apoptotic and antioxidant expression in the IBC cells affects response to classical inducers of apoptosis and whether it can be modulated by ROS accumulation. In order to study this, we characterized efficacy of TNF-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily and an apoptosis-inducing ligand. TRAIL binding to death receptors (DR) 4 and 5 on the cell surface leads to
receptor aggregation, recruitment of adaptor molecule Fas-associated death domain (FADD) protein and activation of caspase-8, which then leads to initiation of the protease cascade, activation of the effector caspases (caspase-3 and -7), and subsequent apoptosis (9). In addition, since TRAIL has the ability to directly induce tumor cell death via death receptors, the status of intracellular sensors such as p53 is less relevant, thereby making it attractive as an anti-cancer agent (10). TRAIL has demonstrated promising therapeutic potential in a variety of cancer cell lines (11, 12) with little or no toxicity to non-transformed cells. Although initial clinical trials using TRAIL reported high hepatotoxicity, newer versions of recombinant TRAIL have been very encouraging, with relatively low toxicity to normal cells (10, 13). It has been shown that repeated TRAIL treatment leads to resistance (14), and multiple mechanisms of resistance have been identified. These include differential expression of death receptors, constitutive activation of Akt and NF-kappaB, overexpression of cFLIP and one or more of the inhibitor of apoptosis proteins (IAP), mutations in the Bax and Bak genes, and defects in the release of mitochondrial proteins (15, 16). Therefore, agents that can sensitize cells to TRAIL-mediated apoptosis are attractive candidates for combination cancer therapy. One of the dominant features of TRAIL resistance observed is XIAP overexpression (17-19).

Interestingly, it has been observed that triple negative breast cancer cells, which includes the SUM149 (EGFR activated, basal-type) IBC cell line isolated from patient primary tumor (20) show higher sensitivity to TRAIL treatment as a single agent compared to a majority of other breast cancers (21, 22). In the present study, we observed that rSUM149, an isogenic acquired resistance model derived from primary basal type IBC line SUM149 which exhibits endogenous XIAP overexpression (6), is less sensitive to TRAIL-mediated apoptosis compared to the
parental SUM149 cells. Alternately, stable exogenous overexpression of XIAP in the parental SUM149 cells decreased TRAIL sensitivity.

Embelin, a natural plant derived agent, has been identified as a cell permeable, small molecule inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine database consisting of 8221 individual herbal products (23). It binds to the BIR3 domain of XIAP with affinity similar to natural Smac/DIABLO peptides, blocking XIAP’s interaction with caspases to promote apoptosis. In addition to XIAP, embelin has been reported to inhibit NFkappaB signaling and to have anti-tumor, anti-inflammatory and analgesic properties (24, 25). In the present study, we observed that in addition to blocking XIAP’s caspase-inhibitory activity, embelin suppressed key antioxidants and increased accumulation of ROS to potentiate TRAIL sensitivity, revealing a new mechanism of embelin action.
Materials and Methods

Cell culture

SUM149 cells were obtained from Asterand, Inc. (Detroit, MI) and were cultured as described previously (5). Asterand characterizes cell lines using short tandem repeat polymorphism analysis. Cells were banked upon receipt and cultured for no more than 6 months prior to use in this study. rSUM149 is an isogenic acquired resistance model established in the lab (6). SUM149 cells stably expressing wtXIAP and FG9 vector control were generated using a lentiviral expression system (kindly provided by Dr. Colin Duckett, University of Michigan) as described in (6).

shRNA-mediated knockdown of XIAP

rSUM149 cells (6) were seeded at 30,000 cells per well into a 12 well plate and allowed to adhere overnight. After 24 h, cells were transfected with a plasmid containing XIAP-targeting shRNA using Mirus TransIT 2020 transfection reagent (Mirus Bio, Madison, WI) according to manufacturer’s instructions. TRAIL treatments (0-1000ng/mL) were applied 48 h post transfection for 24 h, and then cells were harvested for trypan blue exclusion assay. Effective knockdown was confirmed by western immunoblot analysis.

Treatment of cells for determination of viability and signaling analysis

Cells were seeded in 6-well plates (Corning Incorporated, Corning, NY) and allowed to reach 70% confluence. Cells were then treated for 24h in regular growth media with TRAIL (BioMol, Plymouth Meeting, PA), embelin (Sigma), and an SOD mimetic (MnTnHex-2-PyP5+) (26) alone and in combination. The MEK1/2 inhibitor U0126 (Cell Signaling Technologies, Danvers, MA)
was applied to cells for one hour prior to addition of embelin or embelin+TRAIL. Cell viability was determined by trypan blue exclusion as described previously (5).

**Western immunoblot analysis**

Western immunoblot analysis was carried out as described previously (5). Cell lysates were harvested after treatment with TRAIL for 24 h. Membranes were incubated with primary antibodies against XIAP (BD Bioscience, San Jose, CA), procaspase-3, MAPK, JNK, cFLIP, DR5 (Cell Signaling Technologies), DR4, actin or GAPDH (Santa Cruz, Santa Cruz, CA), overnight at 4 °C. Stripping of membranes for detection of total protein was done as described previously (17). Densitometric analysis was performed using the NIH ImageJ software (27).

**Caspase 3/7 activity assay**

Cells were seeded in 6-well plates (Corning Incorporated), and the next day cells were treated with TRAIL for 4h in regular growth media. After incubation, caspase 3/7 activity was determined in 3 μg total cell lysates using the Caspase-Glo® Assay (Promega, Madison, WI) as per the manufacturer’s instructions.

**Glutathione assay**

Reduced glutathione levels were assessed as described previously (7) using the GSHGlo™ Glutathione Assay (Promega) as per the manufacturer’s instructions.

**ROS measurement**
Cells were cultured in six-well plates (Corning Incorporated, Corning, NY) in regular growth media until reaching 70–80% confluence. Cells were treated with embelin or paraquat (Sigma) for 1h, then harvested and incubated for 30 min with 10µM MitoSOX Red dye (Molecular Probes, Carlsbad, CA) to detect mitochondrial superoxide. Cells were then washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry. At least 25,000 events were collected on a FACScalibur flow cytometer (Beckton Dickinson, Rockville, MD) and analyzed using Cellquest (Beckton Dickinson).

**Assessment of cell viability via measurement of mitochondrial membrane potential**

Viability and cell injury were assessed using the mitochondrial membrane potential marker tetramethylrhodamine, ethyl ester, perchlorate (TMRE, Molecular Probes). Cells were treated with the indicated concentrations of embelin for 1h, then harvested and incubated for 30 min with 500nM TMRE. Cells were washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry.

**Drug synergism analysis**

Analysis of drug synergism was performed using the Calcusyn software (Biosoft, Cambridge, UK), which uses the Chou-Talalay method (28) where a combination index (CI) <1 indicates synergism.

**Statistical analysis**

The statistical analyses were performed using Graphpad InStat (Graphpad Software, Inc., La Jolla, CA) Student’s two-tailed t-test. Differences were considered significant at p<0.05.
Results

XIAP overexpression inversely correlates with TRAIL sensitivity in IBC cells

In this study, the role of XIAP in TRAIL sensitivity was evaluated in SUM149 cells [triple negative, ErbB1 activated cell line (20) isolated from primary IBC tumor]. Isogenic cells with differential XIAP expression derived from SUM149 were characterized for TRAIL sensitivity (10-1000 ng/mL) at a 24 h time period. These include parental SUM149, SUM149 wtXIAP (stable XIAP overexpression using a lentiviral construct) and its vector-control counterpart SUM149 FG9 (6), rSUM149 (6), a model of acquired resistance to an ErbB1/2 targeting agent with endogenous high XIAP expression, and rSUM149 with XIAP knockdown (rSUM149 shXIAP). Cell viability determined by trypan blue exclusion assay shows that parental SUM149 have significantly higher sensitivity to TRAIL compared to rSUM149 (Figure 1A) and SUM149 wtXIAP cells (Figure 1B, left panel). Calculated IC₅₀ for cell viability in the presence of TRAIL was approximately 770ng/mL in SUM149 wtXIAP cells, 530ng/mL in endogenously XIAP overexpressing rSUM149 cells, and 45ng/mL (p<0.005) in vector control SUM149 cells. To further investigate the role of XIAP in this observed differential sensitivity to TRAIL, we characterized TRAIL efficacy in the rSUM149 cells with XIAP knockdown (rSUM149 shXIAP) through transfection of a plasmid expressing shRNA against XIAP. Immunoblot analysis is shown in the inset of Figure 1B. Data in Figure 1B (right panel) show that XIAP knockdown in the rSUM149 cells results in higher sensitivity to TRAIL-induced cell death. Knockdown of XIAP in the rSUM149 cells resulted in a pronounced decrease in IC₅₀ from 530ng/mL in rSUM149 cells to approximately 30ng/mL in the rSUM149 shXIAP cells. In addition, XIAP knockdown caused the rSUM149 shXIAP cells to be even more sensitive to TRAIL than the parental SUM149 cells, which have some basal level of XIAP expression. rSUM149 shXIAP
cell viability drops to ~60% upon treatment with 10ng/mL TRAIL, while SUM149 cell viability is about 80% with the same treatment. Cell viability differences were consistent with XIAP downregulation post TRAIL treatment in the SUM149 cells at 50ng/mL compared to the rSUM149 and SUM149wtXIAP cells (Figure 1C). In addition, a functional assay was used to measure the activity of caspases during the apoptotic process; this assay was performed after 4h of TRAIL treatment to ensure that cells were dying but not yet dead, at which point caspase activity would be unreadable. XIAP decrease following treatment in the TRAIL-sensitive SUM149 cells corresponded with increased caspase 3/7 activity (Figure 1D, p<0.005) compared to limited caspase activity post-TRAIL treatment in the rSUM149 and SUM149wtXIAP cells.

These results demonstrate that SUM149wtXIAP and rSUM149 cells with XIAP overexpression compared to SUM149 show significantly reduced sensitivity to TRAIL-mediated apoptosis.

**Embelin enhances TRAIL sensitivity**

Since XIAP overexpression and activity corresponds with decreased TRAIL sensitivity in the SUM149 model, we evaluated the effects of embelin (Figure 2A), a small molecular inhibitor of XIAP, in combination with TRAIL. Combination studies with TRAIL and embelin were conducted at 24 h, and viability was assessed by trypan blue exclusion assay. Data in Figure 2B-D show that embelin at 24h alone at three concentrations (12.5, 25, 50μM) induced a modest concentration-dependent decrease (10-25%) in viability in the three cell lines. TRAIL (50ng/mL) alone at 24 h as described in Figure 1B, shows higher sensitivity in the parental SUM149 cells compared to the XIAP overexpressing SUM149wtXIAP and rSUM149 isogenic lines. Combining increasing concentrations of embelin with TRAIL (50ng/mL) at 24 h caused a significant decrease in cell viability in all three cell lines. Increasing concentrations of TRAIL up
to 100ng/mL in combination with 25μM or 50μM embelin did not have any significantly enhanced response over that seen in Figure 2 (data not shown).

In order to characterize the interaction of these two agents, we analyzed the above results (Figure 2B-D, left panels) using the CalcuSyn program (Biosoft, Cambridge, UK), which employs the Chou-Talalay Method, a derivation of the mass-action law principle (28). When experimental data is entered into the program, it produces graphs in which the X axis represents the dose of each drug alone or in combination, and the Y axis represents treatment efficacy, with 1.0 meaning 100% cell death, while a 0.5 is equal to 50% cell death. From these graphs, the program calculates a combination index (CI) that is a quantitative measurement of the relationship between two agents; a CI greater than 1 indicates antagonism, while a CI of one indicates an additive interaction and a CI less than one indicates synergism. The CI for the interaction between embelin and TRAIL in SUM149, SUM149 wtXIAP and rSUM149 cells were calculated to be 0.077, 0.041, and 0.122 respectively (Figure 2), which is indicative of strong synergism in all three cell lines.

Effect of embelin, TRAIL, or embelin+TRAIL on downstream TRAIL signaling pathway proteins in treated lysates was analyzed by western immunoblot. Immunoblot analysis of death receptors DR4 and DR5 (Figure 3A) in SUM149 cells revealed no significant upregulation of death receptor expression in response to treatment. The same results were seen in the SUM149 wtXIAP cells upon treatment with embelin and/or TRAIL (data not shown). A decrease in cFLIP, a caspase-8 homolog that binds to the death-inducing signaling complex (DISC) to block caspase activation and apoptosis, was observed following treatment with embelin and TRAIL alone as well as in the combination-treated lysates in the parental SUM149 cells (Figure 3B, left panel). However, cFLIP levels remain unchanged in the XIAP overexpressing cells (Figure 3B,
right panel) treated with embelin, TRAIL or embelin+TRAIL, although cell death was significantly increased (Figure 2B). Examination of lysates from SUM149 wtXIAP cells treated with embelin or embelin+TRAIL reveal XIAP downregulation upon treatment with embelin compared to control. In addition, an XIAP cleavage product (30kDa) along with potent inhibition of XIAP levels is detected in cells treated with embelin+TRAIL (Figure 3C). In summary, embelin and TRAIL synergize to increase cell death in the triple negative IBC cell model.

**Embelin modulates extracellular signaling regulated kinase activation**

To further study the mechanisms behind the combinatorial synergism, signaling pathways that are linked to apoptosis were examined in response to treatment. For this purpose, SUM149 and SUM149 wtXIAP cells were treated with embelin, TRAIL, or embelin+TRAIL at the indicated concentrations for 24 h and then examined for the phosphorylation status of extracellular signaling regulated kinase (ERK1/2) and the stress activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (Figure 4). In SUM149 cells, cell death associated with embelin or embelin+TRAIL treatment correlated with a decrease in p-ERK1/2 signaling (Figure 4A). Interestingly, in the SUM149 wtXIAP cells with exogenous XIAP overexpression, the basal levels of ERK1/2 were itself lower than the parental SUM149 cells. Further, an increase in p-ERK1/2 was observed in embelin and embelin+TRAIL treated SUM149 wtXIAP cell lysates compared to vehicle- or TRAIL-treated cells (Figure 4B). To interrogate the nature of ERK1/2 signaling in this system, we added the MEK1/2 inhibitor U0126 to the embelin+TRAIL combination in SUM149 wtXIAP cells to block phosphorylation of ERK1/2. This resulted in further reduction of cell viability from approximately 25% with embelin+TRAIL to less than 1% viable cells in the presence of embelin+TRAIL+U0126 (Figure 4B, graph). No specific change
in p-JNK levels was observed in the various treatments as compared to vehicle control in the SUM149 or SUM149 wtXIAP cells. Representative immunoblot is shown in Figure 4C.

**Embelin induces generation of reactive oxygen species by downregulating superoxide dismutase 1 and oxidizing glutathione**

Recently we reported (7) that the XIAP overexpressing rSUM149 cells which have acquired resistance to therapeutic apoptosis mediated by lapatinib, an ErbB1/2 targeting agent, have lost the ability to accumulate reactive oxygen species in the presence of oxidizing agents like paraquat and hydrogen peroxide; they also have high expression of key antioxidants superoxide dismutase-1, -2, and reduced-glutathione. Interestingly, embelin treatment downregulates SOD1 (Figure 5A, left panel) and consumes reduced glutathione (Figure 5A, right panel, p<0.05) in the rSUM149 cells, inhibiting the detoxification of damaging oxidative species. Decrease in antioxidant expression corresponds with an increase in mitochondrial superoxides as measured by flow cytometry compared to paraquat, a classical ROS generating agent which we have previously reported (7) to have insignificant effect on ROS generation in the rSUM149 cells (Figure 5B).

**SOD mimic/Antioxidant reverses efficacy of embelin+TRAIL combination**

To determine whether the generation of ROS by embelin is specifically contributing to enhanced cell death observed with embelin+TRAIL treatment, we tested the effect of an SOD mimic (MnTnHex-2-PyP5+) (7, 26), which was simultaneously added to the combination at increasing concentrations. This cationic SOD mimic is a potent antioxidant both in vitro and in vivo (26). Addition of the SOD mimic to the embelin+TRAIL combination provided protection against
ROS and resulted in a dose dependent increase in cellular viability (Figure 5C). Together, these results indicate that the concurrent inhibition of XIAP along with the modulation of antioxidant molecules and ROS generation by embelin sensitizes resistant cells to TRAIL-induced apoptosis.
Discussion

We report herein an inverse relationship between XIAP expression and TRAIL sensitivity in isogenic cell lines derived from SUM149, a basal-type IBC cell line isolated from patient primary tumors. SUM149 cells with endogenous and exogenous overexpression of XIAP (rSUM149 and SUM149 wtXIAP respectively) showed increased resistance to TRAIL-induced apoptosis and limited caspase activation compared to parental TRAIL-sensitive SUM149 cells (20). Additionally, knockdown of XIAP in the XIAP-overexpressing rSUM149 cells enhanced TRAIL efficacy; the knockdown rendered the rSUM149 shXIAP cells even more sensitive to TRAIL-induced apoptosis than the parental SUM149 cells, which have basal levels of XIAP expression. Further, we demonstrate that the XIAP inhibitor embelin synergizes with TRAIL to induce apoptosis in XIAP overexpressing IBC lines (SUM149 wtXIAP and rSUM149) and increases the potency of TRAIL in the isogenic TRAIL-sensitive counterpart (SUM149), the first report of this phenomenon in inflammatory breast cancer. We also identified that embelin activated ERK1/2 in SUM149 wtXIAP cells, and this effect was amplified and accompanied by increased cell death when combined with TRAIL. Further, embelin treatment reversed high superoxide dismutase expression and inhibited the enhanced glutathione detoxification capacity observed in the acquired therapeutic resistant IBC isogenic cell model; this led to the accumulation of reactive oxygen species identifying a potential new mechanism of embelin action.

Previous studies evaluating the efficacy of various therapeutic agents (such as gossypol, perifosine, and zerumbone) and chemotherapy that can potentiate TRAIL efficacy have reported upregulation of TRAIL-specific death receptors as an important mechanism of action leading to cellular sensitization to TRAIL (29-31). However, this mechanism was not apparent in the IBC
lines, as no increase in death receptor expression in embelin-treated cells was observed. In addition to XIAP, the anti-apoptotic caspase-8 homolog cFLIP has previously been reported to block apoptosis induced by ligand binding to Fas, TRAIL, TNF, and CD95 receptors (14, 32, 33). Inhibition or downregulation of cFLIP in certain contexts is able to sensitize resistant cells to TRAIL-induced apoptosis (34-39). In the current study, although cFLIP protein levels decreased significantly in TRAIL or embelin+TRAIL treated parental SUM149 cells undergoing cell death, cFLIP levels remained unchanged in embelin+TRAIL treated XIAP overexpressing cells that were also undergoing apoptosis. This observation indicates that cFLIP degradation or downregulation may not be sufficient in determining TRAIL sensitivity in this model.

In the present study, embelin treatment as a single agent in the XIAP overexpressing cell line (SUM149 wtXIAP) caused an increase in ERK1/2 phosphorylation, and in combination with TRAIL, this ERK1/2 phosphorylation was amplified and corresponded with significant cell death. In contrast, ERK phosphorylation in SUM149 cells was decreased following embelin or embelin+TRAIL treatment. Interestingly, the SUM149 wtXIAP cells have very low levels of basal ERK1/2 phosphorylation compared to the parental SUM149 cells. This observation is consistent with a previous study showing that XIAP knockdown in a mouse model corresponded with increase in ERK1/2 phosphorylation (40). ERK signaling is largely proliferative (41) and promotes survival (42); down regulation or inhibition of ERK1/2 is sometimes necessary for apoptosis to take place both in normal and cancerous cells. Previous studies in neuronal (43) and leukemia models (44, 45) have also reported activation of p38-MAPK and JNK in conjunction with ERK inhibition to be critical for induction of apoptosis. However, more recent reports have yielded convincing evidence implicating the Ras/Raf/ERK pathway in pro-apoptotic signaling events such as the expression of death ligands and/or receptors (29), modulation of Bcl-2 family
members to disrupt the mitochondrial membrane, and suppression of anti-apoptotic signaling molecules (46). In fact, the action of many apoptosis-inducing drugs (estradiol, tamoxifen, cephalosporin, etc.) is abrogated through inhibition of the Ras/Raf/ERK pathway (47). In the parental SUM149 cells with basal levels of XIAP expression, treatment with apoptosis inducing agent embelin correlates with decreased ERK1/2 phosphorylation, indicating a pro-survival role of ERK. In the SUM149 cells with XIAP overexpression, which has low basal ERK activation, treatment with potent apoptosis inducing agents correlates with increased ERK1/2 phosphorylation, suggesting that ERK1/2 signaling is a compensatory mechanism to oppose apoptosis. This is further supported by the observation that addition of the MEK1/2 inhibitor U1026 to embelin+TRAIL treatment caused significant cell death over and above embelin+TRAIL combination in the SUM149 wtXIAP cells. U0126 as a single agent has no effect on SUM149 cell proliferation (48).

We recently reported that the rSUM149 cells lack the ability to accumulate reactive oxygen species in the presence of ROS-generating agents due to overexpression of superoxide dismutase (SOD1, 2) and increased glutathione levels (7). Embelin sensitized rSUM149 to apoptosis, caused a significant downregulation of SOD1 and reduced glutathione content, and increased superoxide levels, thereby revealing an ROS modulating mechanism of embelin in IBC cells. Furthermore, addition of an SOD mimic (MnTnHex-2-PyP5+) provided protection against ROS and resulted in an increase in cellular viability in a dose dependent manner with the embelin+TRAIL combination. This is consistent with our previous report that treatment of parental SUM149 cells with an SOD mimic reversed the ability of these cells to accumulate ROS in the presence of oxidizing agents (7). In addition, we compared embelin+TRAIL to 2-methoxyestradiol (2-ME)+TRAIL and observed a significant increase in cell death similar to
embelin+TRAIL (data not shown). 2-ME is an ROS modulator in clinical trials which was previously reported to increase cell death in rSUM149 cells (7). Together, these results indicate that embelin primes IBC cells for TRAIL-mediated apoptosis through direct inhibition of XIAP anti-caspase activity and by shifting the cellular redox balance toward oxidative stress-mediated apoptosis. Thus, ROS modulators may represent a novel promising approach to enhance efficacy of TRAIL-based treatment protocols in IBC.
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References


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Figure Legends

**Figure 1.** Effect of TRAIL on XIAP expression, viability, and caspase activation in SUM149 isogenic lines. A, SUM149 and rSUM149 cells were treated with TRAIL for 24 h, and viability was assessed using the trypan blue exclusion assay. Bars represent mean±SEM viable cells taken as a percentage of the untreated control (n=2-4). B, Left panel: SUM149 wtXIAP and vector control SUM149 FG9 cells were treated with TRAIL for 24 h, and viability was assessed as previously described. Bars represent mean±SEM viable cells taken as a percentage of the untreated control (n=2-4). Right panel: rSUM149 and rSUM149 cells transfected with an XIAP-targeting shRNA plasmid with knockdown of XIAP expression were treated with TRAIL for 24 h, and viability was assessed as previously described (n=2-4). Inset: XIAP expression in rSUM149 cells and rSUM149 shXIAP cells. GAPDH was used as a loading control. C, XIAP immunoblot analysis of rSUM149, SUM149 wtXIAP, and SUM149 FG9 vector control cells treated with TRAIL for 24 h. Treatments are compared to untreated cells. Actin was used as a loading control. D, Caspase 3/7 activity in rSUM149, SUM149 wtXIAP, and SUM149 FG9 vector control cells after treatment with TRAIL for 3 h. Bars represent mean±SEM RLU (n=3). **p<0.005

**Figure 2.** Effect of TRAIL and the XIAP inhibitor embelin alone or in combination on viability of isogenic IBC cells and combinatorial analysis. A, Structure of embelin. SUM149 FG9 vector control (B), SUM149 wtXIAP (C), and rSUM149 (D) cells were treated with embelin (12.5-50µM) and TRAIL (50ng/mL) alone or in combination for 24 h, and viability was assessed using the trypan blue exclusion assay (left panels). Bars represent mean±SEM of viable cells (n=2-8).
*p<0.05, **p<0.005  Right panels are dose effect curves for embelin and TRAIL alone as well as the combination; these were created using CalcuSyn software.

**Figure 3.** Effect of TRAIL and embelin on TRAIL resistance factors. A, Western immunoblot analysis of DR4 and DR5 expression in SUM149 cells treated with embelin, TRAIL, or embelin+TRAIL for 24h. Treatments are compared to untreated cells or DMSO vector control. B, cFLIP western immunoblot analysis of SUM149 (left panel) and SUM149 wtXIAP (right panel) cells treated with embelin, TRAIL, or embelin+TRAIL for 24h. C, XIAP immunoblot analysis of SUM149 wtXIAP cells treated with embelin, TRAIL, or embelin+TRAIL for 24h; full length XIAP and its cleavage product are shown. Numbers represent densitometric analysis of protein normalized to GAPDH (A, C) or β-actin (B) for all blots.

**Figure 4.** Effect of TRAIL and embelin on cellular signaling in IBC cells with differential XIAP expression. A, Phospho-ERK1/2 (MAPK p44/42) western immunoblot analysis of SUM149 cells treated with embelin or embelin+TRAIL for 24 h. B, Upper panel: Phospho-ERK1/2 western immunoblot analysis of SUM149 wtXIAP cells treated with embelin, TRAIL, or embelin+TRAIL. Numbers represent densitometric analysis of p-ERK1/2 normalized to total ERK1/2 protein. Lower panel: Cellular viability as determined by trypan blue viability assay for SUM149 wtXIAP cells treated with 50µM embelin, 50ng/mL TRAIL, or combination, and the combination with the addition of 10µM U0126 MEK1/2 inhibitor. C, Phospho-JNK western immunoblot analysis of SUM149 wtXIAP cells treated with embelin, TRAIL, or embelin+TRAIL for 24 h. Treatments are compared to untreated cells or DMSO vector control. Numbers represent densitometric analysis of p-JNK normalized to GAPDH.
Figure 5. The XIAP inhibitor embelin mediates the generation of ROS in rSUM149 cells to cause cell death. A, Western immunoblot analysis of SOD1 (left panel) in rSUM149 cells treated with 50µM embelin or vector control. Numbers represent densitometric analysis of protein normalized to β-actin. Right panel, glutathione content of cells treated with 50 µM embelin or a vector control. * p< 0.05  
B, Left axis displays accumulation of mitochondrial superoxide in cells treated with 50µM embelin for 1h or 5mM paraquat for 24h as measured by fold increase in MitoSOX Red staining via flow cytometry. Right axis displays decrease in mitochondrial membrane integrity, measured by percent of cells with high TMRE staining via flow cytometry. Inset: Structure of paraquat. C, Cellular viability as determined by trypan blue viability assay for SUM149 wtXIAP cells treated with 50µM embelin, 50ng/mL TRAIL, or combination (gray bars) and the combination with the addition of an SOD mimic (MnTnHex-2-PyP5+) at 10-200µM (black bars). *p<0.05, **p<0.005
Figure 1

A

% Cell Viability

SUM149  rSUM149

TRAIL (ng/mL)

B

% Cell Viability

SUM149 FG9  SUM149 wtXIAP  rSUM149 rSUM149 shXIAP

TRAIL (ng/mL)

C

TRAIL (ng/mL)

SUM149 FG9  rSUM149  SUM149 wtXIAP

D

Caspase 3/7 Activity (10,000 RLU)

SUM149 FG9  SUM149 wtXIAP  rSUM149

TRAIL (ng/mL)
A

Embelin [MW 294]
(2,5-Dihydroxy-3-undecyl-1,4-benzoquinone)

B

% Cell Viability

Embelen (μM)  -  12.5  25  50  -  12.5  25  50
TRAIL (ng/mL)  -  -  -  50  50  50  50

C

% Cell Viability

Embelen (μM)  -  12.5  25  50  -  12.5  25  50
TRAIL (ng/mL)  -  -  -  50  50  50  50

D

% Cell Viability

Embelen (μM)  -  50  -  12.5  25  50
TRAIL (ng/mL)  -  -  50  50  50  50

Effect

Dose

+ TRAIL alone
○ Embelen alone
× Combination

Effect

Dose

+ TRAIL alone
○ Embelen alone
× Combination

Effect

Dose

+ TRAIL alone
○ Embelen alone
× Combination

Effect

Dose

+ TRAIL alone
○ Embelen alone
× Combination
Figure 3

A

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Embelin (µM)

TRAIL (ng/mL)

DR4

DR5

GAPDH

B

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Embelin (µM)

TRAIL (ng/mL)

cFLIP

Actin

C

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XIAP

Cleaved XIAP

GAPDH
Figure 5

A

B

C

Figure 5
Molecular Cancer Therapeutics

XIAP Inhibition and Generation of Reactive Oxygen Species Enhances TRAIL Sensitivity in Inflammatory Breast Cancer Cells


Mol Cancer Ther Published OnlineFirst April 16, 2012.

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