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Cellular FLICE-like inhibitory protein (c-FLIP): A novel target for Taxol-induced apoptosis☆

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Abbreviations:
MT, microtubule
MAPK, smitogen-activated protein kinases
PTK, protein tyrosine kinases
TNF-α, tumor necrosis factor-α
TNFR1, tumor necrosis factor-α receptor 1
TRAIL, tumor necrosis factor-related apoptosis-inducing ligand
AIF, apoptosis-inducing factor
IAP, an inhibitor of apoptosis protein
Apaf1, apoptotic proteinase activating factor-1
PKC, protein kinase C

ABSTRACT

It is known that by binding to the FAS-associated death domain (FADD) protein and/or caspases-8 and -10 at the level of the death-inducing signaling complex (DISC), cellular FLICE-like inhibitory protein (c-FLIP) can prevent apoptosis triggered by death-inducing ligands. We investigated whether the c-FLIP splice variants, c-FLIP long [c-FLIP(L)] and c-FLIP short [c-FLIP(S)], play a role in Taxol-induced apoptosis. Our results showed that low Taxol concentrations triggered caspase-8- and caspase-10-dependent apoptosis in the CCRF-HSB-2 human lymphoblastic leukemia cell line, and induced the down-regulation of c-FLIP(S) and c-FLIP(L). Taxol decreased the expression of c-FLIP by a post-transcriptional and caspase-independent mechanism. To explore the distinct functions of the c-FLIP variants in Taxol-induced apoptosis, we transfected the cells with expression vectors carrying c-FLIP(L) and c-FLIP(S) in the sense orientation or c-FLIP(S) in the antisense orientation [c-FLIP(S)-AS]. Caspases-8 and -10 were more efficiently activated in the c-FLIP(S)-AS strain treated with 5–50 nM Taxol, which revealed that c-FLIP regulates Taxol-induced apoptosis by interacting with these caspases. Furthermore, our data showed that increased expression of c-FLIP(L) or c-FLIP(S) reduced apoptosis at 5–50 nM Taxol concentrations suggesting that both isoforms of c-FLIP prevent Taxol-induced apoptosis. These results revealed that Taxol induces apoptosis by down-regulating c-FLIP(S) and c-FLIP(L) expression.

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PARP, poly(ADP-ribose) polymerase
DFF45, DNA fragmentation factor-45
ROS, reactive oxygen species
c-FLIP, cellular FLICE-like inhibitory protein
PTP, permeability transition pore
PI, propidium iodide

1. Introduction

Taxol (paclitaxel) is one of the most active cancer chemotherapeutic agents and is effective against several human tumors including ovarian, breast, non-small-cell lung tumors, and head and neck carcinomas [1–3]. It promotes microtubule (MT) assembly, inhibits MT depolymerization and changes MT dynamics, resulting in disruption of the microtubule network required for mitosis and cell proliferation [1,2,4]. Therefore, cells treated with Taxol have abnormal cell cycles and are arrested in the G_1 and G_2/M phases [3,5,6]. Different concentrations of Taxol can trigger distinct effects on both the cellular microtubule network and biochemical pathways [5–11]. It is known that low concentrations of Taxol (5–30 nM) alter microtubule dynamics and/or induce G_2/M cell cycle arrest, whereas high concentrations of the drug (0.2–30 μM) cause significant microtubule damage [8]. The main apoptotic mechanisms triggered at high Taxol concentrations include characteristic changes in the gene expression profile and activation of mitogen-activated protein kinases (MAPKs), Raf-1, protein tyrosine kinases (PTK), c-Jun NH(2)-terminal kinase (JNK), cyclin-dependent kinases, and caspases [12,13]. Taxol at low drug concentrations also allows apoptosis to occur, but its mechanisms are largely unknown. Recently, we reported that Taxol at concentrations of 0.1–1 μM induced apoptosis independently of Fas, TNF-α, or TRAIL death receptors, but it is dependent on FADD [14]. Furthermore, the drug induced activation of caspases-3, -6, -8, and -10, and cleaved Bcl-2, Bid, PARP, and lamin B. Moreover, Taxol-induced apoptosis was primarily through caspase-10 activation and was also partially caspase-8 dependent [14]. However, despite the release of cytochrome c from the mitochondria in Taxol-treated cells, caspase-9 was not activated. Inhibitors of caspases-3, -6, or -8 partially inhibited Taxol-induced apoptosis, while the caspase-10 inhibitor, z-AEVD-fmk, completely abrogated this process. Taxol-induced apoptosis was also associated with decreased mitochondrial membrane potential ($\Delta\Psi_m$).

In this report, we investigated the role of c-FLIP in regulating apoptosis of CCRF-HSB-2 cells following treatment with low concentrations of Taxol (≤0.05 μM). Taxol activated caspases-8 and -10, and decreased expression of the cellular FLICE-like inhibitory protein splice (c-FLIP) variants. c-FLIP has been identified as a regulator of death ligand-induced apoptosis downstream of death receptors and FADD, and exists as long [c-FLIP(L)], short [c-FLIP(S)], and [c-FLIP(R)] splice variants [15–20]. While the exact functional role of the c-FLIP variants in apoptosis remains controversial, our data clearly demonstrated that c-FLIP(L) and c-FLIP(S) variants were decreased after treatment with low concentrations of Taxol. In this work, we explored the distinct functions of the c-FLIP(L) and c-FLIP(S) variants in regulating the activity of initiator caspases-8 and -10, and their involvement in Taxol-induced apoptosis in leukemia cells transfected with the expression vectors carrying c-FLIP(L) and c-FLIP(S) in the sense orientation or c-FLIP(S) in the antisense orientation.

2. Materials and methods

2.1. Cell culture

The CCRF-HSB-2 human T-cell lymphoblastic leukemia cell line and MCF-7 human breast cancer cell line were obtained from American Type Culture Collection (ATTC, Manassas, VA). The CCRF-HSB-2 cell line was maintained in DMEM/F12 medium with 15% fetal calf serum (FCS) and 100 ng/ml each of penicillin and streptomycin (Invitrogen, Inc., Carlsbad, CA) at 37 °C in 5% CO_2. MCF-7 cell line was maintained as described for HSB-2 except 10% FCS was used. Paclitaxel, *Taxus* sp. (Taxol) (EMD Biosciences, Inc., La Jolla, CA) was used in this study and was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO). For cell culture experiments, Taxol was added so that the final concentration of DMSO did not exceed 0.1%.

2.2. Plasmids and stable transfections

To acquire the full-length coding region of c-FLIP(S), total RNA from CCRF-HSB-2 was prepared by using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Three micrograms of RNA was used in a reverse transcription reaction with Superscript III reverse transcriptase (Invitrogen) and a c-FLIP(S) gene specific antisense primer 5′-GCGCGGTACCTCACATGGAACAATTTC-CAAG-3′ as described by the manufacturer. The resulting cDNA was used as a template in PCR, along with a c-FLIP(S) specific sense primer 5′-GCGCAAGCTTATGTCTGCTGAAGT-CATCCAT -3′ and antisense primer 5′-GCGCGGTACCTCA-CATGGAACAATTTCCAAG-3′, to amplify the coding region. The full-length c-FLIP(S) cDNA fragment was purified and subcloned into pCR2.1-TOPO using a TA cloning kit (Invitrogen). The c-FLIP(S) cDNA fragment was excised and subcloned into pIRES2-EGFP (BD Biosciences, San Diego, CA) to generate

the plasmid c-FLIP(S)-EGFP. The full length coding region of c-FLIP(L) was obtained as described above, except the gene specific antisense primer 5′-GCGCGGATCCTTATGTGTAGGAGAGGATAAG-3′ was used along with a specific sense primer 5′-AAGAGTCTCAAGGATCCTTCAAATAACTTC-3′ constructed to overlap homologous c-FLIP(S) and c-FLIP(L) coding sequences. The resulting c-FLIP(L) cDNA fragment was subcloned into plasmid c-FLIP(S)-EGFP to produce the plasmid c-FLIP(L)-EGFP. Restriction digestion and automated sequence analysis of both strands confirmed that the cDNAs encoding c-FLIP(S) and c-FLIP(L) were correct.

To construct the c-FLIP(S)-AS plasmid, the full-length c-FLIP(S) fragment was excised from pCR2.1-TOPO (Invitrogen) by digesting with *Eco*RI. The c-FLIP(S) *Eco*RI fragment was subcloned into *Eco*RI digested pIRES-EGFP (BD Biosciences). Restriction digestion and automated sequence analysis of both strands confirmed that the cDNA encoding the c-FLIP(S) fragment was in the antisense orientation.

CCRF-HSB-2 cells were transfected with pIRES-EGFP, p-FLIP(L)-EGFP, pFLIP(S)-EGFP, and FLIP(S)-AS-EGFP by electroporation using the Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA) according to the manufacturer instructions. Briefly, 5×10^6 cells were harvested and rinsed three times with PBS and resuspended in 200 μl DMEM/F12 medium. Five micrograms of plasmid DNA were added to the cells and the mixture was transferred to a 0.2 mm electroporation cuvette (Bio-Rad). The CCRF-HSB-2 cells were electroporated using a pre-set square wave protocol with 140 V and a pulse length of 25 ms. The cells were collected and incubated in DMEM/F12 medium containing 15% FCS for 24 h prior to selection for transfectants by adding 1 mg/ml G418 (Invitrogen) to the growth medium. The G418 was titrated up to a final concentration of 3 mg/ml for maintenance of stable clones.

2.3. RT-PCR and real-time PCR analysis

CCRF-HSB-2 cells were harvested and 3×10^6 cells were plated in 3 ml of medium and incubated for 3 h at 37 °C. Taxol was added to a final concentration of 10 nM and the cells were incubated for an additional 24 h at 37 °C. Total RNA was prepared by using Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. Residual genomic DNA was removed by using the DNA-free kit (Ambion, Inc., Austin, TX). Three micrograms of RNA was used in a reverse transcription reaction with Superscript III reverse transcriptase (Invitrogen) and an OligodT primer (Promega, Madison, WI) according to the manufacturer instructions. To detect c-FLIP(L) and c-FLIP(S) transcripts, 2 μl of cDNA were added to a 50 μl PCR mixture containing 1× PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 10 pmole of each primer, and 1.0 unit of Platinum *Taq* DNA Polymerase (Invitrogen). The specific primers that were used are as follows: c-FLIP(L) forward 5′-AATTCAAGGCTCAGAAGCGA-3′ and reverse 5′-GGCAGAAACTCTGCTGTTCC-3′; c-FLIP(S) forward 5′-GGCCGAGGCAAGATAAGCAAGG-3′ and reverse 5′-GCGCGGTACCTCACATGGAACAATTTCCAAG-3′; β-ACTIN forward 5′-GCCCCCCTGAACCCCAAGGCCAAC-3′ and reverse 5′-CCGCTCGGCCGTGGTGGTGAAGCT-3′. The PCR cycling conditions were as follows: 1 cycle at 95 °C for 4 min, and then 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. Products were separated on a 2% agarose gel containing 0.5 μg/ml of ethidium bromide. For real-time PCR, total RNA was isolated as described above and the expression of c-FLIP(L) and c-FLIP(S) was quantified using an iCycler (Bio-Rad, Hercules, CA). SYBR Green methods were employed according to the manufacturer's protocol. The c-FLIP primers used for Real-time PCR were the same as used for the semi-quantitative RT-PCR analysis. The expression value was normalized to GAPDH. Relative gene expression was determined by assigning the control a relative value of 1.0, with all other values relative to the control.

2.4. Western blotting

CCRF-HSB-2 cells were harvested and 3×10^6 cells were plated in 3 ml of medium and incubated for 3 h at 37 °C. To analyze the effect of Taxol on c-FLIP(L) and c-FLIP(S) levels, Taxol was added to give a final concentration of 0.5, 1, 5, 10, or 50 nM and incubated for an additional 24 h at 37 °C. For all other experiments, Taxol was added to give a final concentration of 5, 10, or 50 nM and incubated for an additional 24 h at 37 °C. MCF-7 cells were harvested and 3×10^6 cells were plated in 3 ml of medium and incubated overnight at 37 °C. To analyze the effect of Taxol on c-FLIP(L) and c-FLIP(S) levels, Taxol was added at a final concentration of 10, 100, or 500 nM and incubated for an additional 48 h at 37 °C. The CCRF-HSB-2 or MCF-7 cells were harvested, rinsed 2× in cold PBS, and lysed in cold RIPA buffer (50 mM Tris–HCl [pH 8.0]), 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, and 1% protease inhibitor cocktail (Sigma) followed by centrifugation ($10{,}000 \times g$, 15 min). Protein concentration was determined by using the BCA/Cu_2SO_4 protein (Sigma) assay as described by the manufacturer. Fifty micrograms of lysate were separated on a NuPAGE 4–12% or 10% bis-Tris Gel (Invitrogen) and transferred to an Immobilon-P (Fisher Scientific, Pittsburgh, PA) membrane. Membranes were incubated in blocking buffer (PBS, 0.1% Tween 20, 5% skim milk) and then incubated first with specific antibodies in blocking buffer followed by the addition of anti-mouse immunoglobulin G (IgG) antibodies (Amersham Biosciences Corp, Piscataway, NJ). Immunoreactive proteins were visualized by using SuperSignal West Pico solutions (Pierce Biotechnology, Rockford, IL). In this study, the following primary antibodies were used: anti-β-actin clone AC-74 (Sigma); anti-c-FLIP clone NF6 (Alexis, San Diego, CA); anti-caspase-10 clone 4C1 (MBL International, Watertown MA), and anti-caspase-8 clone 1C12 (Cell Signaling Technology, Beverly MA).

2.5. Determination of apoptosis and cell death using Annexin V and propidium iodide staining

CCRF-HSB-2 cells were harvested and plated at a density of 2.5×10^5 cells/ml. After incubation for 3 h at 37 °C, Taxol was added to give a final concentration of 5, 10, or 50 nM. The cells were incubated for an additional 24 h, harvested, and stained with fluorescein isothiocyanate-labeled Annexin V (BD Biosciences) and propidium iodide (PI) according to the manufacturer's protocol. The cells were analyzed using a FACScan (BD Biosciences) flow cytometry and CellQuest software (BD Biosciences).

2.6. *Effect of the caspase inhibitors on Taxol-induced apoptosis*

The general caspase inhibitor (z-VAD-fmk), the caspase-8 inhibitor (z-IETD-fmk), and the caspase-10 inhibitor (z-AEVD-fmk) were purchased from R&D Systems, Minneapolis, MN. The inhibitors were dissolved in DMSO and added to the cultured cells so that the final concentration of DMSO was 0.1%. 2.5×10^5 CCRF-HSB-2 cells were pre-incubated with or without 100 μM of inhibitor for 3 h at 37 °C, treated with Taxol at a final concentration of 5, 10, or 50 nM, and incubated for an additional 24 h. The cells were harvested and stained with fluorescein isothiocyanate-labeled Annexin V (BD Pharmingen) and PI as described above. For Western blot analysis of cells treated with the caspase inhibitors, the experiment was executed as described above, except 3×10^6 cells per drug treatment were used.

3. Results

3.1. *Taxol-induced apoptosis of CCRF-HSB-2 cells*

One of the early events in the apoptotic process is the disruption of phospholipid asymmetry in the plasma membrane and the exposure of phosphatidylserine on the outer leaflet of the plasma membrane. Annexin V is an anticoagulant protein that binds to negatively charged phospholipids such as phosphatidylserine and is used to identify apoptotic cells. Double staining the cells with Annexin V and propidium iodide allowed us to detect early apoptosis (Annexin V-positive) and cell death (Annexin V-positive, PI-positive) by flow cytometry. Treating CCRF-HSB-2 cells with 5, 10, and 50 nM Taxol for 24 h induced cell death in 10, 15, and 21% of the cells, respectively (Fig. 1a). Next, we determined whether the induction of overall cell death after Taxol treatment was caspase-dependent. After pretreating the cells with the general caspase inhibitor, z-VAD-fmk for 3 h, followed by 5, 10, and 50 nM Taxol for an additional 24 h, cell death was induced in 8, 10, and 13% of the cells, respectively indicating that caspase-independent cell death mechanisms were also involved since cell death was increased compared to the untreated control (Fig. 1a). Furthermore, analysis of only the Annexin V-positive cells showed that caspase inhibition decreased apoptosis as compared to the Taxol only treated cells but did not completely protect the cells from Taxol's effects, indicating the early apoptotic process also involves a caspase-independent mechanism (Fig. 1b). Collectively, these data show that Taxol induces apoptosis and cell death in CCRF-HSB-2 cells by both caspase-dependent and -independent mechanisms.

3.2. *Taxol-mediated down-regulation of c-FLIP expression*

We recently reported that Taxol-induced apoptosis is FADD-dependent [14]. Moreover, by binding to FADD and/or caspases-8 and -10 at the level of the death-inducing signaling complex (DISC), c-FLIP prevents apoptosis induced by TNF family death receptors [15]. Therefore, we examined whether c-FLIP regulates Taxol-induced apoptosis in CCRF-HSB-2 cells. Immunoblot data presented in Fig. 2a show that CCRF-HSB-2 cells treated with Taxol for 24 h decreased the expression levels of both c-FLIP(L) and c-FLIP(S), and that after 10 nM Taxol treatment, both c-FLIP isoforms were reduced.

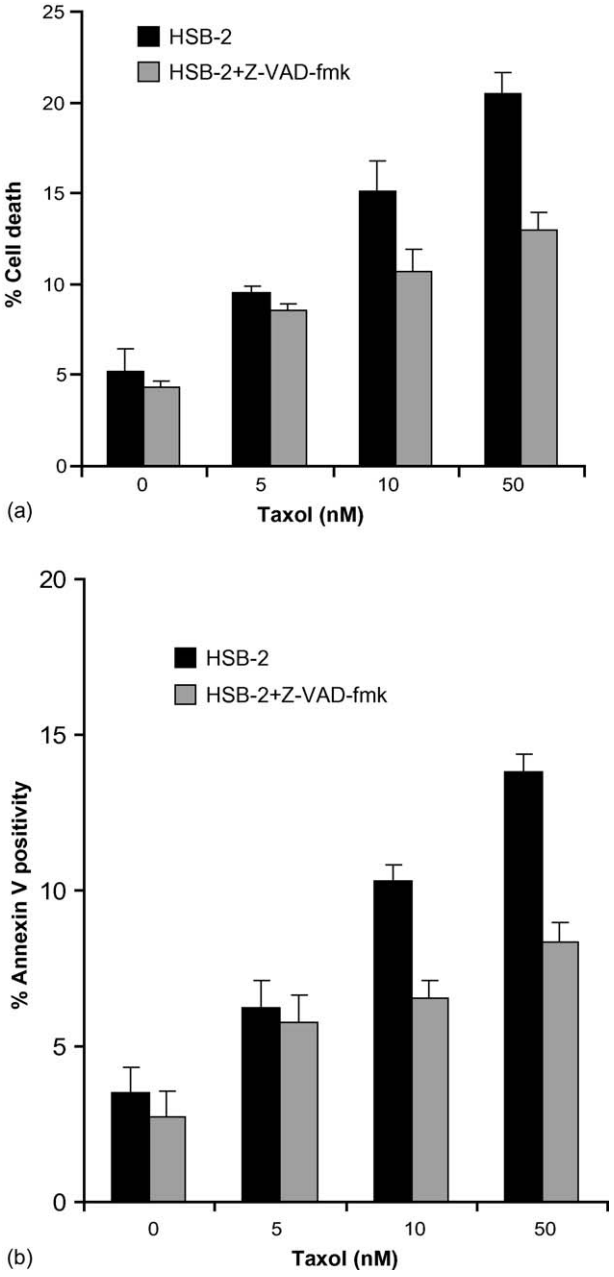

Fig. 1 – Taxol-induced apoptosis of CCRF-HSB-2 cells involves both caspase-dependent and -independent mechanisms. CCRF-HSB-2 cells were treated for 3 h with or without z-VAD-fmk, and Taxol was added at a concentration of 5, 10, or 50 nM for an additional 24 h. Cells were harvested and cell death was determined by FACS analysis as described in the materials and methods section. (a) Percentage of late apoptotic or dead cells stained with Annexin V and PI. (b) Early apoptotic cells stained with Annexin V. Compensation was executed for each experiment using untreated cells stained with Annexin V and propidium iodide. The bar graph demonstrates the mean ± S.D. of triplicate measurements.

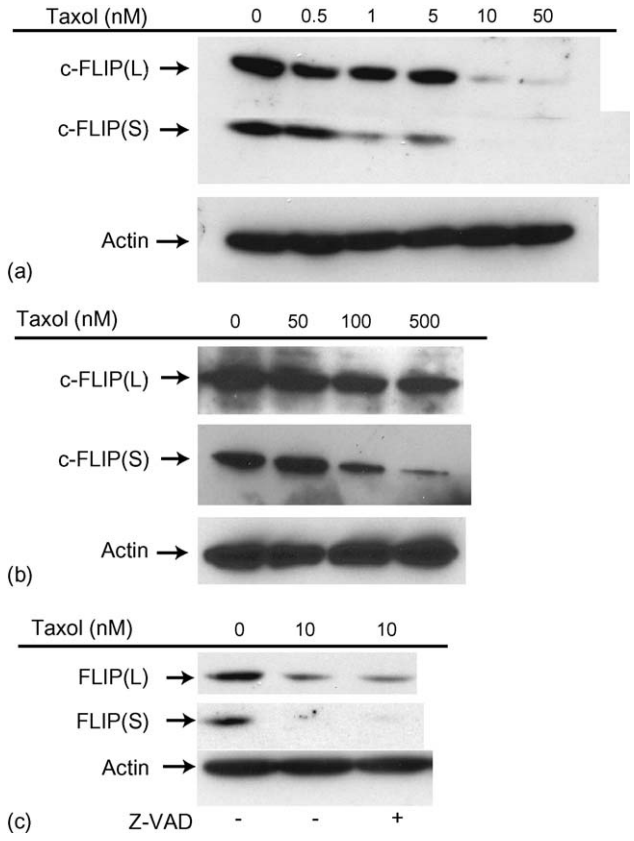

Fig. 2 – Caspase-independent down-regulation of c-FLIP(L) and c-FLIP(S) protein levels by Taxol. (a) CCRF-HSB-2 cells were treated with 0.5, 1, 5, 10, or 50 nM Taxol for 24 h. Cell lysates were subjected to immunoblot analysis using an anti-FLIP antibody. (b) MCF-7 cells were treated with 10, 100, or 500 nM Taxol for 48 h. Cell lysates were subjected to immunoblot analysis using an anti-FLIP antibody. (c) CCRF-HSB-2 cells were treated with 100 μM of z-VAD-fmk for 3 h followed by 10 nM Taxol for an additional 24 h. Immunoblot analysis was conducted on cell lysates treated with or without z-VAD-fmk using anti-c-FLIP antibodies. Equivalent loading was confirmed by reprobing the same blot with anti-β-actin.

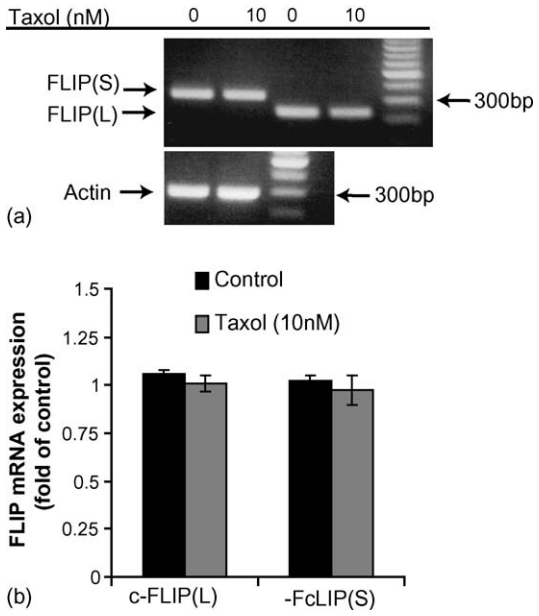

Fig. 3 – Taxol-induced decrease of c-FLIP occurs by a post-transcriptional mechanism. (a) CCRF-HSB-2 cells were treated with or without 10 nM Taxol for 24 h and the total RNA was isolated for semi-quantitative RT-PCR. Equivalent addition of RNA was confirmed by PCR analysis of β-actin. (b) CCRF-HSB-2 cells were treated with or without 10 nM Taxol for 24 h and the expression levels of c-FLIP(L) and c-FLIP(S) mRNA were quantified by real-time PCR. Values are the mean ± S.D. of three independent experiments.

Interestingly, the c-FLIP isoforms were decreased at different Taxol concentrations; c-FLIP(S) was down-regulated at a concentration of 1 nM, however, c-FLIP(L) was not affected until a concentration of 10 nM Taxol was reached (Fig. 2a). We have also found that down-regulation of c-FLIP by Taxol correlated with the Taxol concentrations required to induce apoptosis (see Fig. 1b), indicating the role of c-FLIP in regulating the apoptotic process. In fact, CCRF-HSB-2 cells treated with 10 nM Taxol show a decrease in c-FLIP(L) and c-FLIP(S) protein levels even after 1 h of treatment (data not shown). In order to conclude that a general mechanism of Taxol-triggered apoptosis is due to the down-regulation of c-FLIP isoforms, we also treated the Taxol-sensitive cell line, MCF-7, with 50, 100, and 500 nM Taxol for 48 h and performed Western blot analysis. As shown in Fig. 2b, in MCF-7 cells, c-FLIP(S) and c-FLIP(L) isoforms are down-regulated by Taxol. Thus, these data suggest that one of the mechanisms involved in the Taxol-mediated apoptosis involves a reduction of both c-FLIP isoforms. To determine if caspases were required for the Taxol-mediated decrease in c-FLIP expression, CCRF-HSB-2 cells were treated with the z-VAD-fmk inhibitor for 3 h prior to the addition of 10 nM Taxol. Inhibition of caspase activity did not alter the levels of either c-FLIP(L) or c-FLIP(S), indicating that a caspase-independent mechanism was involved in lowering c-FLIP expression (Fig. 2c).

To determine if the Taxol-mediated decrease in c-FLIP occurred at the transcriptional level, semiquantitative and quantitative RT-PCR was performed. After treating CCRF-HSB-2 cells with 10 nM Taxol for 24 h, no decrease in either c-FLIP(L) or c-FLIP(S) transcript levels were observed (Fig. 3a and b). Our data clearly showed that Taxol mediated the reduction of c-FLIP by a post-transcriptional, caspase-independent mechanism.

3.3. *Genetic manipulation of c-FLIP(L) and c-FLIP(S) regulates Taxol-induced apoptosis of CCRF-HSB-2*

To determine the functional significance of c-FLIP in Taxol-induced apoptosis, we constructed a c-FLIP(S)-AS plasmid (c-FLIP(S)-AS), as well as c-FLIP(L) and c-FLIP(S) in the sense orientation, transfected CCRF-HSB-2 cells with each of these plasmids, and examined their role in Taxol-induced apoptosis. CCRF-HSB-2 stable cell lines harboring the c-FLIP(S)-AS plasmid had a reduction in both c-FLIP(L) and c-FLIP(S) protein

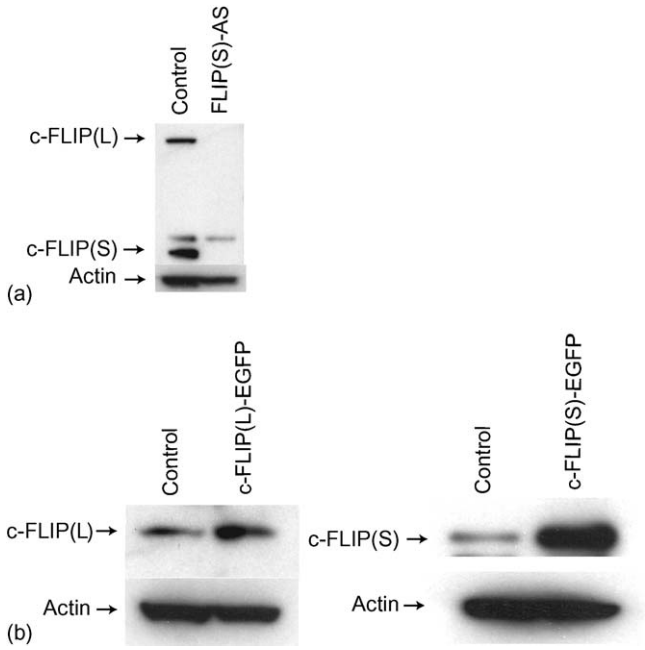

Fig. 4 – Western blot analysis of c-FLIP in c-FLIP(S)-AS, c-FLIP(L)-EGFP, and c-FLIP(S)-EGFP cell lysates. (a) Immunoblot analysis of control and c-FLIP(S)-AS cell lysates using an anti-c-FLIP antibody. (b) Immunoblot analysis of control, c-FLIP(L)-EGFP, and c-FLIP(S)-EGFP cell lysates using an anti-c-FLIP antibody. Equivalent loading was confirmed by reprobing each blot with anti-β-actin.

levels as shown by immunoblot analysis (Fig. 4a). CCRF-HSB-2 stable cell lines harboring c-FLIP(L)-EGFP or c-FLIP(S)-EGFP plasmids contained elevated levels of each c-FLIP isoform as determined by immunoblot analysis (Fig. 4b).

Treatment of the c-FLIP(S)-AS cell line with 5, 10, or 50 nM Taxol for 24 h induced cell death in 35, 37, and 45% of the cells, respectively, but in the control strain only 14, 18, and 27% increases in cell death were observed (Fig. 5a). The c-FLIP(S)-AS strain was more sensitive to Taxol leading to approximately a two-fold increase in cell death as compared to the control strain (Fig. 5a). Furthermore, treating the c-FLIP(S)-AS strain with 5 or 10 nM Taxol induced a two-fold increase in apoptosis over treated control cells, as determined by Annexin V-positive staining, validating c-FLIP's role in regulating the early steps of apoptosis (Fig. 5b). In fact, an induction in total cell death from 8 to 27% was observed after treating control cells with 50 nM Taxol, as compared to 9 to 35% in the c-FLIP(S)-AS strain after only 5 nM Taxol was used, indicating that low c-FLIP levels strongly enhance CCRF-HSB-2 sensitivity to Taxol (Fig. 5a). Following treatment of the FLIP(S)-AS strain with 50 nM Taxol, cell death occurred in 45% of the cells, while in control cells only 27% of the cells were stained with Annexin V and PI (Fig. 5a). The elevated level of dead cells in the c-FLIP(S)-AS strain following 50 nM Taxol treatment was most likely due to the absence of both c-FLIP isoforms (see Fig. 4a), while in the control strain, reduced but functional c-FLIP still modulated the cell death process. These findings also correlate with the immunoblot results that showed a Taxol-mediated reduction of c-FLIP expression (see Fig. 2a). Therefore, low c-FLIP levels increase the sensitivity of the CCRF-HSB-2 cell line to Taxol.

The CCRF-HSB-2 variants expressing either c-FLIP(L)-EGFP or c-FLIP(S)-EGFP were more resistant to Taxol-induced cell death and early apoptosis (Fig. 5c and d). Moreover, treatment of the c-FLIP(L)-EGFP or c-FLIP(S)-EGFP expressing variants with 5, 10, or 50 nM Taxol for 24 h induced cell death in 7, 10, and 12% of the cells, respectively, as compared to 11, 15, and 18% in control cells indicating that both c-FLIP isoforms suppress Taxol's effects (Fig. 5c). The expression of both c-FLIP isoforms also inhibited apoptosis as compared to control cells, as determined by Annexin V-positive staining (Fig. 5d). However, neither c-FLIP(S) nor c-FLIP(L) were able to completely inhibit Taxol's effects, which is not surprising considering that c-FLIP's primary role in regulating apoptosis is binding to and inhibiting the activation of caspases-8 and -10, and would not affect Taxol-mediated cell death or apoptosis via caspase-independent processes. In fact, our results showed that pre-treatment with the general caspase inhibitor z-VAD-fmk lowered, but did not completely inhibit cell death and apoptosis (Fig. 1a and b). Therefore, in the c-FLIP-AS strain, the absence of c-FLIP promotes maximal cell death by elevating caspase activation in addition to a caspase-independent death mechanism, therefore producing an overall enhanced level of Taxol-triggered cell death (27% compared to 45%) (Fig. 5a).

From these data, it can be concluded that c-FLIP plays a major role in inhibiting Taxol-induced apoptosis. Additionally, we showed that by decreasing c-FLIP, Taxol-triggered apoptosis was increased, but cell lines overexpressing either c-FLIP(L) or c-FLIP(S) were less sensitive to Taxol, validating c-FLIP's inhibitory role in the apoptotic process. Overall, these results showed that reducing c-FLIP expression was sufficient to sensitize the CCRF-HSB-2 cell line to Taxol-induced apoptosis. Finally, these experiments illustrate that c-FLIP provides protection to Taxol by preventing apoptotic induction, most likely by binding to and inhibiting caspases-8 and -10.

3.4. *Decreased levels of c-FLIP enhance Taxol-induced activation of caspases*

Next, we tested the activation of caspase-8 in cell lysates isolated from Taxol-treated control, c-FLIP(S)-AS, c-FLIP(L)-EGFP, and c-FLIP(S)-EGFP cells by immunoblotting. After the addition of 5, 10, and 50 nM Taxol, the caspase-8-activated bands p43/41, p28, and p18 were more abundant in the c-FLIP(S)-AS strain as compared to the control strain (Fig. 6a). Following Taxol treatment, the c-FLIP(L)-EGFP and c-FLIP(S)-EGFP cell lysates both showed a decrease in the caspase-8 activated bands p43/p41, and p18 as compared to the control cell lysates (Fig. 6b and c). These data showed that a decrease in c-FLIP induced caspase-8 activation, and that either c-FLIP(L) or c-FLIP(S) suppressed the activation of caspase-8 following Taxol treatment.

To determine if the sensitivity of the c-FLIP(S)-AS strain to Taxol was due to caspase 8 activation, the cells were treated with the caspase-8 inhibitor z-IETD-fmk for 3 h prior to adding Taxol. After treatment with 5, 10, or 50 nM of Taxol, the caspase-8 inhibitor decreased cell death of the control and c-FLIP(S)-AS strains, indicating that caspase-8 activation

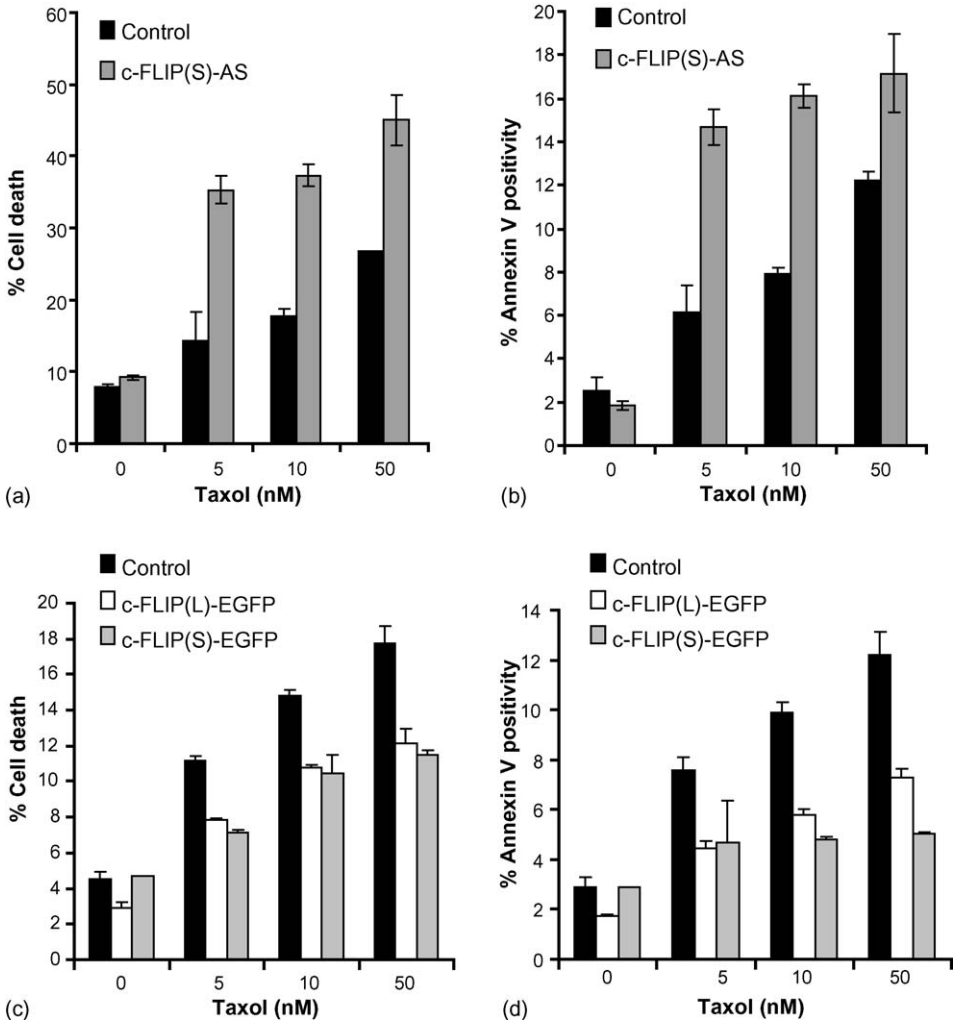

Fig. 5 – Genetic manipulation of c-FLIP(L) and c-FLIP(S) levels affect Taxol-induced apoptosis of CCRF-HSB-2 cells. Control CCRF-HSB-2 cells, c-FLIP(S)-AS, c-FLIP(L)-EGFP, and c-FLIP(S)-EGFP variants were treated with Taxol for 24 h and the level of cell death and/or apoptosis was determined by FACS analysis as described in Section 2. (a and c) Percentages of cell death were obtained by counting the cells in the lower right quandrant (Annexin V^+, PI^-) plus the upper right quandrant (Annexin V^+, PI^+). (b and d) Percentages of apoptotic cells were obtained by counting the cells in the lower right quandrant (Annexin V^+, PI^-). Compensation was executed for each experiment using untreated cells stained with Annexin V and PI. Results are representative of three independent experiments, and error bars show the standard deviation from duplicate measurements.

promotes Taxol-induced cell death in CCRF-HSB-2 (Fig. 7a). Additionally, as shown in Fig. 7b, immunoblot analysis of c-FLIP(S)-AS whole cell lysates confirmed that the caspase-8 inhibitor decreased caspase-8 activation following Taxol treatment. Furthermore, the inhibition of caspase-8 reduced cell death in the c-FLIP(S)-AS strain, which indicated that low levels of c-FLIP promote caspase-8 activation and Taxol-induced cell death. This finding correlates with previous reports showing that c-FLIP can inhibit caspase-8 activation [16,21]. However, at all concentrations of Taxol, the caspase-8 inhibitor decreased cell death of the c-FLIP-AS strain, but not to the levels observed in the control cells treated with the caspase-8 inhibitor, indicating that in addition to caspase-8, other c-FLIP-independent mechanisms were involved in the cell death process (Fig. 7a).

To determine if caspase-10 was activated following Taxol treatment, cell lysates were prepared from Taxol-treated control, c-FLIP(S)-AS, c-FLIP(L)-EGFP, and c-FLIP(S)-EGFP cells for immunoblotting. The caspase-10 activated bands p47 and p43 were increased in the c-FLIP(S)-AS strain as compared to the control strain at all Taxol concentrations (Fig. 8a). Additionally, Taxol-treated cell lysates derived from either c-FLIP(L)-EGFP or c-FLIP(S)-EGFP stable cell lines had a reduction in caspase-10 activation as compared to the control cell lysates (Fig. 8b and c). Both the caspase-10 and the caspase-8 immunoblot results showed that after Taxol treatment, c-FLIP regulated caspases-10 and -8. Furthermore, our data illustrates for the first time that the forced expression of c-FLIP(S) and c-FLIP(L) decreased the levels of procaspase-8, and in particular procaspase-10 (see Figs. 6 and 8b and c).

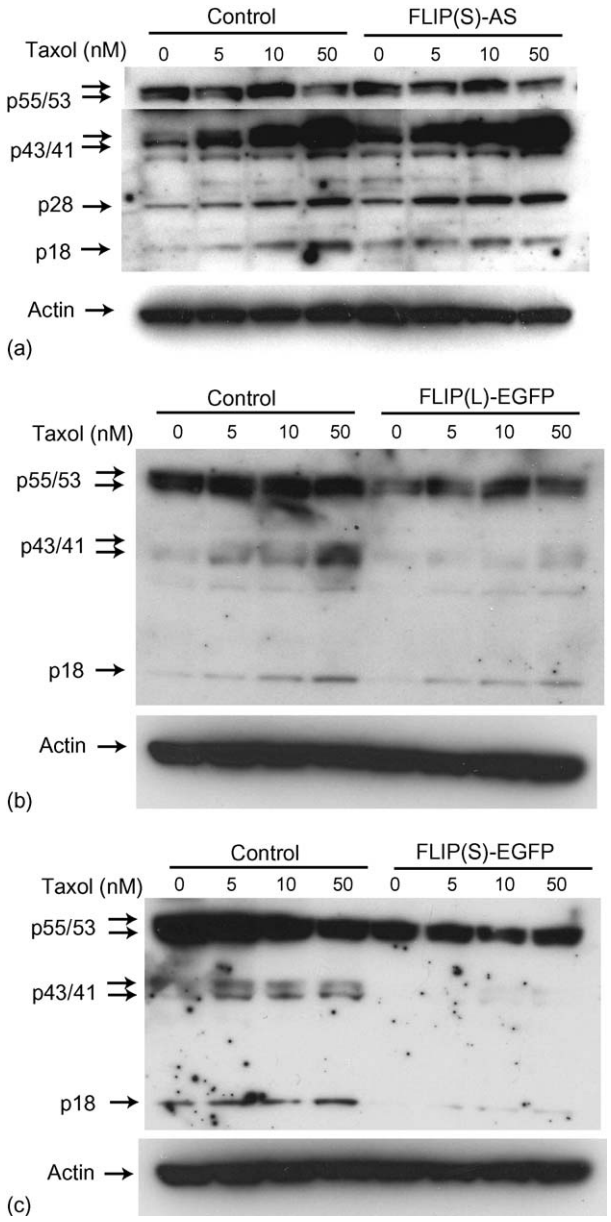

Fig. 6 – c-FLIP inhibits Taxol-induced caspase-8 activation. Whole cell lysates were prepared from control CCRF-HSB-2 cells and (a) c-FLIP(S)-AS, (b) c-FLIP(L)-EGFP, and (c) c-FLIP(S)-EGFP variants 24 h after Taxol treatment. Lysates were analyzed by SDS-PAGE using a caspase-8 specific antibody. The positions of unprocessed and fully processed forms of caspase-8 are indicated. Equivalent loading was confirmed by reprobing each blot with anti-β-actin.

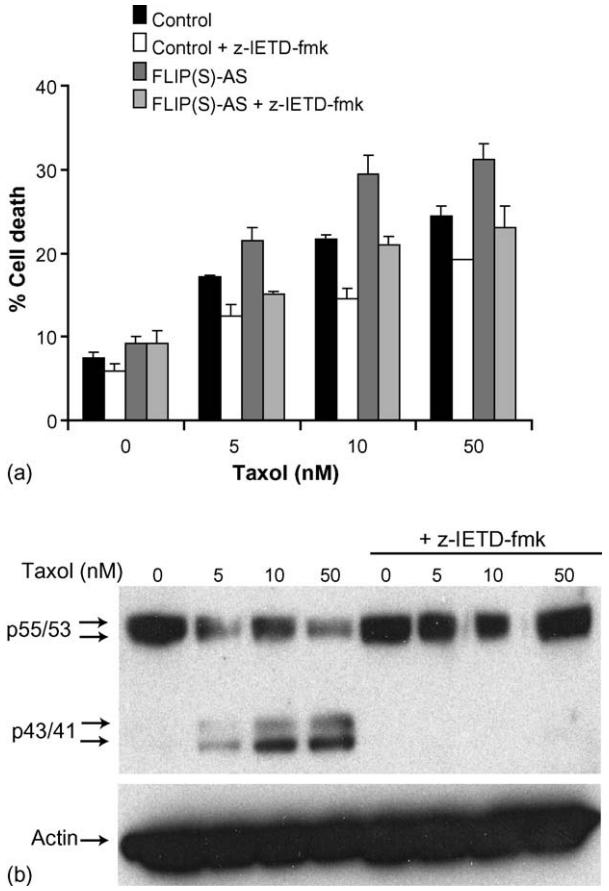

Fig. 7 – Decreased c-FLIP enhances caspase-8 activation and Taxol-induced cell death. Control or c-FLIP(S)-AS cells (2.5×10^5) were incubated with or without 100 μM of caspase-8 inhibitor (z-IETD-fmk) prior to the addition of 5, 10, or 50 nM Taxol. Cells were harvested and cell death was determined by FACS analysis. (a) Percentages were obtained by counting the cells in the lower right quandrant (Annexin V^+, PI^-) plus the upper right quandrant (Annexin V^+, PI^+). Compensation was executed for each experiment using untreated cells stained with Annexin V and PI. Results are representative of three independent experiments, and error bars show the standard deviation from duplicate measurements. (b) Immunoblot analysis of the c-FLIP(S)-AS cell lysates with or without inhibitor treatment using a caspase-8 antibody. Equivalent loading was confirmed by reprobing the blot with anti-β-actin.

Whether the c-FLIP variants directly reduce expression of these procaspases, or indirectly affect this process by activating the cytoprotective pathways known to decrease cellular sensitivity to Taxol, remains to be elucidated.

To determine if the c-FLIP(S)-AS sensitivity to Taxol was mediated by caspase-10 activation, the cells were treated with the specific caspase-10 inhibitor z-AEVD-fmk for 3 h prior to adding Taxol. After treatment with 5, 10, or 50 nM of Taxol, the caspase-10 inhibitor decreased cell death in the control more efficiently than when the caspase-8 inhibitor was used (Figs. 7 and 9a). However, it had no effect on the c-FLIP(S)-AS strain, indicating that caspase-10 activation may play a role in Taxol-induced cell death independent of c-FLIP (Fig. 9a). Immunoblot analysis of c-FLIP(S)-AS cell lysates pre-treated with the casapase-10 inhibitor showed a reduction in caspase-10 activation following Taxol treatment validating the effectiveness of the inhibitor in preventing caspase-10 activation (Fig. 9b). Therefore, even though the caspase-10 was activated more in the c-FLIP(S)-AS strain than in control cells at low Taxol concentrations, this enhanced activation does not

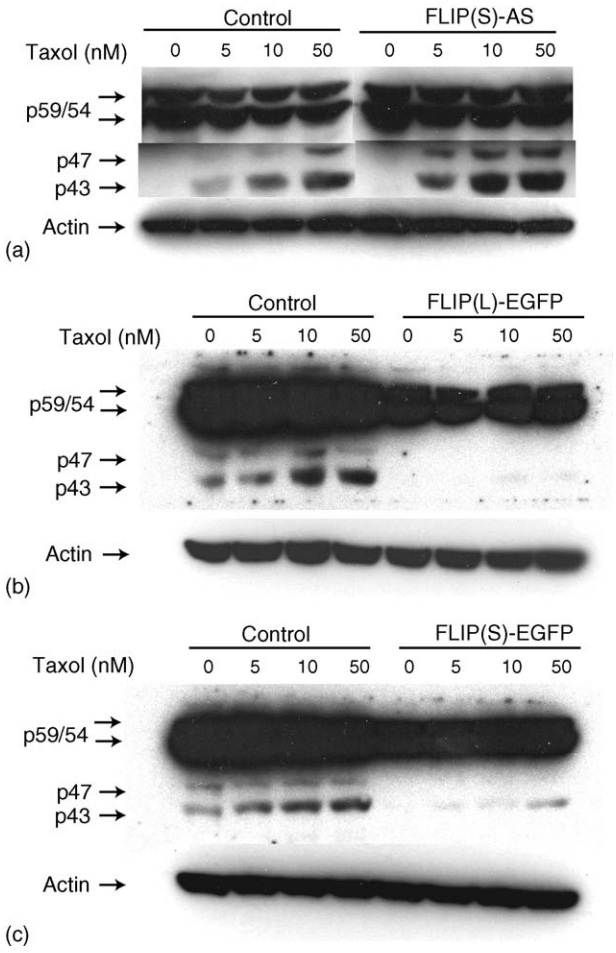

Fig. 8 – c-FLIP inhibits Taxol-induced caspase-10 activation. Whole cell lysates were prepared from control CCRF-HSB-2 cells and (a) c-FLIP(S)-AS, (b) c-FLIP(L)-EGFP, and (c) and c-FLIP(S)-EGFP variants 24 h after Taxol treatment. Lysates were analyzed by SDS-PAGE using a caspase-10 specific antibody. The positions of unprocessed and fully processed forms of caspase-10 are indicated. Equivalent loading was confirmed by reprobing each blot with anti-β-actin.

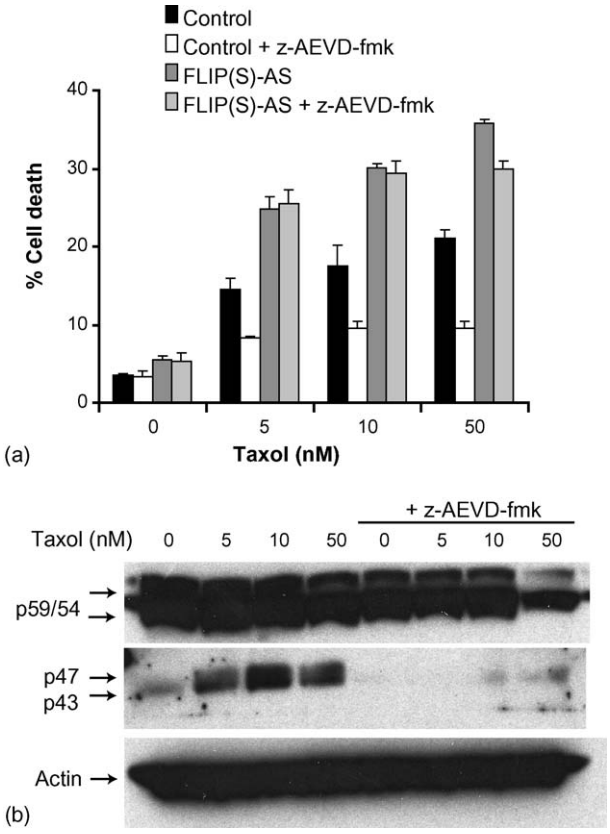

Fig. 9 – Decreased c-FLIP promotes Taxol-induced cell death of CCRF-HSB-2 independently of caspase-10. Control or c-FLIP(S)-AS cells (2.5×10^5) were incubated with or without 100 μM of caspase-10 inhibitor (z-AEVD-fmk) prior to the addition of 5, 10, or 50 nM Taxol. Cells were harvested and cell death was estimated by FACS analysis. (a) Percentages were obtained by counting the cells in the lower right quadrant (Annexin V^+, PI^-) plus the upper right quadrant (Annexin V^+, PI^+). Compensation was executed for each experiment using untreated cells stained with Annexin V and PI. Results are representative of three independent experiments, and error bars show the standard deviation from duplicate measurements. (b) Immunoblot analysis of the c-FLIP(S)-AS cell lysates with or without inhibitor treatment using a caspase-10 antibody. Equivalent loading was confirmed by reprobing the blot with anti-β-actin.

correlate with cell death induction, indicating that the increased cell death in the c-FLIP(S)-AS strain, compared to the control strain, is independent of caspase-10 activation.

4. Discussion

In this report, we demonstrated that low concentrations of Taxol decreased the expression of c-FLIP(S) and c-FLIP(L), induced caspase-8 and -10-dependent activation, triggered apoptosis and cell death in CCRF-HSB-2 leukemia cells. c-FLIP has been identified as a regulator of apoptosis triggered by death receptor ligands [18,19]. The c-FLIP(L) form contains two death effector domains (DEDs) and an inactive caspase domain composed of the p20 and p12 subunits, while c-FLIP(S) lacks the caspase domain, but contains two DEDs [22]. Additionally, c-FLIP(L) is cleaved at position Asp-376 (LEVD) in a caspase-8-dependent manner in the DISC to produce c-FLIP(L) N-terminal (p43) and C-terminal (p12) fragments [16,23,24]. However, our results showed that Taxol decreases c-FLIP(S) and c-FLIP(L) by a caspase-independent mechanism. Moreover, the Taxol-induced decrease in c-FLIP expression was regulated post-transcriptionally. These findings were in agreement with recent findings showing that peroxisome proliferator-activated receptor-gamma (PPARγ) ligands and hemin-mediated differentiation induce c-FLIP down-regulation by a transcription-independent mechanism involving protein ubiquitylation [25,26].

The functional role of the c-FLIP variants in apoptosis triggered by various stimuli remains controversial.

To determine the role of c-FLIP variants in Taxol-induced apoptosis, we first stably transfected CCRF-HSB-2 cells with an antisense c-FLIP(S) construct (c-FLIP(S)-AS) and found that inhibition of c-FLIP variants enhanced Taxol-induced apoptosis and increased the activation of caspases-8 and -10. Conversely, transfection of these cells with either c-FLIP(S) or c-FLIP(L) cDNA cloned in the sense orientation decreased Taxol-induced apoptosis. Therefore, these results revealed that both c-FLIP variants regulated Taxol-triggered cell death. Our preliminary results also showed that overexpression of c-FLIP(S) in MCF-7 breast cancer cells very significantly decreases apoptosis induced by 5–50 nM (data not shown). Experiments to determine whether c-FLIP(L) also reduces Taxol-triggered apoptosis in MCF-7 are underway. In recently published reports, c-FLIP has been shown to interfere with TNF-α, FasL, and TRAIL-induced signaling pathways by binding to FADD and/or caspase-8 in a ligand-dependent fashion, which in turn prevents the formation of DISC and subsequent activation of the caspase cascade [19,22,27–29]. However, it appears that under certain conditions, the binding of c-FLIP to FADD and caspase-8 is not sufficient for its anti-apoptotic function [30,31]. Moreover, studies using c-FLIP-deficient mice support the dual function of c-FLIP and confirm a role for c-FLIP in FasL and TNF-α-induced apoptosis, and reveal that c-FLIP has a similar function to caspase-8 in heart development [32]. Our results revealed that both c-FLIP(S) and c-FLIP(L) decreased Taxol-induced cell death. However, previous studies have shown that each of the c-FLIP variants may have a distinct functional role in cell death [19,24,33]. For example, while c-FLIP(S) has been shown to inhibit TRAIL-induced DISC formation and apoptosis [34,35], c-FLIP(L) has dual functions whereby it inhibits Fas-induced caspase-8 activation when it is highly expressed, but enhances caspase-8 activation when its expression is low [24,33,36,37]. Interestingly, recent in vitro studies have also demonstrated that c-FLIP(L) activates caspases-8 and -10 by forming heterodimeric enzyme molecules with substrate specificity and catalytic activity indistinguishable from caspase-8 homodimers [38]. Furthermore, a recent study using small interfering RNA (siRNA) specific for c-FLIP(L) showed that decreasing c-FLIP(L) induced caspase-8 activation, indicating that endogenous c-FLIP(L) primarily plays an inhibitory role in TRAIL-induced death receptor-mediated apoptosis [21]. These results are consistent with our data, that inhibition of c-FLIP expression by c-FLIP(S)-AS increases Taxol-induced caspase-8 and -10 activation. Moreover, an inhibitor of caspase-8 decreased cell death induced by Taxol in c-FLIP(S)-AS transfected cells, indicating that the interaction of c-FLIP with caspase-8 decreases activation of this caspase and reduces cell death. Our results are in agreement with a recently published report showing that downregulation of c-FLIP sensitizes cancer cells to oxaliplatin, 5-FU, and CPT-11-induced apoptosis [39].

Derived from the results of this study, we have proposed roles of c-FLIP in Taxol-induced apoptosis in CCRF-HSB-2 cells. First, we have shown that Taxol-induced apoptosis in CCRF-HSB-2 cells occurs by caspase-dependent and -independent pathways. Second, the Taxol-mediated decrease in c-FLIP leads to caspases-8 and -10 activation and induction of cell death. Third, low levels of c-FLIP in the antisense variant promote Taxol-mediated caspase-8 activation and cell death in CCRF-HSB-2 cells. Fourth, high levels of c-FLIP in the c-FLIP-sense expressing variants decreased the Taxol-mediated activation of caspases-8 and -10, thereby protecting the CCRF-HSB-2 cells from cell death. Fifth, Taxol promoted apoptosis even in the presence of elevated levels of c-FLIP in the c-FLIP-sense expressing variants due to caspase-independent mechanisms. The results from this study demonstrate the expression levels of c-FLIP isotypes are important regulators of Taxol-induced apoptosis in CCRF-HSB-2 cells. Based on this study, development of agents that target and decrease c-FLIP may prove to be a clinically relevant method to enhance sensitivity of certain cancers to Taxol's death-inducing signals.

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