

# Triptolide sensitizes human breast cancer cells to tumor necrosis factor- $\alpha$ -induced apoptosis by inhibiting activation of the nuclear factor- $\kappa$ B pathway

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**Abstract.** Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) can act as either a tumor promoter, linking inflammation with carcinogenesis, or a tumor inhibitor, inducing cancer cell death. However, several types of cancer, including breast cancer, are resistant to TNF- $\alpha$  therapy. Triptolide, a diterpene triepoxide, has been reported to exert anti-inflammatory and antiproliferative effects, associated with the inhibition of nuclear factor- $\kappa$ B (NF- $\kappa$ B). The present study investigated the effects of triptolide sensitization on human breast cancer cells to TNF- $\alpha$ -induced apoptosis by inhibiting activation of the NF- $\kappa$ B pathway. Human breast cancer MDA-MB-231 cells and MCF-7 cells were treated with different concentrations of triptolide, with or without 10 ng/ml TNF- $\alpha$ , for different durations, followed by measurement of cell proliferation using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay, apoptosis induction, through determination of caspase-3 activity and poly (ADP-ribose) polymerase (PARP) cleavage, and NF- $\kappa$ B pathway activation, through determination of inhibitor of NF- $\kappa$ B (I $\kappa$ B) and the NF- $\kappa$ B downstream genes, X-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein1/2 (cIAP1/2)] using Western blot and reverse transcription-quantitative polymerase chain

reaction analyses. TNF- $\alpha$ , when combined with triptolide, was observed to inhibit the activation of I $\kappa$ B $\alpha$ , increase the level of cleaved PARP, and further activate caspase-3 in the breast cancer cells. Triptolide also inhibited the expression levels of the downstream anti-apoptotic genes of NF- $\kappa$ B activation, XIAP and cIAP1/2. The results of the present study demonstrated that triptolide sensitized human breast cancer cells to TNF- $\alpha$ -induced apoptosis, which may provide a promising combination strategy for human breast cancer therapeutics.

## Introduction

Breast cancer is the second leading cause of cancer-associated mortality, with high morbidity and mortality rates in women worldwide. Chemotherapy remains an alternative therapeutic option for breast cancer to surgery and radiation, however, multidrug resistance and an unfavorable systemic toxicity against normal cells limit their clinical effects, which highlights the urgent requirement to identify novel therapeutic strategies and agents (1).

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is one of the major inflammatory cytokines, which can act as either a tumor promoter, by linking inflammation with carcinogenesis, or as a tumor inhibitor, through the induction of cancer cell death. TNF- $\alpha$  signaling occurs via two cell-surface receptors, TNF- $\alpha$  factor receptor 1 and 2 (TNFR1 and TNFR2). Complex I is formed when TNF- $\alpha$  binds to TNFR1, and this event leads to activation of nuclear factor (NF)- $\kappa$ B, mitogen-activated protein kinases and Complex II, ultimately leading to apoptosis (2,3). Of note, Complex I and Complex II have disparate and opposing effects on apoptosis. Complex I leads to activation of the NF- $\kappa$ B signaling pathway, whereas Complex II leads to activation of caspase-3 and caspase-9. Activated NF- $\kappa$ B can upregulate the expression levels of downstream genes, which include anti-apoptotic genes, including X-linked inhibitor of apoptosis (XIAP) and cellular inhibitors of apoptosis 1 and 2 (cIAP1/2). Therefore, the balance between Complex I and Complex II results in cell resistance to TNF- $\alpha$ -mediated apoptosis. In the tumor microenvironment, sustained NF- $\kappa$ B

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activation can have an anti-apoptotic effect on tumor cells, which depends on Complex I signaling, whereas Complex II can attenuate TNF- $\alpha$ -induced NF- $\kappa$ B activation.

Triptolide, a natural and biologically active compound as a diterpenoid triepoxide, was originally purified from the Chinese herb *Tripterygium wilfordii* Hook F (4). This natural product has been used in traditional Chinese medicine for centuries, and has a myriad of therapeutic applications against inflammation and autoimmune diseases (5,6). Previous studies have reported that triptolide has antitumor, anti-inflammatory and immunosuppressive activities. In particular, triptolide has been investigated for several different types of cancer cells *in vitro* and *in vivo* (7-9). The inhibitory effects of triptolide in the growth of cholangiocarcinoma cells in hamsters (10), and the growth of xenografts in human breast cancer, bladder cancer, melanoma and gastric carcinoma, have been shown in nude mice (9). It has also been demonstrated that the antiproliferative properties of triptolide may be involved in inhibiting NF- $\kappa$ B activity and inducing cell apoptosis (11). Taken together, these previous studies have demonstrated that triptolide may be clinically effective for tumor chemotherapy.

The TNF- $\alpha$  signaling pathway is important in tumor development, and its antitumor properties may be exploited with avoidance of its tumorigenic properties (2). Based on the essential requirement for an inflammatory microenvironment in tumor formation, the present study hypothesized that triptolide sensitizes human breast cancer cells to TNF- $\alpha$ -induced apoptosis by inhibiting activation of the NF- $\kappa$ B pathway. The results of the present study demonstrated that TNF- $\alpha$  combined with triptolide effectively sensitized human breast cancer cells to triptolide-mediated induction of apoptosis, by targeting inhibitor of NF- $\kappa$ B (I $\kappa$ B), an effective signaling pathway of TNF- $\alpha$ . Due to the inhibitory activities of I $\kappa$ B, triptolide inhibited the NF- $\kappa$ B signaling pathway and further promoted the activation of caspase-3. Finally, increased activation of caspase-3 resulted in apoptosis, which contributed to its anti-inflammatory and anticancer activities. These observations suggested that this may be a promising combination strategy for use in human breast cancer therapeutics.

## Materials and methods

**Reagents.** Triptolide was purchased from A.G. Scientific, Inc. (San Diego, CA, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Stock solutions (50 mM) were prepared, and aliquots were stored at -20°C for further use in the subsequent experiments. L15/1640, penicillin and streptomycin were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Gibco (Thermo Fisher Scientific, Inc). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; BTN111105), RNaseA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) kits were purchased from Promega Corporation (San Luis Obispo, CA, USA). Ac-Asp-Glu-Val-Asp-AMC and Z-Gly-Gly-Leu-AMC, the substrates for caspase-3 and chymotrypsin (CT)-like activities, respectively, were obtained from Calbiochem, Inc. (San Diego, CA, USA). Mouse monoclonal anti-poly(ADP-ribose)polymerase (PARP) (46D11; dilution, 1:1,000), anti-caspase-3 (8G10; dilution, 1:1,000);

anti-caspase-9 (9502; dilution, 1:1,000), anti-XIAP (2042; dilution, 1:1,000) and anti-IAP1/2 (4952; dilution, 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal anti-ubiquitin (P4D1; sc-8017; dilution, 1:1,000), rabbit polyclonal anti-I $\kappa$ B- $\alpha$  (C-15; sc-203; dilution, 1:1,000), goat polyclonal anti- $\beta$ -actin (C-11; sc-1615; dilution, 1:5,000), and secondary goat anti-rabbit IgG-horse-radish peroxidase (dilution, 1:5,000; sc-2054) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Velcade was purchased from A.G. Scientific, Inc..

**Cell culture and whole cell extract preparation.** Human breast cancer MDA-MB-231 and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in L15/1640 supplemented with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (5.0-8.0x10<sup>5</sup> cells/ml). All cells were maintained at 37°C with 5% CO<sub>2</sub>. Whole cell extracts were prepared, as described previously (12,13). Briefly, cells were harvested, washed with phosphate-buffered saline (PBS) and homogenized for 30 min at 4°C in lysis buffer, containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol (Invitrogen; Thermo Fisher Scientific, Inc.). The lysates were immediately centrifuged at 12,000 x g for 12 min at 4°C, and the supernatants were collected as whole cell extracts. Protein concentration was determined using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). Bovine serum albumin (BSA) was used as a standard (Invitrogen; Thermo Fisher Scientific, Inc.).

**MTT assay.** MTT assays were performed in 96-well plates. Briefly, 3.5x10<sup>5</sup> MDA-MB-231/MCF-7 cells were seeded per well and incubated overnight at 37°C. The cells were treated with triptolide (13, 25, 50, 100, 200 or 400 nM), alone or in combination with TNF- $\alpha$  (10 ng/ml) for 48 h. DMSO (0.1%)-treated cells were used as a control. Inhibition of cell proliferation was determined using an MTT assay (10  $\mu$ l MTT/100  $\mu$ l cells), as described previously (12). Absorbance was measured using a Wallac Victor3™ multilabel counter (PerkinElmer, Walltham, MA, USA) at 540 nm, and cell viability was determined relative to the DMSO-treated control cells. In individual experiments, each treatment condition was set up with four repeats, and each experiment was repeated three times independently.

**Caspase-3 activity determination, CT-like activity assay and Western blot analysis.** Caspase-3 activity was determined by measuring the release of the AMC groups from the caspase-3-specific substrate, Ac-Asp-Glu-Val-Asp-AMC. The MDA-MB-231/MCF-7 cells (5.0-8.0x10<sup>5</sup>/well) were plated and, following overnight incubation at 37°C, were treated with indicated concentrations of triptolide, either alone or in combination with TNF- $\alpha$  (10 ng/ml), for 24 h, followed by preparation of whole cell extracts. The cell extracts (25 mg) were then incubated in a 96-well plate in 100  $\mu$ l assay buffer (50 mM Tris-HCl; pH 7.5) with 40  $\mu$ M Ac-Asp-Glu-Val-Asp-AMC. The reaction mixture was incubated at 37°C for 2 h and the hydrolyzed fluorescent AMC groups were quantified, as previously described (12,13). The production of AMC groups was measured using a Victor3 Multilabel Counter (PerkinElmer)

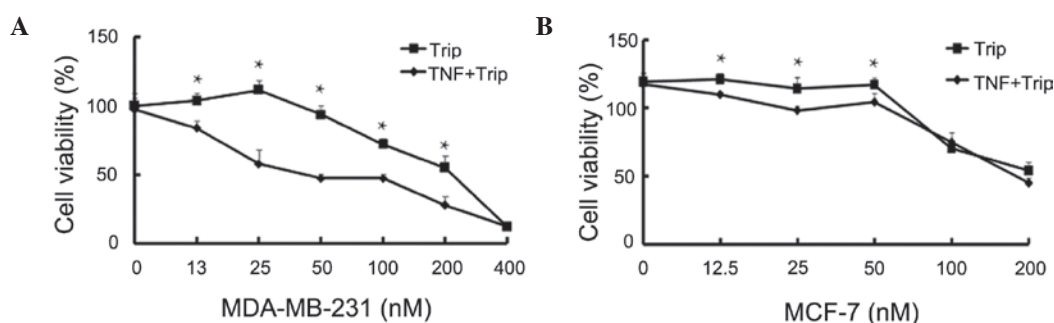


Figure 1. Effect of dose on TNF- $\alpha$ +triptolide on cell viability. (A) MDA-MB-231 cells and (B) MCF-7 cells were treated either with different concentrations of triptolide, or with TNF- $\alpha$  (10 ng/ml) and different concentrations of triptolide for 48 h, followed by measurement of cell viability using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay. MDA-MB-231 cells: IC<sub>50</sub> triptolide=209 nM; IC<sub>50</sub> triptolide+TNF- $\alpha$ =131 nM. MCF-7 cells: IC<sub>50</sub> triptolide=171 nM; IC<sub>50</sub> triptolide+TNF- $\alpha$ =153 nM. Data are expressed as the mean  $\pm$  standard deviation of three experiments. \*P<0.05. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Trip, triptolide; IC<sub>50</sub>, half maximal inhibitory concentration.

with an excitation filter of 380 nm and an emission filter of 460 nm.

Proteasomal CT-like activity was determined by measuring the release of the AMC groups from the CT-like specific substrate, Z-Gly-Gly-Leu-AMC. The MDA-MB-231 and MCF-7 cells ( $5.0\text{--}8.0 \times 10^5$ /well) were plated in 96-well plates and treated with triptolide at the desired final concentrations, either alone or in combination of TNF- $\alpha$  (10 ng/ml), for 8 h, or for the indicated time periods, and incubated with Z-Gly-Gly-Leu-AMC (at 40  $\mu$ M) for an additional 2 h at 37°C. The production of hydrolyzed AMC groups was measured, as previously described (12,13).

For Western blot analysis, the cells ( $5.0\text{--}8.0 \times 10^5$ /ml) were plated in 60 mm dishes and treated with the desired final concentration of triptolide, either alone or in combination with TNF- $\alpha$ , for different time periods, followed by preparation of whole cell extracts. Protein was extracted from the whole cell lysates using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with 10 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride and 1X Roche Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA). After the determination of protein concentration, 5  $\mu$ g protein extracted from cultured cells was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrically transferred onto polyvinylidene difluoride membranes. The blots were blocked with 5% fat milk for 1 h at room temperature, and then incubated with specific antibodies for 1 h at room temperature. After washing, membranes were incubated for 1 h at room temperature with goat anti-rabbit IgG-horseradish peroxidase and exposed to X-ray film. Western blots were analyzed using enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Inc.), as previously described (12,13).

**RT-qPCR.** Cells ( $5 \times 10^6$ ) were pretreated with TNF- $\alpha$  (10 ng/ml) at the desired final concentrations, either alone or in combination of triptolide for 12 h at 37°C. Total RNA was extracted using TRIzol reagent ( $5 \times 10^6$  cells/ml; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The genomic DNA was digested using RNase-free DNaseI, and the concentration of the RNA was detected using UV spectroscopy as previously described (12). Single-stranded

cDNA was generated using random hexamer primers with a Prime Script<sup>®</sup> RT Master mix first strand synthesis system for RT-qPCR (A1250; Promega Corporation). Following RT, qPCR was performed with SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Tli RNaseH Plus). The 20.0  $\mu$ l reaction mixture contained 2.0  $\mu$ l cDNA template, 10.0  $\mu$ l SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (2x), 0.8  $\mu$ l PCR forward primer, 0.8  $\mu$ l PCR reverse primer, 0.4  $\mu$ l ROX Reference Dye II and 6.0  $\mu$ l dH<sub>2</sub>O. A GAPDH primer set was used as an internal control. The products were detected by agarose gel electrophoresis. The PCR primers used were as follows: XIAP, forward ACATGGCTGTCAAGAAGG-AGAT and reverse ACTGCAGCCTCGAAGCTTCTG (180 bp); cIAP1/2, forward TTCCGTGGCTCTTATTCAAAC and reverse GCACAGTGGTAGGAAGCTTCTCAT (96 bp); 18sr, forward CCTGGATACCGCAGCTAGGA and reverse GCG GCGCAATACGAATGCCCC (112 bp). Amplification cycles were as follows: 95°C for 2 min, then 40 cycles at 95°C for 15 s, 60°C for 1 min, followed by 95°C for 10 min, and 60°C for 1 min. The fluorescent signal of the PCR product was detected by ABI PRISM<sup>®</sup> 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Statistical analysis was performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Data are expressed as the mean  $\pm$  standard deviation of the mean. Student's t-test was applied to evaluate differences between the treated groups and controls. Data from multiple groups were analyzed using one-way analysis of variance, followed by the Tukey-Kramer multiple comparison test. For all tests, P<0.05 was considered to indicate a statistically significant difference between groups.

## Results

**TNF- $\alpha$  sensitizes breast cancer cells to triptolide-induced cell death.** The antiproliferative effects of the combination of TNF- $\alpha$  and triptolide against MDA-MB-231 and MCF-7 human breast cancer cells were examined using an MTT assay. As shown in Fig. 1, TNF- $\alpha$ , at a low concentration of 10 ng/ml, had no significant effects on cell proliferation of either human breast cancer cell lines. Exposure to different concentrations of triptolide (25–200 nM) with or without TNF- $\alpha$  for 24 h induced a dose-dependent inhibition in cell proliferation. As

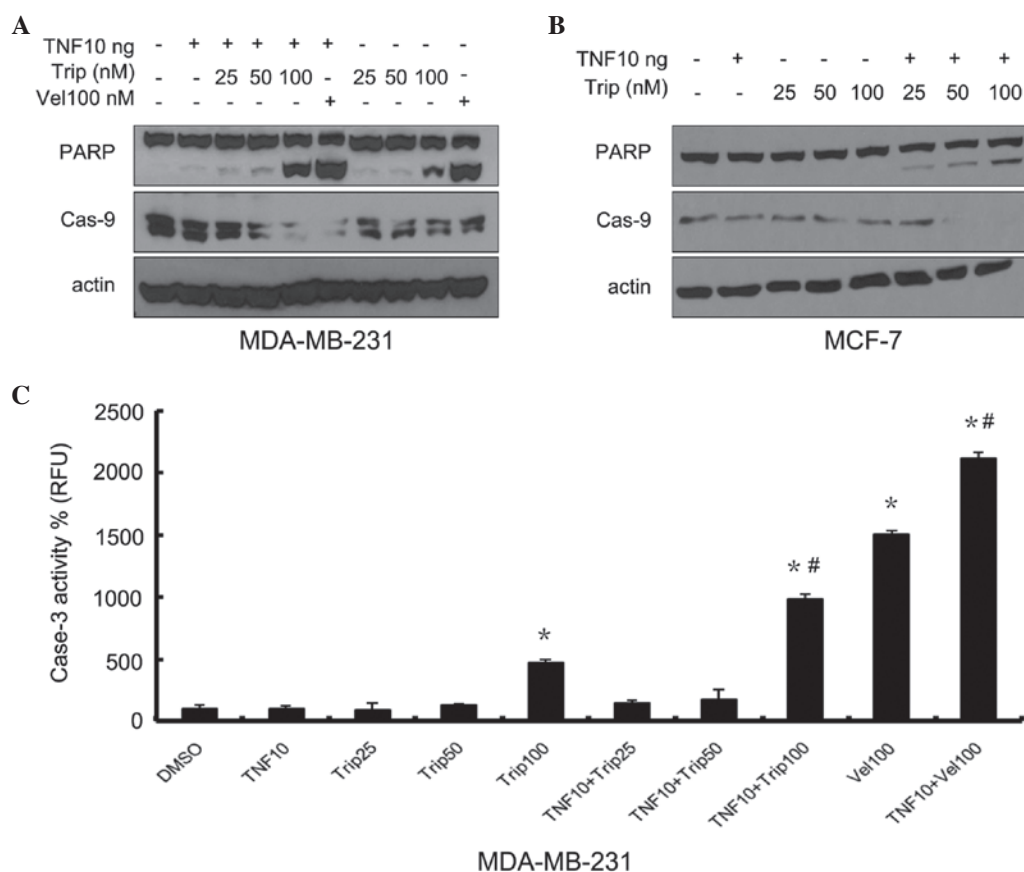


Figure 2. Effects of different concentrations of triptolide in combination with TNF- $\alpha$  on PARP cleavage, and caspase-3 and -9 activity. The MDA-MB-231 cells and MCF-7 cells were treated either with different concentrations of triptolide, or with TNF- $\alpha$  (10 ng/ml)+different concentrations of triptolide. Velcade(100 nM) was used as a positive control. After 24 h, the protein levels of PARP and caspase-9 were determined in the (A) MDA-MB-231 cells and (B) MCF-7 cells by Western blot analysis using specific antibodies. (C) Caspase-3 activity was determined in the MDA-MB-231 cells. Data are expressed as the mean  $\pm$  standard deviation of three experiments. \* $P < 0.05$ , vs. DMSO-treated group; # $P < 0.05$  Trip/Vel-treated group, vs. Trip/Vel+TNF- $\alpha$  group. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Trip, triptolide; DMSO-dimethyl sulfoxide; Vel, Velcade; PARP, poly (ADP-ribose) polymerase; Cas, caspase.

shown in Fig. 1A, triptolide exerted inhibitory effects when combined with or without TNF- $\alpha$ , and the inhibitory effects were more marked in the combination group. The combination of TNF- $\alpha$  and triptolide exhibited increased cytotoxic effects against the MDA-MB-231 human breast cells at triptolide concentrations between 25 and 200 nM. The combined effects were not marked when a higher concentration of triptolide (400 nM) was used for treatment of the MDA-MB-231 cells. The combination treatment had decreased cytotoxic effects against the MCF-7 human breast cells (Fig. 1B), however, the combination of TNF- $\alpha$  and triptolide showed potent antiproliferative effects against the two human breast cancer cell lines.

*TNF- $\alpha$  sensitizes breast cancer cells to triptolide-induced apoptosis.* The present study then determined whether the cell death induced by TNF- $\alpha$ +triptolide corresponded to the apoptotic response. The MDA-MB-231 and MCF-7 cells were treated, respectively, with triptolide (25, 50 and 100 nM), combined with or without TNF- $\alpha$  (10 ng/ml) for 24 h. The nuclear enzyme, PARP, which is one of the primary cleavage targets of caspase-3; and caspase-9, which activates caspase-3, were detected. Velcade, the first proteasome inhibitor to become a therapeutic modality for multiple myeloma, was used as a positive control, as it has been reported to induce the apoptosis of several types of cancer cell (14). As shown

in Fig. 2A, TNF- $\alpha$ , at a low concentration of 10 ng, markedly increased the effect of velcade on PARP cleavage. As shown in Fig. 2A and B, PARP cleavage was enhanced in the triptolide+TNF- $\alpha$ -treated cells in a dose-dependent manner and, to a lesser degree, in the triptolide-treated cells. Triptolide also exhibited marked effects on the levels of caspase-9 in a dose-dependent manner. Caspase-9 decreased markedly when the concentration of triptolide reached 100 nM. Following treatment with TNF- $\alpha$  combined with triptolide, the degradation of caspase-9 was more marked, compared with triptolide-only treatment in the two cell lines. As shown in Fig. 2C, a marked increase in caspase-3 activity was observed in the cells treated with TNF- $\alpha$ +triptolide, compared with the cells treated with triptolide alone, at triptolide concentrations up to 100 nM. Caspase-3 activity was not assessed in the MCF-7 cells, as MCF-7 cells do not express caspase-3. These data demonstrated that the combination of TNF- $\alpha$  and triptolide induced apoptosis in breast cancer cells, associated with the activation of caspase-3.

*TNF- $\alpha$  sensitizes breast cancer cells to triptolide-induced apoptosis.* The kinetic effects of the combination of TNF- $\alpha$  and triptolide were further evaluated in the present study. As shown in Fig. 3A and B, PARP fragments and caspase-9 reduction were not detected up to 24 h when treated with



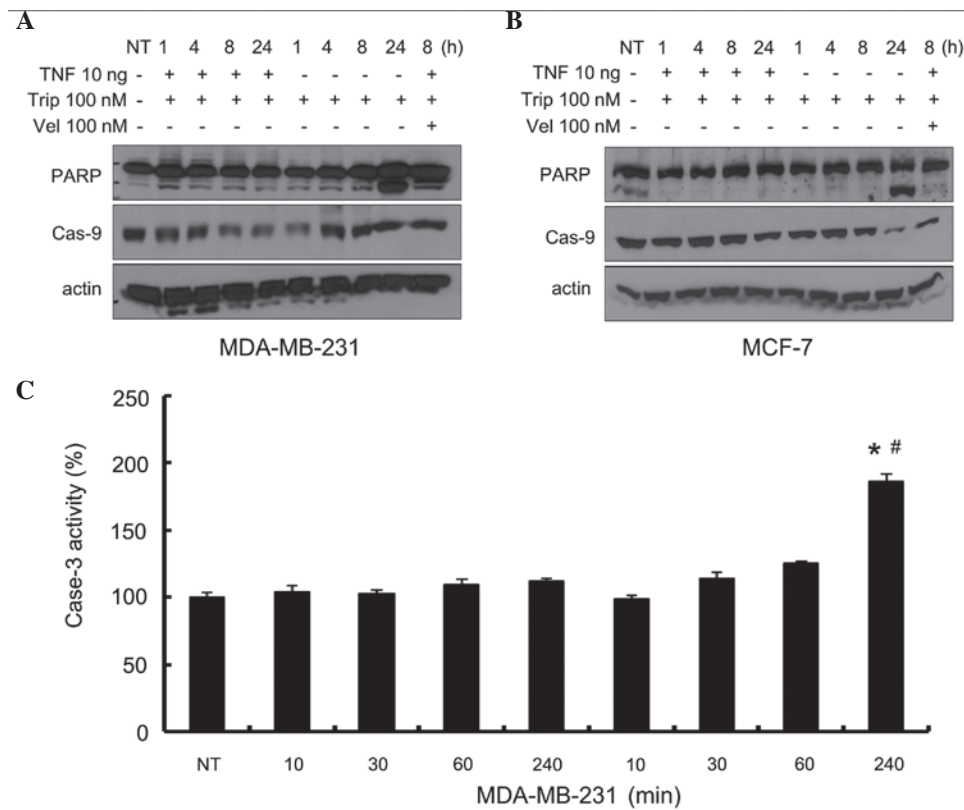


Figure 3. Kinetic effects of TNF- $\alpha$ +triptolide on PARP cleavage, and caspase-3 and -9 activities. The (A) MDA-MB-231 cells and (B) MCF-7 cells were treated with 100 nM triptolide or with TNF- $\alpha$  (10 ng/ml)+triptolide for the indicated durations. Velcade (100 nM) was used as a positive control. The protein levels of PARP and caspase-9 were determined via Western blot analysis using specific antibodies. (C) Caspase-3 activity was determined in the MDA-MB-231 cells. Data are expressed as the mean  $\pm$  standard deviation of three experiments. \* $P$ <0.05, vs. NT group; # $P$ <0.05 Trip/Vel-treated group, vs. Trip/Vel+TNF- $\alpha$  group. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Trip, triptolide; NT, no treatment; Vel, Velcade; PARP, poly (ADP-ribose) polymerase; Cas, caspase.

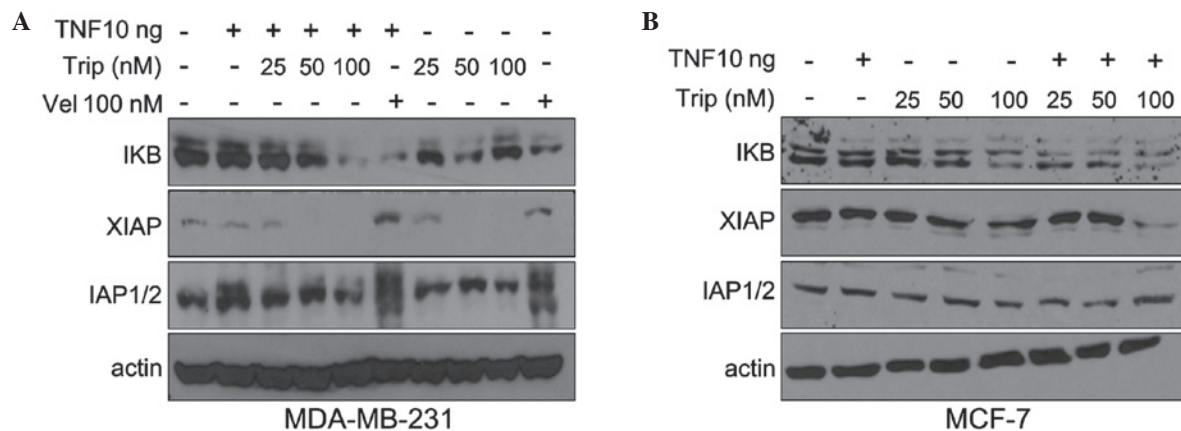


Figure 4. Effects of different concentrations of triptolide combined with TNF- $\alpha$  on the levels of NF- $\kappa$ B-targeting genes coding proteins. The (A) MDA-MB-231 and (B) MCF-7 cells were treated with either different concentrations of triptolide or with TNF- $\alpha$  (10 ng/ml)+different concentrations of triptolide, Velcade (100 nM) was used as a positive control. After 24 h, the protein levels of I $\kappa$ B, XIAP and cIAP1/2 were determined via Western blot analysis using specific antibodies. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Trip, triptolide; Vel, Velcade; I $\kappa$ B; inhibitor of NF- $\kappa$ B; XIAP; X-linked inhibitor of apoptosis protein; IAP1/2, inhibitor of apoptosis protein 1/2.

triptolide, with or without TNF- $\alpha$ , in either of the cell lines. At 24 h, the combination of TNF- $\alpha$  and triptolide markedly enhanced quantity of PARP fragments, and reduced the quantity of caspase-9 in the two cell lines, which were consistent with the results of Fig. 2. As shown in Fig. 3C, there were no changes in the caspase-3 activity of the MDA-MB-231 cells prior to 240 mins when treated with triptolide alone, however,

levels of activity increased at 240 mins when treated with TNF- $\alpha$ +triptolide. These data indicated that the combination of TNF- $\alpha$  with triptolide promoted the activation of caspase-3.

*Effects of the combination of TNF- $\alpha$  with triptolide on the NF- $\kappa$ B pathway.* Previous reports have demonstrated that triptolide is able to suppress the production of inflammatory

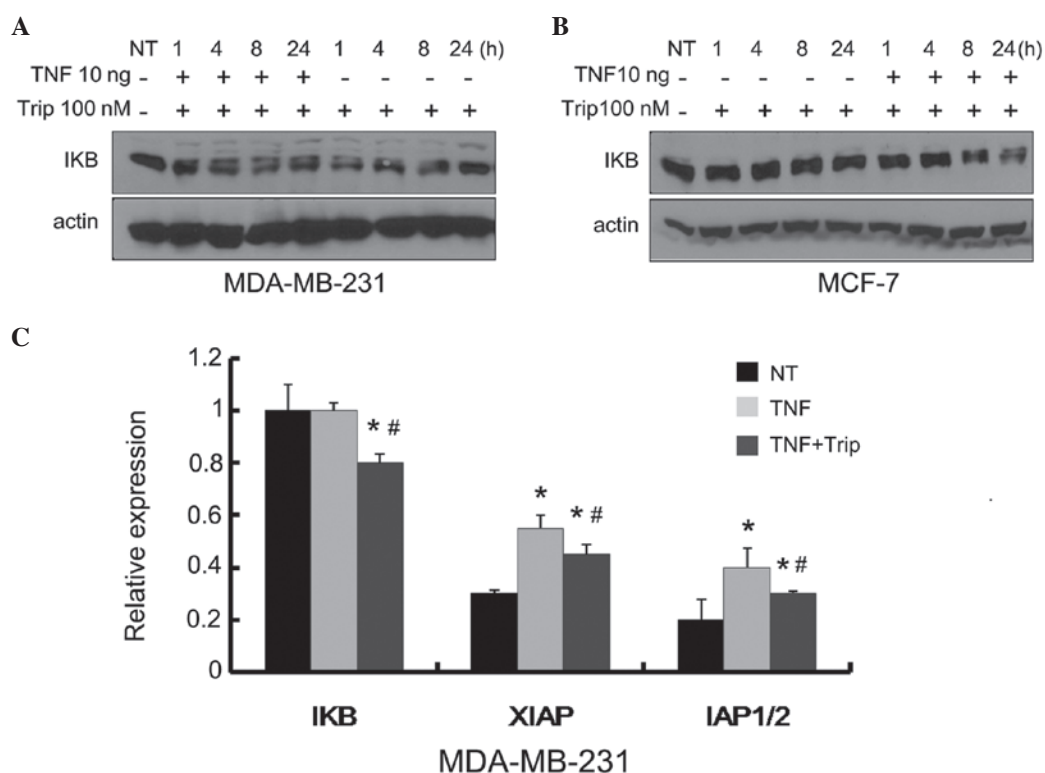


Figure 5. Kinetic effects of TNF- $\alpha$ +triptolide on the levels of I $\kappa$ B, and the mRNA levels of NF- $\kappa$ B-targeting genes. The (A) MDA-MB-231 cells and (B) MCF-7 cells were treated with 100 nM triptolide, or with TNF- $\alpha$  (10 ng/ml)+triptolide (100 nM) for the indicated durations. Velcade (20 nM) was used as a positive control. The protein levels of I $\kappa$ B, XIAP and cIAP1/2 were determined via Western blot analysis using specific antibodies. (C) MDA-MB-231 cells were treated with TNF- $\alpha$  (10 ng/ml), or with TNF- $\alpha$  (10 ng/ml)+triptolide (100 nM) for 12 h, followed by measurement of the mRNA levels of I $\kappa$ B, XIAP and cIAP1/2 via reverse transcription-quantitative polymerase chain reaction analysis. Data are expressed as the mean  $\pm$  standard deviation of three experiments. \* $P$ <0.05, vs. NT group; # $P$ <0.05 Trip/Vel treated group, vs. Trip/Vel+TNF- $\alpha$  group. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Trip, triptolide; Vel, velcade; NT, no treatment; I $\kappa$ B; inhibitor of NF- $\kappa$ B; XIAP; X-linked inhibitor of apoptosis protein; IAP1/2, inhibitor of apoptosis protein 1/2.

mediators induced by stimuli in various cell types (5,12,15). As TNF- $\alpha$  and the NF- $\kappa$ B pathway are important in inflammation and are involved in the anti-inflammatory effect of triptolide, intracellular I $\kappa$ B, which is important in the NF- $\kappa$ B pathway was further examined. MDA-MB-231 and MCF-7 cells were treated, respectively, with triptolide (25, 50 and 100 nM), with or without TNF- $\alpha$  (10 ng/ml), for 24 h. As shown in Fig. 4A and B, exposure of the cells to triptolide, either alone or with TNF- $\alpha$ , inhibited the expression of I $\kappa$ B in a dose-dependent manner. This inhibitory effect was more marked when triptolide was used in combination with TNF- $\alpha$ . The inhibitory effect of triptolide on I $\kappa$ B may be due to its inhibition of inflammatory pathways, which is consistent with previous reports (11,12).

According to previous reports, XIAP and cIAP1/2 are target genes of NF- $\kappa$ B (16) and exert effects on the caspase-9 and caspase-3 apoptotic pathways (17,18). Therefore, the effects of TNF- $\alpha$  combined with triptolide on the levels of XIAP and cIAP1/2 were further examined in the present study. As shown in Fig. 4, in the MDA-MB-231 and MCF-7 cells, exposure to TNF- $\alpha$  and triptolide had no significant effect on the levels of cIAP1/2. However, triptolide caused a reduction in XIAP, and TNF- $\alpha$  aggravated these effects.

**Time-dependent effects of TNF- $\alpha$ +triptolide on I $\kappa$ B.** The present study also examined the kinetic effects of TNF- $\alpha$ +triptolide treatment on I $\kappa$ B. The MDA-MB-231 and MCF-7 cells were

treated, respectively, with triptolide (100 nM), with or without TNF- $\alpha$  (10 ng/ml), and incubated for different durations. As shown in Fig. 5A and B, the levels of I $\kappa$ B and caspase-9 gradually decreased with increasing duration, and the decrease in the levels of I $\kappa$ B in the TNF- $\alpha$ +triptolide-treated cells were more marked, compared with those in the triptolide only-treated cells. The results of the kinetic effects confirmed that TNF- $\alpha$  combined with triptolide inhibited the expression of I $\kappa$ B and promoted the degradation of caspase-9.

**Effects of TNF- $\alpha$  combined with triptolide on the transcription of NF- $\kappa$ B target genes.** In order to investigate whether the inhibition of I $\kappa$ B, XIAP and IAP1/2 were caused by inhibiting gene expression, the mRNA expression levels of I $\kappa$ B, XIAP and IAP1/2 were analyzed using RT-qPCR. The MDA-MB-231 breast cancer cells were treated with TNF- $\alpha$  (10 ng/ml) or TNF- $\alpha$ +triptolide (100 nM) for 6 h. As shown in Fig. 5C, TNF- $\alpha$  resulted in a marginal, but not significant increase in the mRNA level of I $\kappa$ B. This increase may be a feedback effect of the cells to TNF- $\alpha$ . TNF- $\alpha$ +triptolide caused a significant decrease in the mRNA level of I $\kappa$ B, which may have been due to inhibition of the NF- $\kappa$ B pathway by triptolide. The expression levels of XIAP and IAP1/2 increased when the MDA-MB-231 cells were treated with TNF- $\alpha$  alone. Treatment with TNF- $\alpha$ +triptolide reduced the expression levels of XIAP and IAP1/2, with the effects on XIAP being more marked, compared with the effects on IAP1/2. These

results suggested that the inhibition of I $\kappa$ B, XIAP and IAP1/2 may due to inactivation of the NF- $\kappa$ B pathway.

## Discussion

Resistance to conventional chemotherapy and systemic toxicity remains a significant obstacle in improving the long-term survival rates of patients with breast cancer. Therefore, it is necessary to identify novel therapeutic strategies and agents to improve treatment. In the present study, it was demonstrated that a low dose of TNF- $\alpha$  sensitized MDA-MB-231 and MCF-7 cells to triptolide, leading to apoptosis. The results showed that treatment of cells with TNF- $\alpha$  (10 ng/ml) combined with triptolide (50-200 nM) increased triptolide-mediated cell death (Fig. 1), suggesting that low doses of TNF- $\alpha$  promoted triptolide to activate cell death. As shown in Fig. 2, TNF- $\alpha$ +triptolide activated caspase-3, and the activation of caspase-3 coincided with an increase in the cleavage of PARP (Figs. 2 and 3). Caspase-3 led to activation of downstream events, and promoted cell apoptosis. These observations confirmed that combining TNF- $\alpha$  with triptolide may provide a novel therapeutic combination for the treatment of patients with breast cancer.

However, the mechanism underlying the combinational effect of TNF- $\alpha$  with triptolide to induce apoptosis remains to be fully elucidated. The response of cells to TNF- $\alpha$ , an important modulator of I $\kappa$ B, is key in the resistance to caspase-3-mediated apoptosis in several types of cancer cell (2,3). I $\kappa$ B is the predominant inhibitory protein of NF- $\kappa$ B. Activation of I $\kappa$ B kinases (I $\kappa$ K $\alpha$ , I $\kappa$ K $\beta$  and the NF- $\kappa$ B essential modulator, I $\kappa$ K $\gamma$ ) results in phosphorylation of inhibitory I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  proteins bound to NF- $\kappa$ B. NF- $\kappa$ B is consequently released, and translocates to the nucleus where it interacts with other transcription factors and transcriptional co-factors to regulate the expression of an array of genes, several of which are involved in inflammatory signaling, proliferation and apoptosis (19). The classical activation of the NF- $\kappa$ B pathway can be initiated by a wide range of extracellular stimuli. These agents can activate the cells and mediate the phosphorylation of I $\kappa$ B, resulting in its degradation and rendering the NF- $\kappa$ B dimer-free to translocate to the nucleus to regulate several target genes (20), including XIAP/cIAP1/2 (21). NF- $\kappa$ B is activated by a variety of pro-inflammatory agents, including TNF- $\alpha$ , phorbol esters and several growth factors (22,23). Of these agents, the tumor microenvironment has been implicated in TNF- $\alpha$ -mediated NF- $\kappa$ B activation through the phosphorylation of I $\kappa$ B. In the present study, it was found that triptolide significantly inhibited the level of I $\kappa$ B induced by TNF- $\alpha$ . Following the use of a low dose of TNF- $\alpha$  to imitate the tumor microenvironment, it was found that the combination of TNF- $\alpha$  and triptolide was more effective, compared with triptolide alone, at inhibiting the expression of I $\kappa$ B (Fig. 4).

XIAP and cIAP-1/2 can inhibit death receptor-mediated apoptosis (24). These polypeptides belong to the IAP family, a group of intracellular proteins containing one or more zinc-binding baculovirus IAP repeat domains. Several IAPs, including XIAP, cIAP-1 and cIAP-2, also contain a carboxy-terminal RING domain with ubiquitin E3 ligase properties (25). Although all IAPs can potentially bind to caspases, only XIAP is a direct inhibitor of caspases-9, -3 and -7 (17,18),

whereas cIAP-1 and cIAP-2 are considered to regulate receptor-mediated signaling pathways upstream of mitochondria, through their interactions with TNF receptor-associated factor (TRAF)1 and TRAF2 (26). The present study further examined the association between TNF- $\alpha$  with triptolide, and the levels of I $\kappa$ B, caspase-9, XIAP, and cIAP-1/2. The results demonstrated that I $\kappa$ B, caspase-9, XIAP and cIAP1/2 were downregulated by the combination of TNF- $\alpha$  and triptolide in the breast cancer cells (Figs. 4 and 5). As XIAP is a direct inhibitor of caspases-9, -3 and -7 (17,18), the lower levels of XIAP and cIAP1/2, may contribute to the increased degradation of caspase-9. Therefore, the present study evaluated the effect of TNF- $\alpha$  combined with triptolide on the mRNA expression levels of I $\kappa$ B, XIAP and cIAP1/2. The results revealed that TNF- $\alpha$ +triptolide markedly downregulates the mRNA levels of I $\kappa$ B, XIAP and cIAP1/2 in the breast cancer cells (Fig. 5A). This downregulation of I $\kappa$ B eliminated the activation of the NF- $\kappa$ B-pathway that was induced by TNF- $\alpha$ . In the presence of a low dose of TNF- $\alpha$ , the suppressive effects of triptolide on XIAP and cIAP1/2 became necessary for the apoptotic pathway to facilitate caspase-3 activation.

In conclusion, the present study found that the cytotoxic effects of triptolide on breast cancer cells were enhanced in the presence of TNF- $\alpha$ . The investigation of TNF- $\alpha$  in the tumor microenvironment revealed that the levels of TNF- $\alpha$  are generally higher in the tumor microenvironment, and that TNF- $\alpha$  is important in the development of tumors. The results of the present study provided evidence for the effect of the natural anti-inflammatory drug, triptolide, in the treatment of inflammatory tumors, which holds important implications for the therapeutic strategies used in the treatment of breast cancer.

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