

Cellular FLICE-inhibitory protein regulates chemotherapy-induced apoptosis in breast cancer cells

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Abstract

Combination treatment regimens that include topoisomerase-II – targeted drugs, such as doxorubicin, are widely used in the treatment of breast cancer. Previously, we showed that IFN- γ and doxorubicin cotreatment synergistically induced apoptosis in MDA435 breast cancer cells in a signal transducer and activator of transcription 1 – dependent manner. In this study, we found that this synergy was caspase-8 dependent. In addition, we found that IFN- γ down-regulated the expression of the caspase-8 inhibitor cellular FLICE-inhibitory protein (c-FLIP). Furthermore, IFN- γ down-regulated c-FLIP in a manner that was dependent on the transcription factors signal transducer and activator of transcription 1 and IFN regulatory factor-1. However, IFN- γ had no effect on c-FLIP mRNA levels, indicating that c-FLIP was down-regulated at a posttranscriptional level following IFN- γ treatment. Characterization of the functional significance of c-FLIP modulation by small interfering RNA gene silencing and stable overexpression studies revealed it to be a key regulator of IFN- γ – and doxorubicin-induced apoptosis in MDA435 cells. Analysis of a panel of breast cancer cell lines indicated that c-FLIP was an important general determinant of doxorubicin- and IFN- γ – induced apoptosis in breast cancer cells. Furthermore, c-FLIP gene silencing sensitized MDA435 cells to other chemotherapies, including etoposide, mitoxantrone, and SN-38. These results suggest that c-FLIP plays a pivotal role in modulating drug-induced apoptosis in breast cancer cells. [Mol Cancer Ther 2007;6(5):1544 – 51]

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Introduction

The IFNs are a family of widely expressed cytokines with pleiotropic biological effects that are mediated by the regulation of gene expression (1–3). The active form of IFN- γ is a homodimer that exerts its effects by interacting with its cell surface receptor, IFN- γ receptor. IFN- γ receptor consists of two polypeptides: IFNGR1, responsible for ligand binding, and IFNGR2, which has a minor role in ligand binding but is required for IFN- γ signaling. IFN- γ binding triggers oligomerization of IFNGR1 and IFNGR2 causing them to associate with Janus-activated kinase 1 and Janus-activated kinase 2, respectively (4). The activation of the Janus-activated kinases induces phosphorylation of key tyrosine residues within the IFNGR1 intracellular domain, which are recognized by the SH2 domain of the transcription factor signal transducer and activator of transcription 1 (STAT1). STAT1 molecules are subsequently phosphorylated and activated to form homodimers that translocate to the nucleus, where they bind to specific gene promoter elements and initiate gene transcription (1, 3–5). Many of the genes induced are transcription factors [such as *IFN regulatory factor-1 (IRF1)*] that propagate a further wave of transcription (3). Several proapoptotic IFN-stimulated genes have been identified, such as *tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)*, *Fas*, and *caspase-8* (6); hence, the IFN family is now regarded as apoptosis-inducing cytokines (7).

Several investigators have reported promising antitumor responses when IFN- γ was used in combination regimens. For example, it has been found that treating with IFN- γ sensitizes cancer cells to apoptosis induced by a variety of chemotherapeutic agents (8–12). Anthracyclines, such as doxorubicin, are among the most active agents used in the treatment of breast cancer (13). Doxorubicin (Adriamycin) inhibits the DNA topoisomerase-II enzyme causing DNA double-strand breaks that trigger cell death (14). Monti et al. (15) reported a synergistic interaction between IFN- γ and doxorubicin in human MDR colon cancer cells. Furthermore, we reported recently that IFN- γ and doxorubicin interacted synergistically to induce apoptosis in MDA435 breast cancer cells in a STAT1-dependent manner (9).

Death receptors, such as Fas, are members of the tumor necrosis factor receptor superfamily (16). On binding of Fas ligand (FasL), the Fas receptor recruits the adaptor molecule Fas-associated protein with death domain (FADD/MORT1), which then recruits the initiator caspases, caspase-8 and caspase-10. Caspase-8 is the main initiator caspase and, together with FADD, forms the death-inducing signaling complex (DISC). Formation of the DISC results in cleavage and activation of caspase-8, which in turn cleaves and activates downstream effector caspases, such as caspase-3 and caspase-7 (17, 18). Another major death receptor pathway is mediated by TRAIL.

Currently, four human TRAIL receptors (TRAIL-R) have been identified, death receptor (DR)4/TRAIL-R1, DR5/TRAIL-R2, decoy receptor (DcR) 1/TRAIL-R3, and DcR2/TRAIL-R4. TRAIL-bound DR4 and DR5 can recruit FADD, which in turn recruits caspase-8 forming the DISC in a similar manner to Fas. The decoy receptors, DcR1 and DcR2, lack the cytoplasmic domain necessary to recruit FADD and so do not form DISCs (19, 20). Cellular FLICE-inhibitory protein (c-FLIP; FLAME-1/Casper) is a key inhibitor of Fas- and TRAIL-mediated apoptosis. It has homology to caspase-8 and caspase-10 but lacks their protease activity due to the absence of key NH₂ acid residues at the active site (21). Multiple splice variants of c-FLIP have been reported; however, only two main forms are detected at the protein level, c-FLIP_{Long} (c-FLIP_L) and c-FLIP_{Short} (c-FLIP_S; ref. 22). c-FLIP_L and c-FLIP_S have been shown to inhibit death receptor-mediated apoptosis by being recruited to the DISC and inhibiting caspase-8 and caspase-10 activation (22). Several investigators have reported that down-regulating c-FLIP expression confers sensitivity to TRAIL- and Fas-induced apoptosis (23–26). Furthermore, recent work from our laboratory has shown that c-FLIP down-regulation sensitized colorectal cancer cells to chemotherapy (26).

The purpose of this study was to investigate the mechanism involved in mediating the enhanced apoptotic phenotype that we reported previously in MDA435 cancer cells treated with IFN- γ and doxorubicin (9). Our findings indicate that c-FLIP plays a key role in mediating the synergistic interaction between these agents.

Materials and Methods

Materials and Routine Cell Culture

All cells were maintained at 5% CO₂ at 37°C and grown as a monolayer culture in DMEM (PAA Laboratories) supplemented with 10% FCS, 1 mmol/L sodium pyruvate, and 1 mmol/L L-glutamine, all obtained from Invitrogen. Doxorubicin was purchased from Pharmacia and Upjohn. Mitoxantrone was purchased from Sigma. Etoposide (VP-16) was purchased from Bristol-Myers Squibb. SN-38 was purchased from Abatara Technology. IFN- α , IFN- β , and IFN- γ were purchased from Calbiochem.

Western Blot Analysis

Equal protein concentrations of whole-cell lysates were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Following transfer, blots were incubated with primary antibody overnight at 4°C in 5% nonfat dried milk 1 \times PBS containing 0.1% Tween 20. Western blot analyses were done using specific primary antibodies for c-FLIP, caspase-8 (Alexis Corp.), IRF1 (Santa Cruz Biotechnology, Inc.), poly(ADP-ribose) polymerase (PARP; PharMingen, BD Biosciences), or β -actin (Sigma). Horseradish peroxidase-linked secondary antibody (anti-mouse or antirabbit; Amersham Biosciences) was applied at a dilution of 1:2,000 for 1 h at room temperature. Blots were developed using the SuperSignal chemiluminescent (Pierce) or Enhanced Chemiluminescence Plus (Amersham Biosciences) detection systems and exposed to X-ray film (Fuji).

Cell Viability Assays

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay. Two thousand cells were seeded per well on 96-well tissue culture plates. Twenty-four hours later, the cells were treated with IFN- γ and/or doxorubicin or transfected with dual FLIP-targeted or scrambled control small interfering RNAs (siRNA) for 72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well at a final concentration of 500 μ g/mL and the cells were incubated at 37°C for a further 2 to 3 h. The culture medium was removed and formazan crystals were reabsorbed in 200 μ L DMSO. Cell viability was determined by reading the absorbance of each well at 570 nm using a microplate plate reader (Biotrak II visible plate reader, Amersham Biosciences).

Flow Cytometric Analysis

Cells were seeded in a six-well tissue culture plate at a density of 1×10^5 cells per well and allowed to grow for 24 h, after which time IFN- γ or doxorubicin was added. Apoptosis was determined by evaluating the percentage of cells with DNA content <2N. For the analysis of cell surface receptor expression, cells were treated with the indicated doses of IFN- γ or doxorubicin for 48 h and then harvested, washed in buffer (PBS with 0.1% sodium azide and 0.2% bovine serum albumin), and stained with a phycoerythrin-conjugated anti-DR5 monoclonal antibody (eBiosciences) or a FITC-conjugated monoclonal human anti-Fas antibody (DakoCytomation) according to the manufacturer's instructions. Nonspecific staining was assessed using phycoerythrin-conjugated (eBiosciences) and FITC-conjugated (DakoCytomation) isotype control antibodies. All analyses were conducted on an EPICS XL flow cytometer (Coulter) using CellQuest software (BD Biosciences).

siRNA Transfections

Details of the siRNAs used to down-regulate c-FLIP and STAT1 have been described previously (9, 26). Caspase-8-targeted siRNA was purchased from Qiagen (synthesized by Xeragon). Other sequences used were GCCGAGAUGC UAAGAGCAAGG (IRF1-targeted siRNA) and AAUUCUCCG AACGUGUCACGU (scrambled control sequence). siRNA transfections were done on subconfluent cells (seeded 24 h previously in the absence of antibiotic) incubated in Opti-MEM 1 using the Oligofectamine reagent (both from Invitrogen) according to the manufacturer's instructions.

Caspase-8 Spectrofluorimetric Activity Assay

A spectrofluorimetric caspase-8 enzyme activity assay was used (Sigma) to quantify caspase-8 activity. The assay is based on the release of 7-amino-4-methylcoumarin by caspase-8. Cells were seeded on a six-well tissue culture plate at a density of 2×10^5 cells per well and, after 24 h, treated with IFN- γ and/or doxorubicin. After a further 48 h, samples were suspended and lysed, and 20 μ L lysate was transferred (in duplicate) to a 96-well fluorimetric plate, which also contained a caspase-8-positive control sample. The fluorimetric assay was carried out according to the manufacturer's instructions. Fluorescence was

measured on a fluorescent plate reader (Cytofluor 4000, Applied Biosystems) at 37°C using an excitation wavelength of 360 nm and an emission wavelength of 440 nm, at 5-min intervals. Caspase-8 activity was calculated using a 7-amino-4-methylcoumarin calibration curve analyzed on the same plate. The specificity of the reaction for caspase-8 was confirmed by simultaneously analyzing duplicate samples in the presence of a caspase-8 inhibitor (Ac-IETD-CHO).

Real-time Quantitative Reverse Transcription-PCR Analysis

Real-time quantitative reverse transcription-PCR was done using a DNA Engine Opticon 2 (Genetic Research Instrumentation Ltd.). RNA was collected using STAT-60 reagent (Tel-Test) according to the manufacturer's instructions, and cDNA was synthesized using 1 μ g RNA and a reverse transcriptase kit (Invitrogen). Gene expression was analyzed using a DyNAmo SYBR Green Q-PCR kit (Finnzymes). PCR conditions consisted of an initial denaturation step of 95°C for 15 min followed by 45 cycles of 95°C for 15 s, 54°C for 30 s, 72°C for 1 min, and 75°C for 1 s. A melting curve was included at the end of each run to check specificity of the amplified product. On completion of the run, the PCR products were electrophoresed on a 1.2% agarose gel to confirm their sized matched that of the expected amplicon. Primers used were as follows: c-FLIP, 5'-TTACGGGAAAGAGGTAAG-3' (forward) and 5'-TGGGTCTCCACAGCTTTT-3' (reverse); 18S, 5'-CATTTCGTATGCGCCGCTA-3' (forward) and 5'-CGACGGTATCTGATCGTCT-3' (reverse). c-FLIP mRNA levels were quantified using the comparative threshold cycle (C_T) method using 18S rRNA as the calibrator (27). Experiments were done in triplicate.

Generation of c-FLIP – Overexpressing Cell Lines

c-FLIP_L- and c-FLIP_S-overexpressing cell lines were generated as described previously (26).

Statistical Analyses

The nature of the interaction between IFN- γ and doxorubicin was determined by calculating combination index (CI) values using the CalcuSyn program, which is based on the median-effect principle proposed by Chou and Talalay (28). The program returns a CI value quantifying the degree of synergy between two agents that both induce a pharmacologic response. CI values between 0.3 to 0.7 are defined as synergistic, with values <0.3 considered strongly synergistic and those between 0.7 and 1.0 as moderately or slightly synergistic. All experiments are representative of at least three separate experiments.

Results

IFN- γ Enhances Chemotherapy-Induced Apoptosis in MDA435 Cells

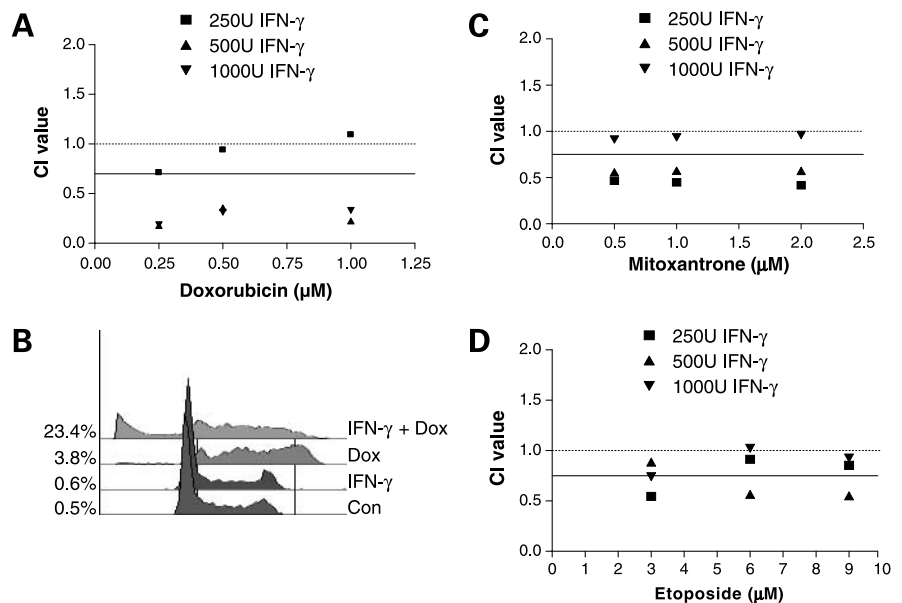
We found previously that cotreatment of MDA435 breast cancer cells with doxorubicin and IFN- γ synergistically enhanced apoptosis as determined by PARP cleavage and caspase-3 activation (9). These results were confirmed by cell viability assays, which indicated that cotreatment with

doxorubicin and 500 units/mL IFN- γ decreased the doxorubicin IC_{50(72h)} dose by ~5.5-fold from 0.67 to 0.12 μ mol/L. Calculation of CI values from the cell viability assays revealed that cotreatment with 0.25 to 1 μ mol/L doxorubicin and 500 or 1,000 units/mL IFN- γ generated CI values <0.7, indicative of a synergistic interaction between these two agents (Fig. 1A). Flow cytometric analyses of cells cotreated with 500 units/mL IFN- γ and 1 μ mol/L doxorubicin for 48 h revealed that a significant proportion of cells (23.4%) were in the sub-(G₀-G₁) apoptotic fraction compared with treatment with either agent alone (Fig. 1B). In addition to doxorubicin, we also analyzed two other topoisomerase-II–targeted agents used in the treatment of breast cancer: mitoxantrone and VP-16. Cell viability assays indicated that IFN- γ also interacted synergistically with both mitoxantrone (Fig. 1C) and VP-16 (Fig. 1D). These data were corroborated by Western blot analyses (data not shown). Furthermore, IFN- γ was also found to interact synergistically in MDA435 cells with the antimetabolite 5-fluorouracil and the topoisomerase-I inhibitor SN-38 (data not shown).

IFN- γ - and Doxorubicin-Induced Apoptosis Is Mediated by Caspase-8

We next analyzed the mechanism of cell death induced by doxorubicin and IFN- γ . Spectrofluorimetric determination of caspase-8 activity in doxorubicin- and IFN- γ -cotreated cells showed a ~9-fold increase in caspase-8 activity compared with ~1.6-fold for IFN- γ alone and ~2.5-fold for doxorubicin alone (Fig. 2A). This suggested that IFN- γ - and doxorubicin-induced apoptosis involved caspase-8, but to test whether this was indeed the case, we used a caspase-8–targeted siRNA. As expected, cotreatment of cells transfected with the scrambled control siRNA with IFN- γ and doxorubicin resulted in apoptosis as determined by cell cycle analysis (Fig. 2B) and Western blotting (data not shown). In contrast, in cells transfected with the caspase-8–targeted siRNA, the apoptosis induced by cotreatment with IFN- γ and doxorubicin was significantly reduced (Fig. 2B). Similar results were obtained with a caspase-8–specific inhibitor (Z-IETD-fmk; data not shown). These data indicated an important role for caspase-8 in mediating the synergistic effect of IFN- γ and doxorubicin; thus, we next examined the expression of the death receptors that activate caspase-8. DR4 was not expressed on the MDA435 cell membrane (data not shown). Fas was constitutively expressed and significantly up-regulated by IFN- γ treatment, although doxorubicin treatment had less of an effect (Fig. 2C). DR5 was also constitutively expressed on the MDA435 cell membrane, and this expression was significantly increased following treatment with doxorubicin (Fig. 2C). The induction of DR5 in response to combined treatment with IFN- γ and doxorubicin seemed to result in slightly lower cell surface expression than with doxorubicin alone. Similarly, the induction of Fas in response to the combined treatment seemed lower than with IFN- γ alone. This may be the result of apoptosis in IFN- γ - and doxorubicin-cotreated cells, which may be most efficiently induced in those cells expressing the highest levels of the

Figure 1. Cotreatment with IFN- γ and chemotherapeutic agents synergistically induces apoptosis in MDA435 cells. **A**, cells were cotreated with 250 to 1,000 units/mL IFN- γ and 0.25 to 1 μ mol/L doxorubicin for 72 h, after which time 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to assess cell viability. The synergistic interaction between the two agents was quantified by calculating CI values using the CalcuSyn program. Values between 0.3 and 0.7 are defined as synergistic, values <0.3 are defined as highly synergistic, and values between 0.7 and 1.0 are defined as moderately or slightly synergistic. **B**, flow cytometric cell cycle analysis of MDA435 cells treated with 500 units/mL IFN- γ and 1 μ mol/L doxorubicin (*Dox*) for 48 h. IFN- γ synergizes with mitoxantrone (**C**) and VP-16 (**D**) as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assays; synergy was determined by calculating CI values using the CalcuSyn program. Data are representative of at least three independent experiments.



two death receptors. These results further implicate death receptor signaling in mediating apoptosis induced by IFN- γ and doxorubicin in MDA435 cells.

IFN- γ Down-regulates Expression of the Caspase-8 Inhibitor c-FLIP

We next examined the role of the caspase-8 inhibitor c-FLIP. Cells were treated with IFN- γ (500 units/mL) or doxorubicin (1 μ mol/L). After 24 h, expression of c-FLIP_L was unchanged by IFN- γ treatment, but levels of c-FLIP_S seemed to be slightly up-regulated (Fig. 3A). When MDA435 cells were treated with doxorubicin for 24 and 48 h, c-FLIP_L was up-regulated by ~2-fold, whereas c-FLIP_S expression was not significantly altered. The most interesting observation came after 48-h treatment with IFN- γ , which dramatically down-regulated expression of both splice forms of c-FLIP (Fig. 3A). Real-time quantitative reverse transcription-PCR analysis of *c-FLIP* gene expres-

sion revealed that it was not significantly altered by IFN- γ treatment (Fig. 3B), indicating that the effects of IFN- γ on c-FLIP protein expression occurred at the posttranscriptional level. Doxorubicin treatment increased c-FLIP mRNA levels somewhat at 24 h (by ~2-fold), which may be the cause of increased c-FLIP_L protein expression in response to this agent. IFN- α and IFN- β did not alter expression of c-FLIP (data not shown). Our previous data described an important role for STAT1 in mediating apoptosis of IFN- γ - and doxorubicin-cotreated MDA435 cells, as *STAT1* gene silencing abrogated apoptosis (9). To further investigate the role of STAT1 signaling in drug-treated MDA435 cells, we investigated the effect of STAT1 and its downstream target IRF1 on c-FLIP expression. Of note, both STAT1- and IRF1-targeted siRNAs blocked IFN- γ -induced down-regulation of c-FLIP (Fig. 3C). Furthermore, *IRF1* gene silencing abrogated apoptosis induced by IFN- γ and doxorubicin

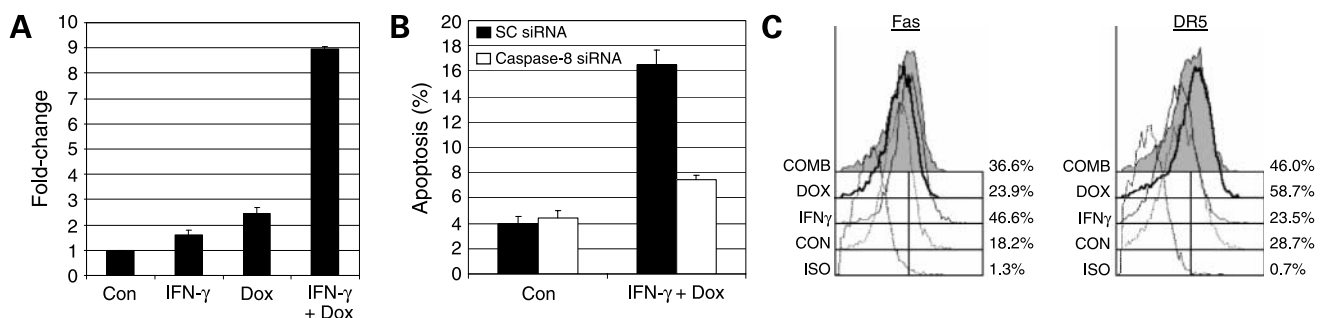


Figure 2. Role of caspase-8 in regulating IFN- γ - and doxorubicin-induced apoptosis. **A**, caspase-8 activity in IFN- γ - and doxorubicin-treated MDA435 cells was measured using a spectrofluorimetric assay. Caspase-8 activity was assessed 48 h after drug treatment. The fold change in activity relative to untreated controls is plotted. **B**, flow cytometric analysis of apoptosis in 500 units/mL IFN- γ -cotreated and 1 μ mol/L doxorubicin-cotreated MDA435 cells transfected with 10 nmol/L caspase-8 or nonsilencing control [scrambled control (SC)] siRNA. Cells were assayed 48 h after drug treatment. **C**, cell surface expression of Fas and DR5 in MDA435 cells treated with no drug (*Con*), 500 units/mL IFN- γ , 1 μ mol/L doxorubicin, or both IFN- γ and doxorubicin for 48 h.

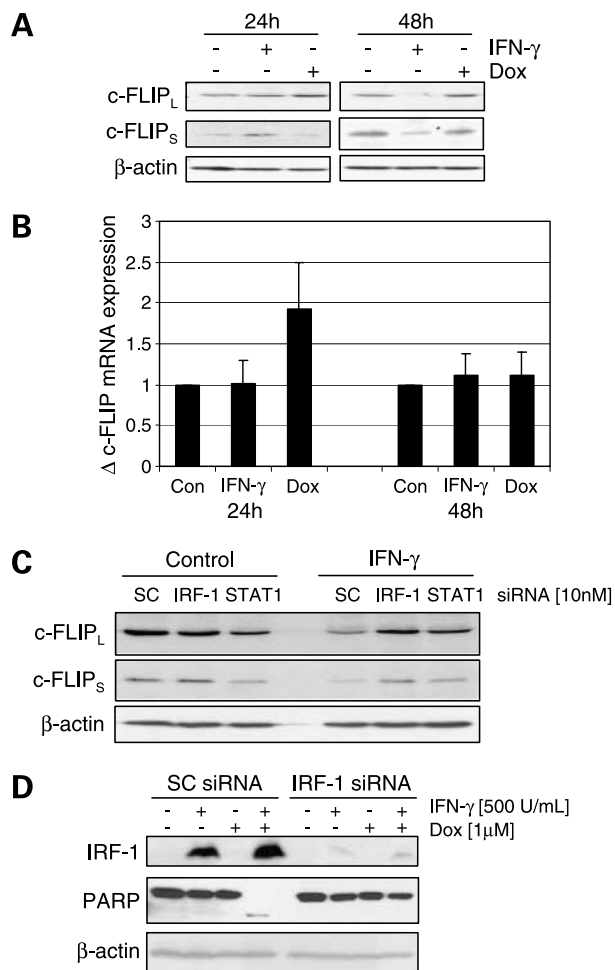


Figure 3. IFN- γ down-regulates c-FLIP protein expression. **A**, MDA435 cells were treated with 500 units/mL IFN- γ or 1 μ mol/L doxorubicin for 24 and 48 h and expression of c-FLIP expression was assessed by Western blotting. **B**, transcriptional regulation of c-FLIP in response to 500 units/mL IFN- γ and 1 μ mol/L doxorubicin was assessed by real-time quantitative reverse transcription-PCR analysis of c-FLIP mRNA levels 24 and 48 h after drug treatment. Expression was normalized to that of 18S rRNA and fold changes were calculated. **C**, Western blot analysis of c-FLIP expression in IFN- γ -treated MDA435 cells transfected with 10 nmol/L IRF1- or STAT1-targeted siRNAs. Cells were harvested 48 h after treatment with 500 units/mL IFN- γ . **D**, Western blot analysis of IRF1 expression and PARP cleavage in MDA435 cells transfected with 10 nmol/L IRF1-targeted or control (SC) siRNAs and cotreated with 500 units/mL IFN- γ and 1 μ mol/L doxorubicin for 48 h. β -Actin was used as a loading control in Western blotting analyses.

cotreatment as indicated by PARP cleavage analysis (Fig. 3D). These results indicate that both the synergy between IFN- γ and doxorubicin and the IFN- γ -mediated down-regulation of c-FLIP are STAT1 and IRF1 dependent.

c-FLIP Regulates Chemotherapy-Induced Apoptosis

The results described above suggested that c-FLIP may be involved in regulating the interaction between IFN- γ and doxorubicin. To determine the importance of c-FLIP in regulating chemotherapy-induced apoptosis in MDA435 cells, we used a c-FLIP-targeted siRNA to simultaneously silence gene expression of both c-FLIP splice forms

(Fig. 4A). In cells transfected with FLIP-targeted siRNA, the degree of PARP cleavage induced by doxorubicin alone was significantly increased compared with cells transfected with scrambled control siRNA (Fig. 4B, compare lanes 3 and 7), indicating that c-FLIP silencing sensitizes MDA435 cells to doxorubicin-induced apoptosis. Interestingly, c-FLIP silencing also increased apoptosis induced by IFN- γ alone (Fig. 4B, compare lanes 2 and 6). Synergy between FLIP-targeted siRNA and doxorubicin was calculated by cell viability assays. It was found that doxorubicin synergized with FLIP-targeted siRNA to a high degree, especially at lower concentrations of the chemotherapy and higher siRNA concentrations (5 and 10 nmol/L; Fig. 4C). Collectively, these results indicate that c-FLIP is an inhibitor of doxorubicin-induced cell death and that IFN- γ may sensitize MDA435 cells to doxorubicin by down-regulating c-FLIP.

To complement the siRNA experiments, we investigated the effects of stably overexpressing the two splice forms of c-FLIP in MDA435 cells. Two clones FL-D24 and FS-F13, which overexpress c-FLIP_L and c-FLIP_S, respectively

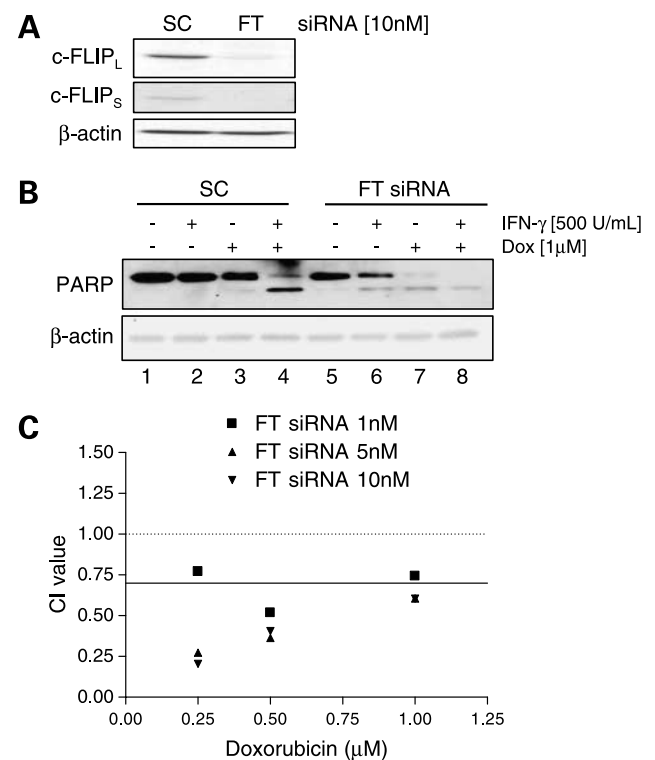


Figure 4. Down-regulation of c-FLIP expression enhances doxorubicin-induced apoptosis. **A**, Western blot analysis of c-FLIP_L and c-FLIP_S expression following transfection of MDA435 cells with 10 nmol/L c-FLIP-targeted (FT) siRNA for 24 h. **B**, Western blot analysis of PARP cleavage in MDA435 cells transfected with 10 nmol/L FLIP-targeted siRNA and treated with 500 units/mL IFN- γ and/or 1 μ mol/L doxorubicin for 48 h. β -Actin was used as a loading control. **C**, FLIP-targeted siRNA synergistically interacts with doxorubicin as determined by cell viability assays; synergy was determined by calculating CI values using the CalcuSyn program. Data are representative of at least three independent experiments.

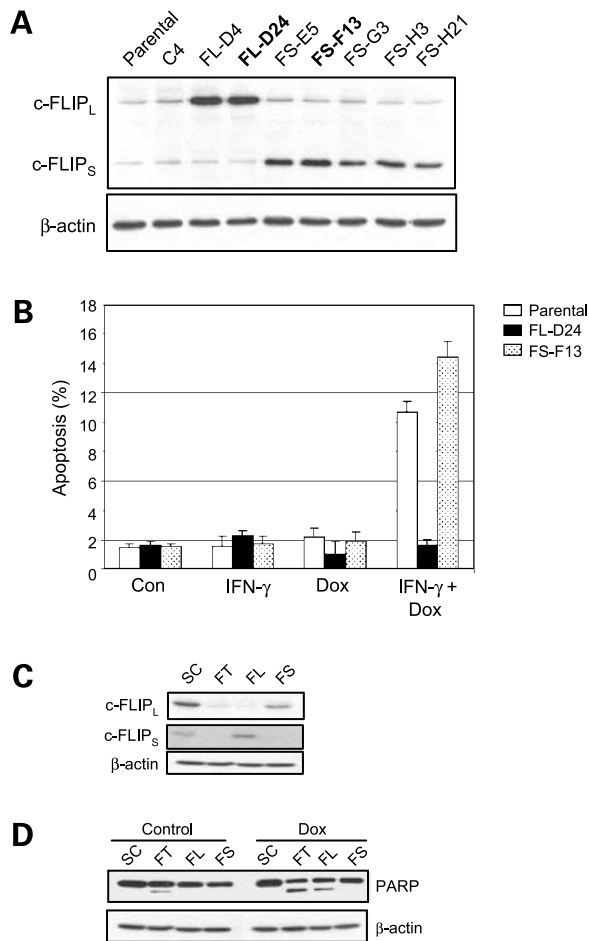


Figure 5. **A**, MDA435 cells were stably transfected with c-FLIP_L or c-FLIP_S expression constructs. Screening of stable c-FLIP-overexpressing cell lines by Western blotting identified the cell lines that were used for the phenotypic studies: FL-D24 and FS-F13. **B**, effect of overexpressing each of the c-FLIP splice forms on IFN- γ and doxorubicin-induced apoptosis was determined by flow cytometric analysis of propidium iodide-stained cells. Cells were treated with 500 units/mL IFN- γ and/or 1 μ M doxorubicin for 48 h. **C**, MDA435 cells were transfected for 24 h with 10 nmol/L siRNA targeted specifically to c-FLIP_L (FL), c-FLIP_S (FS), or both splice forms (FT), and c-FLIP expression was analyzed by Western blot. **D**, MDA435 cells transfected with 10 nmol/L scrambled control, FLIP-targeted, c-FLIP_L, and c-FLIP_S siRNAs were treated with 1 μ M doxorubicin for 48 h and apoptosis was assessed by PARP cleavage analysis. β -Actin was used as a loading control.

(Fig. 5A), were taken forward for phenotypic analyses. Flow cytometry revealed that overexpressing the c-FLIP_L splice form completely abrogated apoptosis induced by IFN- γ and doxorubicin cotreatment, whereas c-FLIP_S overexpression had no effect (Fig. 5B). To further elucidate which c-FLIP splice form was the more important in the regulation of doxorubicin-induced apoptosis, we used siRNAs that specifically down-regulated either c-FLIP_L or c-FLIP_S (Fig. 5C). Both FLIP-targeted and c-FLIP_L siRNAs enhanced doxorubicin-induced apoptosis, although c-FLIP_S siRNA had no effect (Fig. 5D). Collectively, these results suggest that c-FLIP_L rather than c-FLIP_S is the more

important regulator of doxorubicin-induced apoptosis in MDA435 cells.

We next examined whether c-FLIP gene silencing sensitized MDA435 cells to the other chemotherapies that synergized with IFN- γ . When c-FLIP expression was silenced, enhanced PARP cleavage and caspase-8 activation were evident in cells treated with SN-38 and VP-16 and, to a lesser extent, mitoxantrone (Fig. 6A). Caspase-8 activation was assessed by the appearance of the p41/43 cleavage fragment. These data indicate that c-FLIP also inhibits apoptosis induced by these agents, providing a mechanistic basis for the synergy observed between IFN- γ and these chemotherapies. Finally, we investigated the role of c-FLIP in regulating response to chemotherapy in a wider panel of breast cancer cell lines. FLIP-targeted siRNA was effective in down-regulating c-FLIP expression in each cell line (data not shown). Down-regulating c-FLIP sensitized MDA-MB-157, MDA-MB-231, and MCF-7 cells to IFN- γ , although MDA-MB-468 cells remained insensitive to IFN- γ (Fig. 6B). However, down-regulating c-FLIP expression sensitized MDA-MB-468 cells to doxorubicin-induced apoptosis, whereas MDA-MB-157, MDA-MB-231, and MCF-7 cells were also all sensitized to doxorubicin-induced apoptosis by c-FLIP gene silencing. In all four cell lines, high levels of apoptosis were observed in FLIP-targeted transfected cells cotreated with IFN- γ and doxorubicin. From these results, it seems that c-FLIP plays an important role in regulating both IFN- γ - and doxorubicin-induced apoptosis in breast cancer cells.

Discussion

Several studies (including our own) have reported synergistic activation of apoptosis following cotreatment with IFN- γ and a range of agents, including chemotherapies such as doxorubicin and 5-fluorouracil (8–10, 15, 29, 30). Synergy has been attributed to the enhanced expression of certain proapoptotic genes, such as *FasL*, *IRF1*, and *TRAIL* (10, 31, 32). We found previously that STAT1 plays a key role in regulating the synergy between doxorubicin and IFN- γ in MDA435 breast cancer cells (9). To further elucidate the molecular mechanisms that link STAT1 activation with the potentiation of apoptosis in response to these two agents, we investigated the regulation of various components of the death receptor apoptotic signaling pathway. Using Western blotting and enzyme activity assays, we found that cotreatment with IFN- γ and doxorubicin synergistically enhanced activation of caspase-8. Our data and that of others suggest that IFN- γ can prime cells for death receptor-mediated apoptosis by up-regulating caspase-8 (8–10). We confirmed the importance of caspase-8 in the enhanced apoptotic phenotype using caspase-8-targeted siRNA and a caspase-8 inhibitor, both of which abrogated the apoptosis induced by IFN- γ and doxorubicin.

We have made the novel observation that IFN- γ can potentially down-regulate expression of the caspase-8 inhibitor c-FLIP. Both splice variants of c-FLIP were down-regulated by IFN- γ (but not IFN- α or IFN- β) in MDA435

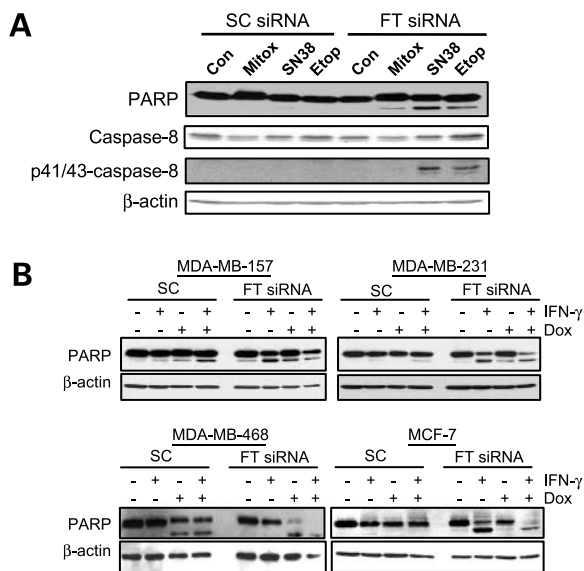


Figure 6. **A**, Western blot analysis of PARP cleavage and caspase-8 activation in MDA435 cells transfected with 10 nmol/L FLIP-targeted or scrambled control siRNA and treated with mitoxantrone (*Mitox*), VP-16 (*Etop*), and SN-38. Cells were treated with \sim IC₅₀ doses of each chemotherapeutic drug for 48 h. Caspase-8 activation was assessed by the appearance of the p41/43 cleavage fragment. β -Actin was used as a loading control. **B**, a panel of breast cancer cell lines were transfected with 10 nmol/L FLIP-targeted or control (SC) siRNA and treated with 500 units/mL IFN- γ and/or 0.5 μ mol/L doxorubicin for 48 h. Apoptosis induction in each cell line was analyzed by Western blotting for PARP. β -Actin was used as a loading control.

cells. Importantly, we found that *c-FLIP* gene silencing enhanced both doxorubicin- and IFN- γ -induced cell death in these cells. Furthermore, cells overexpressing c-FLIP_L were more resistant to apoptosis induced by cotreatment with IFN- γ and doxorubicin, whereas specific silencing of c-FLIP_L was sufficient to sensitize MDA435 cells to doxorubicin. These results indicate that c-FLIP (particularly c-FLIP_L) is an important regulator of the synergistic interaction between IFN- γ and doxorubicin. IFN- γ also increased cell surface expression of Fas, whereas doxorubicin treatment up-regulated DR5. Thus, the combined effects of c-FLIP down-regulation and death receptor up-regulation seem to lead to the synergistic activation of caspase-8, resulting in induction of apoptosis in response to cotreatment with IFN- γ and doxorubicin in MDA435 cells. In other breast cancer lines, we found that *c-FLIP* gene silencing also sensitized the cells to IFN- γ - and/or doxorubicin-induced apoptosis. In addition, *c-FLIP* gene silencing sensitized MDA435 cells to other chemotherapeutic drugs, including VP-16 and SN-38. Collectively, our data indicate that c-FLIP may be an important regulator of IFN- γ - and chemotherapy-induced cell death in breast cancer cells.

We observed increased IRF1 protein expression following treatment of MDA435 cells with IFN- γ . Porta et al. (33) showed recently that IRF1-targeted siRNA diminished IFN- γ -mediated apoptosis. In agreement with these studies, we have shown that doxorubicin- and IFN- γ -induced apopto-

sis is IRF1 dependent. This correlates with our previous finding that *STAT1* gene silencing attenuates IFN- γ - and doxorubicin-induced apoptosis (9). It is notable that both *STAT1* and IRF1 knockdown not only attenuated IFN- γ /doxorubicin-induced apoptosis but also abrogated IFN- γ -mediated down-regulation of c-FLIP, providing further evidence that c-FLIP plays a central role in regulating the effects of IFN- γ on doxorubicin sensitivity. Real-time quantitative reverse transcription-PCR analysis indicated that IFN- γ -mediated down-regulation of c-FLIP occurred at a posttranscriptional level. c-FLIP_L is cleaved by caspase-8 when recruited to the DISC to generate a p43 fragment (22). We found that IFN- γ -induced down-regulation of c-FLIP was not dependent on caspase-8 enzyme activity (data not shown), indicating that processing of c-FLIP by caspase-8 at the DISC is not responsible for the observed down-regulation of c-FLIP in response to IFN- γ . It is possible that IFN- γ stimulates transcription of a *STAT1*/IRF1-dependent gene that regulates c-FLIP protein turnover. Alternatively, IFN- γ may stimulate expression of a *STAT1*/IRF1-regulated micro-RNA that inhibits c-FLIP mRNA translation, resulting in decreased c-FLIP protein expression. These possibilities are currently being examined.

Signal transduction pathways provide potential targets for therapeutic intervention in the treatment of cancer, either alone or in combination with chemotherapeutic agents (34). The data uncovered by this study indicate that activating signal transduction pathways (such as *STAT1*/IRF1) involved in the regulation of apoptotic signaling can potentiate drug-induced apoptosis. Our findings suggest that the link between enhanced *STAT1* activation in response to IFN- γ and doxorubicin and the subsequent potentiation of apoptosis that we observed previously (9) is mediated by death receptor-mediated activation of caspase-8, which is facilitated by *STAT1*- and IRF1-dependent down-regulation of c-FLIP. Moreover, our data suggest that targeting c-FLIP in conjunction with anticancer therapies may have therapeutic potential by enhancing breast cancer cell death.

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