*Preclinical Development*

Molecular Cancer Therapeutics

# 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 in 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 extracellular signal–regulated kinase (ERK)1/2 and ROS accumulation, which correlated with downregulation of antioxidant protein 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 the anti-caspase activity of XIAP 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. *Mol Cancer Ther; 11(7); 1518–27. ©2012 AACR.*

## Introduction

Inflammatory breast cancer (IBC) is an aggressive subtype of breast cancer that is highly invasive and often acquires resistance to chemotherapy, targeted therapy, and radiotherapies, resulting in a relatively low disease-free survival rate compared with locally advanced breast cancer (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 protein (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 (SOD)1/2], making them cross-resistant to other oxidative stressors (7). Furthermore, 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 antioxidant capacity of the cell 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 antiapoptotic and antioxidant expression in the IBC cells affects response to classical inducers of apoptosis and whether it can be modulated by ROS accumulation. To study this, we characterized efficacy of TRAIL, a member of the TNF 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,

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and activation of caspase-8, which then leads to initiation of the protease cascade, activation of the effector caspases (caspase-3/7), and subsequent apoptosis (9). In addition, as 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 anticancer agent (10). TRAIL has shown promising therapeutic potential in a variety of cancer cell lines (11, 12) with little or no toxicity to nontransformed 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-κB, overexpression of cFLIP and one or more of the IAPs, 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 include the SUM149 (EGF receptor–activated, basal-type) IBC cell line isolated from patient primary tumor (20) show higher sensitivity to TRAIL treatment as a single agent compared with 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 that exhibits endogenous XIAP overexpression (6), is less sensitive to TRAIL-mediated apoptosis than 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 8,221 individual herbal products (23). It binds to the BIR3 domain of XIAP with affinity similar to natural Smac/DIABLO peptides, blocking the interaction of XIAP with caspases to promote apoptosis. In addition to XIAP, embelin has been reported to inhibit NF-κB signaling and to have antitumor, anti-inflammatory, and analgesic properties (24, 25). In the present study, we observed that in addition to blocking the caspase-inhibitory activity of XIAP, 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. 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 before use in this study. rSUM149 is an isogenic-acquired resistance model established in the laboratory (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, Ann Arbor, MI) as described in the work of Aird and colleagues (6).

### Short hairpin RNA–mediated knockdown of XIAP

rSUM149 cells (6) were seeded at 30,000 cells per well into a 12-well plate (Corning Incorporated) and allowed to adhere overnight. After 24 hours, cells were transfected with a plasmid containing XIAP-targeting short hairpin RNA (shRNA) using Mirus TransIT 2020 transfection reagent (Mirus Bio) according to manufacturer's instructions. TRAIL treatments (0–1,000 ng/mL) were applied 48 hours posttransfection for 24 hours 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) and allowed to reach 70% confluency. Cells were then treated for 24 hours in regular growth media with TRAIL (BioMol), embelin (Sigma), and an SOD mimetic ($MnTnHex\text{-}2\text{-}PyP^{5+}$; ref. 26) alone and in combination. The mitogen-activated protein kinase kinase inhibitor (MEK1/2) U0126 (Cell Signaling Technologies) was applied to cells for 1 hour before 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 conducted as described previously (5). Cell lysates were harvested after treatment with TRAIL for 24 hours. Membranes were incubated with primary antibodies against XIAP (BD Bioscience), procaspase-3, MAPK, c-*jun*–$NH_2$–kinase (JNK), cFLIP, DR5 (Cell Signaling Technologies), DR4, actin, or glyceraldehyde–3–phosphate dehydrogenase (GAPDH; Santa Cruz), overnight at 4°C. Stripping of membranes for detection of total protein was done as described previously (17). Densitometric analysis was conducted using the NIH ImageJ software (27).

### Caspase-3/7 activity assay

Cells were seeded in 6-well plates, and the next day, cells were treated with TRAIL for 4 hours in regular growth media. After incubation, caspase-3/7 activity was determined using the Caspase-Glo Assay (Promega) as per the manufacturer's instructions.

### Glutathione assay

Reduced glutathione levels were assessed as described previously (7) using the GSH-Glo Glutathione Assay (Promega) as per the manufacturer's instructions.

### ROS measurement

Cells were cultured in 6-well plates in regular growth media until reaching 70% to 80% confluency. Cells were treated with embelin or paraquat (Sigma) for 1 hour and then harvested and incubated for 30 minutes with 10 μmol/L MitoSOX Red dye (Molecular Probes) to detect mitochondrial superoxide. Cells were then washed twice with 1% bovine serum albumin (BSA)/PBS and analyzed for fluorescence by flow cytometry. At least 25,000 events were collected on a FACSCalibur flow cytometer (Beckton Dickinson) 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 1 hour and then harvested and incubated for 30 minutes with 500 nmol/L TMRE. Cells were washed twice with 1% BSA/PBS and analyzed for fluorescence by flow cytometry.

### Drug synergism analysis

Analysis of drug synergism was conducted using the CalcuSyn software (Biosoft), which uses the Chou–Talalay method (28) where a combination index (CI) <1 indicates synergism.

### Statistical analysis

The statistical analyses were conducted using Graphpad InStat (Graphpad Software, Inc.) Student 2-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 (ref. 20) isolated from primary IBC tumor]. Isogenic cells with differential XIAP expression derived from SUM149 were characterized for TRAIL sensitivity (10–1,000 ng/mL) at a 24 hour 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 than to rSUM149 (Fig. 1A) and SUM149 wtXIAP cells (Fig. 1B, left). Calculated $IC_{50}$ value for cell viability in the presence of TRAIL was approximately 770 ng/mL in SUM149 wtXIAP cells, 530 ng/mL in endogenously XIAP-overexpressing rSUM149 cells, and 45 ng/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 Fig. 1B. Data in Fig. 1B (right) 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 $IC_{50}$ from 530 ng/mL in rSUM149 cells to approximately 30 ng/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 approximately 60% upon treatment with 10 ng/mL TRAIL, whereas SUM149 cell viability is about 80% with the same treatment. Cell viability differences were consistent with XIAP downregulation after TRAIL treatment in the SUM149 cells at 50 ng/mL compared with the rSUM149 and SUM149 wtXIAP cells (Fig. 1C). In addition, a functional assay was used to measure the activity of caspases during the apoptotic process; this assay was conducted after 4 hours of TRAIL treatment to ensure that cells were dying but not yet dead, at which point caspase activity would be undetectable. XIAP decrease following treatment in the TRAIL-sensitive SUM149 cells corresponded with increased caspase-3/7 activity (Fig. 1D, $P < 0.005$) compared with limited caspase activity after TRAIL treatment in the rSUM149 and SUM149 wtXIAP cells. These results show that SUM149wtXIAP and rSUM149 cells with XIAP overexpression compared with SUM149 show significantly reduced sensitivity to TRAIL-mediated apoptosis.

### Embelin enhances TRAIL sensitivity

Because XIAP overexpression and activity corresponds with decreased TRAIL sensitivity in the rSUM149 model, we evaluated the effects of embelin (Fig. 2A), a small-molecular inhibitor of XIAP, in combination with TRAIL. Combination studies with TRAIL and embelin were conducted at 24 hours, and viability was assessed by trypan blue exclusion assay. Data in Fig. 2B–D show that embelin at 24 hours alone at 3 concentrations (12.5, 25, 50 μmol/L) induced a modest concentration-dependent decrease (10%–25%) in viability in the 3 cell lines. TRAIL (50 ng/mL) alone at 24 hours as described in Fig. 1B shows higher sensitivity in the parental SUM149 cells than in the XIAP-overexpressing SUM149wtXIAP and rSUM149 isogenic lines. Combining increasing concentrations of embelin with TRAIL (50 ng/mL) at 24 hours caused a significant decrease in cell viability in

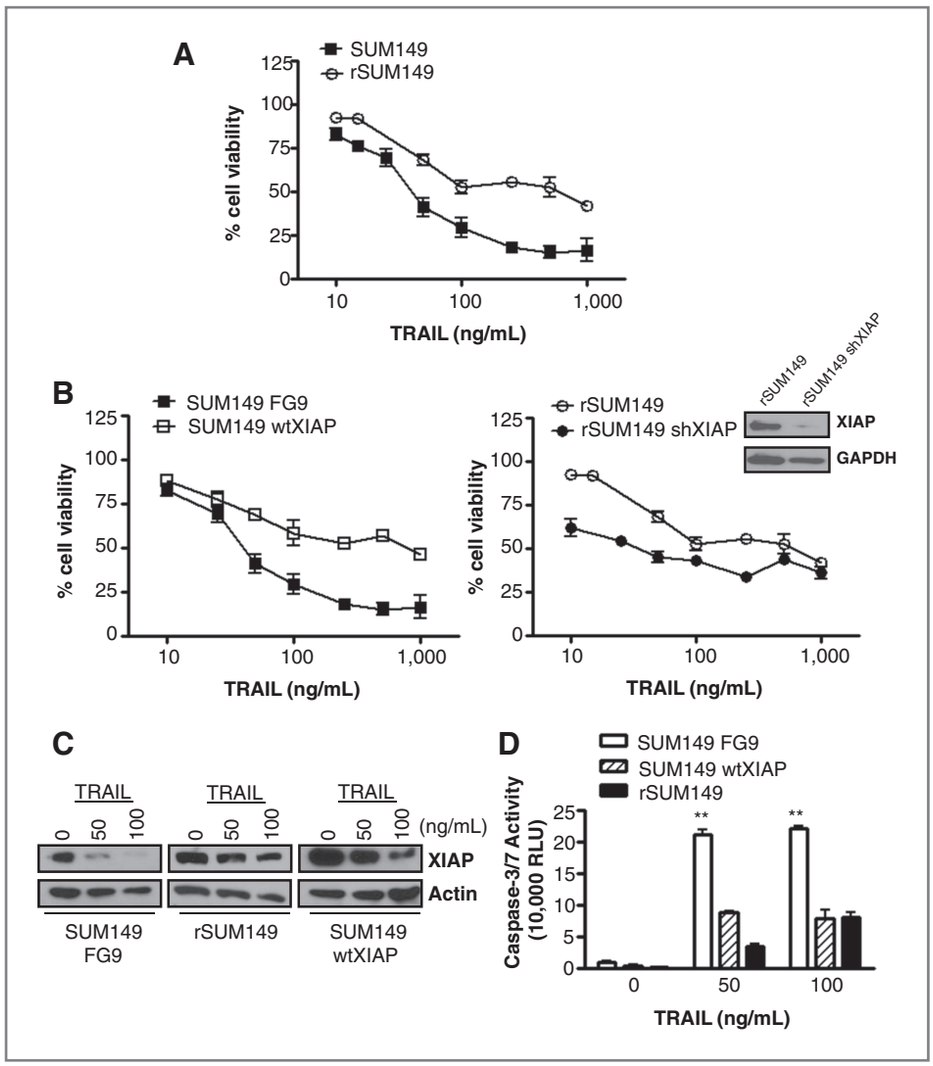


Figure 1. Effect of TRAIL on XIAP expression, viability, and caspase activation in SUM149 isogenic lines A, SUM149 and rSUM149 cells were treated with TRAIL for 24 hours, and viability was assessed using the trypan blue exclusion assay. Bars represent mean ± SEM viable cells taken as a percentage of the total cells ($n = 2$–4). B, left, SUM149 wtXIAP and vector control SUM149 FG9 cells were treated with TRAIL for 24 hours, 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, rSUM149 and rSUM149 cells transfected with an XIAP-targeting shRNA plasmid with knockdown of XIAP expression were treated with TRAIL for 24 hours, 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 hours. Treatments are compared with 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 hours. Bars represent mean ± SEM relative light units (RLU; $n = 3$). **, $P < 0.005$.

all 3 cell lines. Increasing concentrations of TRAIL up to 100 ng/mL in combination with 25 or 50 μmol/L embelin did not have any significantly enhanced response over that seen in Fig. 2 (data not shown).

To characterize the interaction of these 2 agents, we analyzed the above results (Fig. 2B–D, left) using the CalcuSyn program (Biosoft), which uses the Chou–Talalay Method, a derivation of the mass–action law principle (28). When experimental data are 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 whereas 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 2 agents; a CI greater than 1 indicates antagonism, whereas 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 (Fig. 2), which is indicative of strong synergism in all 3 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 DR4 and DR5 (Fig. 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 (Fig. 3B, left). However, cFLIP levels remain unchanged in the XIAP-overexpressing cells (Fig. 3B, right) treated with embelin, TRAIL or embelin + TRAIL, although cell death was significantly increased (Fig. 2C). Examination of lysates from SUM149 wtXIAP cells treated with embelin

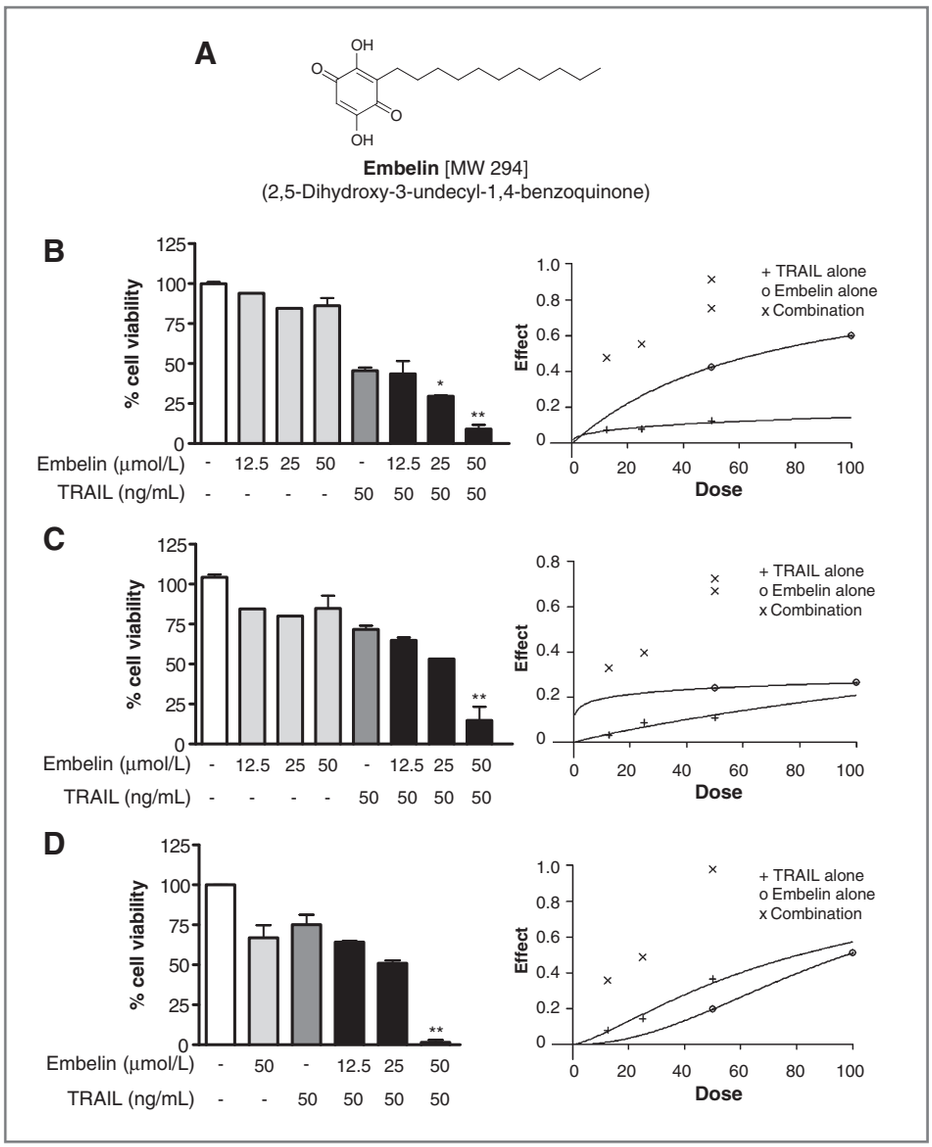


**Figure 2.** Effect of TRAIL and the XIAP inhibitor embelin alone or in combination on viability of isogenic IBC cells and combinatorial analysis. A, structure of embelin. SUM149 FG9 vector control (B), SUM149 wtXIAP (C), and rSUM149 (D) cells were treated with embelin (12.5–50 μmol/L) and TRAIL (50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

or embelin + TRAIL reveal XIAP downregulation upon treatment with embelin compared with control. In addition, an XIAP cleavage product (30 kDa) along with potent inhibition of XIAP levels is detected in cells treated with embelin + TRAIL (Fig. 3C). In summary, embelin and TRAIL synergize to increase cell death in the triple-negative IBC cell model.

### Embelin modulates ERK 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 hours and then examined for the phosphorylation status of ERK1/2 and the stress-activated protein kinase (SAPK)/JNK (Fig. 4). In SUM149 cells, cell death associated with embelin or embelin + TRAIL treatment correlated with a decrease in p-ERK1/2 signaling (Fig. 4A). Interestingly, in the SUM149 wtXIAP cells with exogenous XIAP overexpression, the basal levels of ERK1/2 phosphorylation were itself lower than the parental SUM149 cells. Furthermore, an increase in p-ERK1/2 was observed in embelin- and embelin + TRAIL–treated SUM149 wtXIAP cell lysates compared with vehicle- or TRAIL-treated cells (Fig. 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 (Fig. 4B, graph). No specific change in p-JNK levels was observed in the various treatments as compared

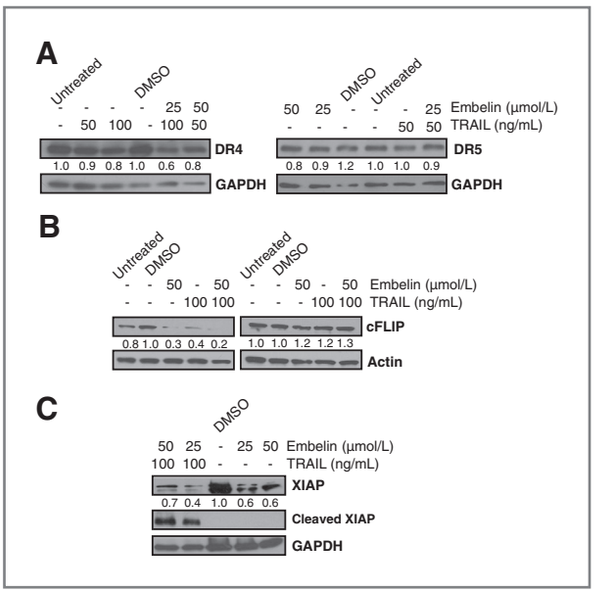


Figure 3. Effect of TRAIL and embelin on TRAIL resistance factors. A, Western immunoblot analysis of DR4 and DR5 expression in SUM149 cells treated with embelin, TRAIL, or embelin + TRAIL for 24 hours. Treatments are compared with untreated cells or dimethyl sulfoxide (DMSO) vehicle control. B, cFLIP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

with vehicle control in the SUM149 or SUM149 wtXIAP cells. Representative immunoblot is shown in Fig. 4C.

## Embelin induces generation of ROS by downregulating SOD1 and oxidizing glutathione

Recently, we reported (7) that the XIAP-overexpressing rSUM149 cells that have acquired resistance to therapeutic apoptosis mediated by lapatinib, an ErbB1/2-targeting agent, have lost the ability to accumulate ROS in the presence of oxidizing agents such as paraquat and hydrogen peroxide; they also have high expression of key antioxidants SOD1, SOD2, and reduced glutathione. Interestingly, embelin treatment downregulates SOD1 (Fig. 5A, left) and consumes reduced glutathione (Fig. 5A, right; $P < 0.05$) 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 with paraquat, a classical ROS-generating agent that we have previously reported (7) to have insignificant effect on ROS generation in the rSUM149 cells (Fig. 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-PyP$^{5+}$; refs. 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 (Fig. 5C). Together, these results indicate that the concurrent inhibition of

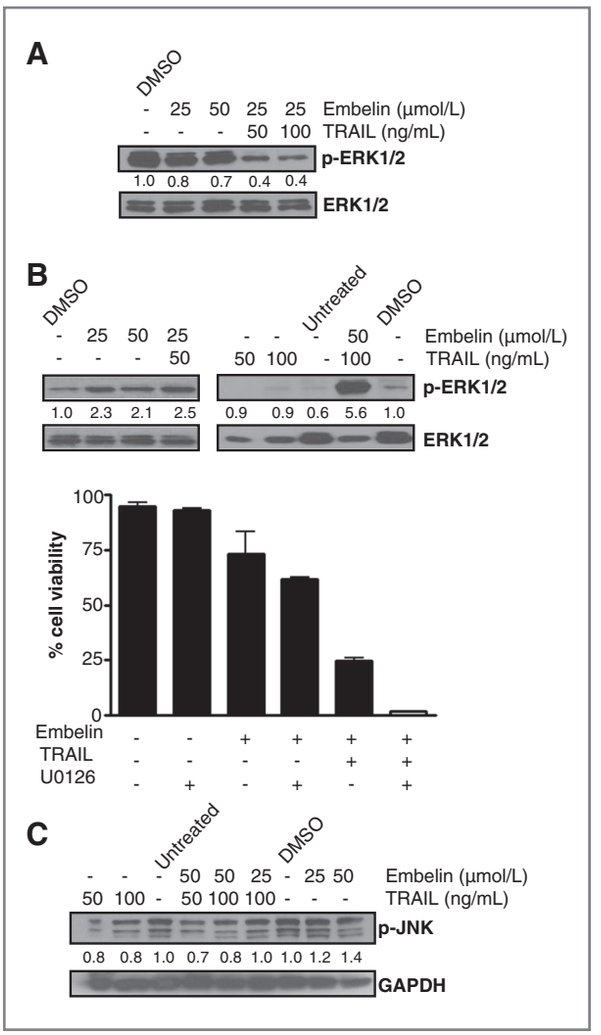


Figure 4. Effect of TRAIL and embelin on cellular signaling in IBC cells with differential XIAP expression. A, phospho-ERK1/2 (MAPK p44/42) Western immunoblot analysis of SUM149 cells treated with embelin or embelin + TRAIL for 24 hours. B, top, p-ERK1/2 Western immunoblot analysis of SUM149 wtXIAP cells treated with embelin, TRAIL, or embelin + TRAIL. Numbers represent densitometric analysis of p-ERK1/2 normalized to total ERK1/2 protein. Bottom, cellular viability as determined by trypan blue viability assay for SUM149 wtXIAP cells treated with 50 μmol/L embelin, 50 ng/mL TRAIL, or combination, and the combination with the addition of 10 μmol/L U0126 MEK1/2 inhibitor. C, p-JNK Western immunoblot analysis of SUM149 wtXIAP cells treated with embelin, TRAIL, or embelin + TRAIL for 24 hours. Treatments are compared with untreated cells or dimethyl sulfoxide (DMSO) vehicle control. Numbers represent densitometric analysis of p-JNK normalized to GAPDH.

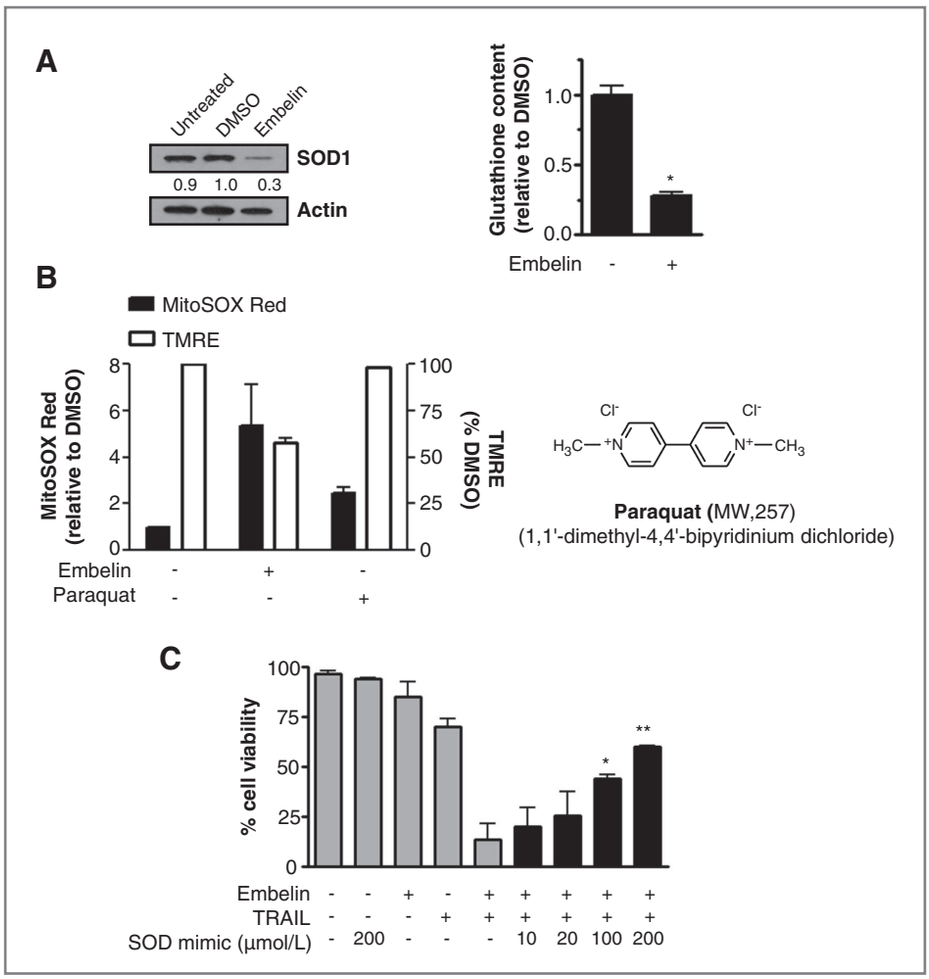


**Figure 5.** The XIAP inhibitor embelin generates ROS in rSUM149 cells to cause cell death. A, Western immunoblot analysis of SOD1 (left) in rSUM149 cells treated with 50 μmol/L embelin or vehicle control. Numbers represent densitometric analysis of protein normalized to β-actin. Right, glutathione content of cells treated with 50 μmol/L embelin or a vehicle control. *, $P < 0.05$. B, left axis displays accumulation of mitochondrial superoxide in cells treated with 50 μmol/L embelin for 1 hour or 5 mmol/L paraquat for 24 hours as measured by fold increase in MitoSOX Red staining via flow cytometry. Right axis displays decrease in mitochondrial membrane integrity, measured by percentage 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 50 μmol/L embelin, 50 ng/mL TRAIL, or combination (gray bars), and the combination with the addition of an SOD mimic (MnTnHex-2-PyP$^{5+}$) at 10 to 200 μmol/L (black bars). *, $P < 0.05$; **, $P < 0.005$.

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 with parental TRAIL-sensitive SUM149 cells (20). In addition, 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. Furthermore, we show 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 IBC. 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. Furthermore, embelin treatment reversed high SOD expression and inhibited the enhanced glutathione detoxification capacity observed in the acquired therapeutic resistant IBC isogenic cell model; this led to the accumulation of ROS, 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 antiapoptotic 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 with 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); downregulation 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 proapoptotic 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) 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 prosurvival role of ERK. In the SUM149 cells with XIAP overexpression, which have 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 ROS in the presence of ROS-generating agents due to overexpression of SOD1, SOD2, 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-PyP$^{5+}$) 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 that 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.

## Disclosure of Potential Conflicts of Interest

I. Batinic-Haberle has ownership interest in, and is a consultant to BioMimetrix Pharmaceutical Inc., and has given expert testimony for BioMarin.

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