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4-(4-Chloro-2-methylphenoxy)-*N*-hydroxybutanamide (CMH) targets mRNA of the c-FLIP variants and induces apoptosis in MCF-7 human breast cancer cells

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Abstract

Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) is a major resistance factor for the tumor necrosis factor-related apoptosis-inducing ligand TRAIL and in drug resistance in human malignancies. c-FLIP is an antagonist of caspases-8 and -10, which inhibits apoptosis and is expressed as long (c-FLIP$_L$) and short (c-FLIP$_S$) splice forms. c-FLIP is often overexpressed in various human cancers, including breast cancer. Several studies have shown that silencing c-FLIP by specific siRNAs sensitizes cancer cells to TRAIL and anticancer agents. However, systemic use of siRNA as a therapeutic agent is not practical at present. In order to reduce or inhibit c-FLIP expression, small molecules are needed to allow targeting c-FLIP without inhibiting caspases-8 and -10. We used a small molecule inhibitor of c-FLIP, 4-(4-chloro-2-methylphenoxy)-*N*-hydroxybutanamide (CMH), and show that CMH, but not its inactive analog, downregulated c-FLIP$_L$ and c-FLIP$_S$ mRNA and protein levels, caused poly(ADP-ribose) polymerase (PARP) degradation, reduced cell survival, and induced apoptosis in MCF-7 breast cancer cells. These results revealed that c-FLIP is a critical apoptosis regulator that can serve as a target for small molecule inhibitors that downregulate its expression and serve as effective targeted therapeutics against breast cancer cells.

Keywords

c-FLIP; Breast cancer; Apoptosis; 4-(4-Chloro-2-methylphenoxy)-*N*-hydroxybutanamide (CMH); Caspases; Death receptors

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Introduction

A key inhibitor of death receptor signaling is c-FLIP, which is expressed in various cancers. c-FLIP is expressed as 55-kDa long (c-$FLIP_L$) and 26-kDa short (c-$FLIP_S$) splice forms and is an antagonist of caspases-8 and -10 that inhibits apoptosis downstream of the death receptors TNF-receptor 1, Fas, DR4, DR5, and FADD [1–3]. Moreover, c-FLIP expression is associated with enhanced tumorgenicity and poor clinical outcome in various cancers because it plays a major role in chemotherapeutic drug and TRAIL resistance [3–10]. Previous studies have revealed that high c-FLIP expression is associated with unresponsive tumors, and downregulating its expression should be considered for death receptor targeted and chemotherapy [3, 11–13]. c-FLIP isoforms contain two N-terminal DED domains, but only c-$FLIP_L$ contains a C-terminal caspase domain with structural similarity to caspase-8. In the caspase-like domain of c-$FLIP_L$, the catalytically active cysteine is replaced by tyrosine, rendering the molecule proteolytically inactive [14]. c-FLIP isoforms are recruited to the DISC by their DED domains and compete with the initiator caspases for FADD binding sites [3, 10, 15]. c-$FLIP_S$ inhibits death signaling at the DISC by inhibiting caspase-8 and -10 activation [3, 16]. At high concentration in the cells, the function of c-$FLIP_L$ at the DISC can be anti-apoptotic like c-$FLIP_S$, but at low levels it acts pro-apoptotically by directly heterodimerizing and activating caspase-8 [17–21]. The pro-apoptotic function of c-FLIP is supported by studies showing that c-FLIP-deficient mice die early in embryonic development due to heart failure, which is a defect also seen in caspase-8- and FADD-deficient mice [22,–23]. However, many studies in various cancer types have found that the role of c-$FLIP_L$ is generally anti-apoptotic in cancer cells. Importantly, we [24] and others [12, 25, 26] have shown that silencing of c-FLIP expression using small interfering RNAs (siRNAs) promotes spontaneous apoptosis in breast cancer, colorectal, lung, and lymphoma cancer cell lines, and augments TRAIL-, anti-DR5 monoclonal antibody-, and drug-induced apoptosis in various cancer cell types [12, 25–29]. We recently discovered that c-$FLIP_L$ interacts with DR5, FADD, and caspase-8 forming an apoptotic inhibitory complex (AIC) in MCF-7 breast cancer cells [24]. Moreover, silencing the c-FLIP gene by a specific siRNA leads to death ligand-independent but DR5-, FADD-, and caspases-8- and -9-dependent apoptosis in these cells. Furthermore, we showed that the knockdown of c-FLIP expression triggers spontaneous apoptosis by activating both the death receptor and mitochondrial pathways and inhibiting breast cancer cell proliferation [24]. Moreover, a recent report identified a checkpoint of the autophagy pathway where cellular and viral FLIPs could limit the Atg3-mediated step of LC3 ubiquitin-like protein conjugation to regulate autophagosome biogenesis. Furthermore, the FLIP-derived short peptides induced growth inhibition and cell death with autophagy by binding to and effectively suppressing Atg3-FLIP interaction, representing new agents as potential anti-cancer therapies [30].

In addition to c-FLIP's key role in preventing apoptosis, c-FLIP variants have also been shown to be involved in proliferation, cell cycle progression, and carcinogenesis. The overexpression of c-$FLIP_L$ inhibited the proteosomal degradation of β-catenin, and the elevated β-catenin levels were shown to either promote Wnt signaling or induce cyclin D expression, leading to the proliferation and cell cycle progression of cancer cells [31, 32]. In

both studies, the c-FLIP/β-catenin signals contributing to cell growth were reversed by the selective silencing of c-FLIP expression. Recent results also suggest a role for nuclear c-$FLIP_L$ in modulating Wnt signaling [33]. Caspase-8-dependent cleavage of c-FLIP has been shown to produce an N-terminal p22 fragment that directly induced NFκB activation and promoted the proliferation of lymphocytic cells [34]. Furthermore, several studies have shown that c-FLIP overexpression can promote carcinogenesis and aggressiveness of endometrial and cervical cancers [3, 35–38]. These studies highlight the functional importance of c-FLIP in the proliferation of cancer cells.

Inhibition of c-FLIP either by compounds that degrade it or c-FLIP-specific small interfering RNA (siRNA) sensitizes a wide range of cancer cell types to TNF-related apoptosis-inducing ligand (TRAIL) and chemotherapy-induced apoptosis [3, 11–13, 27, 39]. We recently reported that an apoptotic inhibitory complex (AIC) comprised of DR5, FADD, caspase-8, and c-$FLIP_L$ exists in MCF-7 cells, and the absence of c-$FLIP_L$ from this complex induces ligand-independent caspase-8 activation in the death-inducing signaling complex (DISC), leading to apoptosis [24]. These results clearly show that c-FLIP prevents death signaling in MCF-7 cells. Similarly, ectopic expression of c-FLIP variants decreased apoptosis caused by anticancer agents [3, 12, 13, 16], indicating that over-expression of these proteins may cause resistance to multiple anticancer drugs. Therefore, c-FLIP is a critical apoptosis regulator that can serve as a target for small molecule inhibitors that downregulate its expression and serve as effective targeted therapeutics for cancer treatment. In oder to support this hypothesis, our in vivo results showed that injecting liposomal complexes of c-FLIP-specific siRNA into MCF-7 xenografts eliminated the neoplastic cells without affecting the normal stromal and fibroblastic cells [28].

There does not appear to be a “handle” to inhibit c-FLIP function with small molecule ligands since as mentioned above, c-FLIP has significant structural similarity to caspase-8 [3]. This resemblance with caspase-8 makes c-FLIP protein a very difficult target for drugs to inhibit its function, since small molecules capable of blocking c-FLIP’s recruitment to the DISC could also inhibit the recruitment of caspase-8 and as a result inhibit apoptosis. Therefore, to reduce or inhibit c-FLIP expression, small molecules which target c-FLIP without inhibiting caspases-8 and -10 are needed. Employing a high-throughput chemical screening strategy, a small molecule inhibitor of c-FLIP, 4-(4-chloro-2-methylphenoxy)-*N*-hydroxybutanamide (CMH) (5809354), that downregulates c-FLIP mRNA expression has been identified [40, 41]. Our results show that CMH, but not its inactive analog 4-(4-chloro-2-methylphenoxy)-*N*-(3-ethoxypropyl) butanamide (CMB) (6094911), downregulates c-$FLIP_L$ and c-$FLIP_S$ mRNA and protein levels in MCF-7 cells, caused PARP degradation, reduced cell survival, and induced apoptosis.

Materials and methods

Cell culture, materials, reagents and antibodies

The MCF-7 human breast cancer cell lines were obtained from American Type Culture Collection (ATTC, Manassas, VA). MCF-7 human breast cancer cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 100 ng/ml each of penicillin and streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2. The MCF-7 multidrug

resistant variant MCF-7/ADR20 cells were selected for resistance to increasing concentrations of doxorubicin (DOX) in the growth medium. These cells were maintained in 10 nM DOX and kept in the growth medium without DOX 1–2 weeks before performing experiments.

4-(4-Chloro-2-methylphenoxy)-*N*-hydroxybutanamide (CMH) (5809354) and its inactive analog 4-(4-chloro-2-methylphenoxy)-*N*-(3-ethoxypropyl) butanamide (CMB) (6094911) were purchased from ChemBridge Corporation (San Diego, CA). Stock solutions (50 mM) of these agents were prepared in dimethyl sulfoxide (DMSO) and stored at 4°C in polystyrene tubes. The final concentration of DMSO in growth medium for survival and apoptosis assays was less than 0.05%.

The following primary antibodies were used: anti-c-FLIP clone G-11 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-FLIP clone Dave-2 (ProSci, Poway, CA), anti-DR5 clone B-D37 (IgG_{2b}), anti-Bcl-2 antibody, anti-PARP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-DR5 (Cell Signaling Technology, Danvers, MA), anticaspase-8 clone 3-1-9 (BD Biosciences, San Jose, CA), and anti-*β*-actin clone AC-74 (Sigma-Aldrich, St. Louis, MO).

Western blotting

Cells were harvested, rinsed in cold PBS, and lysed in an M-PER (Pierce Biotechnology, Rockford, IL) containing 1% protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined using the BCA/Cu_2SO_4 protein assay (Sigma-Aldrich) as described by the manufacturer. Protein lysates (70 μg) were separated on a 10% Bis–Tris gel (Invitrogen) and transferred to an Immobilon-P membrane (Fisher Scientific, Pittsburgh, PA). Membranes were incubated in blocking buffer (PBS, 0.05% Tween 20, 5% skim milk) with specific antibodies, followed by the addition of horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Piscataway, NJ). For detecting the anti-c-FLIP clone Dave-2 antibody, a horseradish peroxidase-conjugated goat anti-rat secondary antibody was used (Santa Cruz Biotechnology).

Detection of apoptosis by DAPI staining

DAPI staining was performed on untreated and CMH-treated MCF-7 cells as described previously (42). Briefly, prior to staining, the cells were fixed with 4% paraformaldehyde for 30 min at RT. After washing with PBS by centrifugation at 650×*g*, DAPI was added to the fixed cells for 5 min, and then the cells were examined by fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of nuclei. A minimum of 300 cells were counted for each treatment, and the percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted × 100. The DAPI staining experiments were performed in triplicate.

Determination of apoptosis and cell death using annexin V and propidium iodide staining

Cells were plated at a density of 2×10^5 cells/ml and treated with 30–100 μM CMH for 48 h, harvested, and stained with fluorescein isothiocyanate-labeled annexin V (BD Biosciences) and propidium iodide according to the manufacturer's protocol. Cells were

analyzed using FAC-Scan (BD Biosciences) flow cytometer and CellQuest software (BD Biosciences). Apoptotic and necrotic cells were analyzed by quadrant statistics on the propidium iodide-positive, annexin V-positive, and the propidium iodide-negative and annexin V-positive cells, respectively.

Effects of the general caspase inhibitor z-VAD-fmk on CMH-induced apoptosis

MCF-7 cells (2×10^4/well in 8-well glass slide) were pretreated with or without 100 μM z-VAD-fmk for 2 h. After that cells were grown in the presence of 10–100 μM PMH at 37°C for 48 h. Numbers of apoptotic cells in the z-VAD-fmk treated and control untreated cells were determined by DAPI staining as described.

RNA isolation and RT-PCR analysis

Total RNA from treated and untreated MCF-7 cells was isolated by Tri Reagent TR-118 (Molecular Research Center, Cincinnati, OH) as described by the manufacturer. One microgram of total RNA was used in reverse transcription reactions with M-MLV reverse transcriptase and oligo (dT) 15 primer (Promega, Madison, WI) as described by the manufacturer. Two microliters of the resulting total cDNA were then used as the template in PCR to measure the mRNA level of interest by using following primers: for c-$FLIP_L$ forward, 5′-GCTGAAGTTATCCATCAGGT-3′; reverse, 5′-CATACTGAGATGCAAGAATT-3′; a 840-bp band was produced. For c-$FLIP_S$, the forward primer was the same as c-$FLIP_L$; reverse, 5′-GATCAGGACAATGGGCATAG-3′; a 662-bp band was produced. For β-actin, the forward primer was 5′-CAGAGCAAGAGAGGCATCCT-3′; reverse 5′-TTGAAGGTCTCAAACATGAT-3′; these produce a 200-bp band. The reactions were performed at 94°C for denaturation, 58°C for annealing, and 72°C for extension for 30 cycles. β-Actin mRNA levels were used as internal controls. The amplified fragments were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Statistical analysis

Data are expressed as the mean ± standard deviation (SE). The statistical significance of intergroup differences was determined using Student's *t* test. *P* values <0.05 were considered statistically significant.

Results

CMH induces robust apoptosis

We used the small molecule inhibitor of c-FLIP, CMH, and showed that CMH, but not its inactive analog CMB (Fig. 1a), robustly reduces cell survival, but the inactive analog had little effect on cell survival (Fig. 1b). The concentration of the drug that reduced cell survival by 50% (IC_{50} value) in MCF-7 cells was obtained from the CMH dose–response curves using methylene blue cytotoxicity assay after 72-h treatment with increasing concentrations of the drug; the IC_{50} was approximately 30 μM. We also determined whether CMH inhibits cell survival in MCF-7/ADR20, a drug resistant variant of MCF-7 which expresses 20-, 2.5-, and 1.9-fold resistance to DOX, Taxol, and vincristine, respectively. The data shown in Fig. 1c demonstrate that CMH had even a greater inhibitory effect on cell

survival in these drug resistant cells. The IC_{50} value for 72 h treatment with CMH for MCF-7/ADR20 was 10 μM.

Next, we determined the levels of CMH-induced apoptosis in MCF-7 cells by DAPI staining. Apoptotic cells were identified by condensation and fragmentation of nuclei. The data shown in Fig. 2a clearly show CMH induced apoptosis visualized by DAPI staining of MCF-7 cells treated with 10–100 μM CMH. Quantitation of apoptosis assessed by DAPI staining revealed that treating the cells with 10–100 μM for 48 h induced 5–28% apoptosis, compared to untreated MCF-7 cells (Fig. 2a). Similar results were obtained when apoptosis was quantified by annexin V binding assay (Fig. 2b). Moreover, treating the cells with 100 μM of the general caspase inhibitor z-VAD-fmk for 48 h significantly inhibited CMH-induced apoptosis as shown in Fig. 3A and B.

CMH inhibits mRNA expression of c-FLIP$_L$ and c-FLIP$_S$ variants

In order to determine if the CMH-mediated decrease in c-FLIP variant expression occurred at the mRNA level, semiquantitative RT-PCR was performed as we previously described (16). After treating MCF-7 cells with 10–100 μM CMH for 72 h, a dose-dependent decrease in c-FLIP$_S$ transcript level was observed (Fig. 4a–c). Moreover, treatment with 50 and 100 μM CMH for 72 h very effectively decreased the c-FLIP$_L$ transcript level (Fig. 3A–C). Our data clearly showed that CMH mediated the reduction of both c-FLIP variants at the mRNA level.

The molecular mechanism by which CMH downregulates c-FLIP$_L$ and c-FLIP$_S$ mRNA in MCF-7 cells is not known. Therefore, we investigated whether CMH-induced downregulation of c-FLIP$_L$ and c-FLIP$_S$ occurs at the transcription level or by altering the stability of their mRNA. We treated the cells with the transcription inhibitor actinomycin D, or actinomycin D plus CMH, as described in the legend of Fig. 5a and b. In order to suppress apoptosis while inhibiting mRNA expression with actinomycin D or actinomycin D plus CMH, we pre-treated the cells with 100 μM of the general caspase inhibitor z-VAD-fmk in DMSO for 2 h and collected them after 6, 12, and 22 h of treatment. RNA from these samples was then prepared, and semiquantitative RT-PCR analysis of c-FLIP$_L$ and c-FLIP$_S$ mRNA expression was carried out as previously described [16]. Our mRNA analysis showed that actinomycin D treatment inhibited c-FLIP$_L$ and c-FLIP$_S$ mRNA expression by about 50% after 22 h treatment. Further-more, mRNA levels in the cells treated with actinomycin D plus CMH were similar to the level of mRNA in the cells treated with actinomycin D alone (Fig. 5a, b). These data and the results shown in Fig. 4a indicate that CMH inhibits c-FLIP$_L$ and c-FLIP$_S$ mRNA expression at the transcription level and does not affect c-FLIP$_L$ mRNA stability.

CMH inhibits expression of c-FLIP$_L$ and c-FLIP$_S$ variants at the protein level

The results of Western blot analysis corroborated our RT-PCR data and showed that treating MCF-7 cells with 10–100 μM CMH for 72 h reduced levels c-FLIP$_S$ and c-FLIP$_L$ in MCF-7 cells (Fig. 6a). In order to determine whether CMH only affects c-FLIP variants or it inhibits expression of another anti-apoptotic protein, we treated the cells with 10–100 μM CMH for 72 h and performed Western blotting to detect BCL-2 expression. These results were

consistent with degradation of poly(ADP-ribose) polymerase (PARP) as shown in Fig. 6b. The results in Fig. 6b clearly show that CMH treatment did not affect the expression of BCL-2.

Discussion

The overexpression of c-FLIP variants in tumor cells is a determinant of resistance to death ligands such as TRAIL and many chemotherapeutic agents in variety of tumors [3]. Moreover, silencing c-FLIP variants with specific siRNAs has sensitized various resistant tumor cells from different types of cancer to these agents [18, 19, 26, 27, 43]. However, in vivo use of siRNA as a systemic therapeutic agent is not feasible because of the lack of appropriate delivery vehicles into the cells. Therefore, agents causing degradation of c-FLIP at the protein level or therapeutics that directly target c-FLIP mRNA are potentially useful modalities for treating tumors resistant to cytokines and chemotherapeutic agents. Moreover, our previous results [28] and the current understanding of c-FLIP action in normal tissues [15, 44, 45] support the notion that c-FLIP-targeted cancer therapy will be well tolerated. However, it is not possible to inhibit c-FLIP function with small molecule ligands, since the cytoprotective DISC binding is mediated by highly conserved DEDs which function by homotypic binding. However, a small molecule CMH (5809354) that induces c-FLIP downregulation and sensitize neoplastic cells to apoptosis induction by the cytokine TRAIL have been identified in global, unbiased chemical screens [40]. CMH causes anoikis in PPC-1 prostate cancer cells cultured in suspension but not in adherent cultures [41]. In order to downregulate c-FLIP variants expression at the mRNA level and trigger apoptosis in MCF-7 and MCF-7/ADR20 cells, we used CMH, which has been shown to sensitize cells to Fas and mediated anoikis [41].

We found that CMH decreased c-$FLIP_L$ and c-$FLIP_S$ mRNA and protein expression and thereby sensitized MCF-7 and MCF-7/ADR20 cells to apoptosis. A previous report showed that CMH was only able to cause anoikis in PPC-1 prostate cancer cells cultured in suspension and did not affect the adherent cells [41]. However, our previous data using c-$FLIP_L$-specific siRNA [24] and the data shown here using CMH demonstrated that these agents induce apoptosis in adherent cells as well. In this study, we also investigated mechanism by which CMH downregulates c-$FLIP_L$ and c-$FLIP_S$ at the mRNA level. Our data suggest that CMH decreased transcription of the c-$FLIP_L$ and c-$FLIP_S$ mRNA.

We also investigated the apoptosis mechanism by which decreased the level of c-$FLIP_L$ and c-$FLIP_S$ sensitized cells. We found that CMH at lower concentrations (10–30 μM) caused little inhibition of the c-FLIP variants while it decreased cell survival and caused cell death. Moreover, CMH triggered more cell death in the resistant variant compared to sensitive MCF-7 cells at these concentrations. However, CMH-induced cell death occured via a caspase 8-dependent mechanism only at 100 μM. Moreover, PARP degradation was observed at 50 and 100 μM CMH treatment. Our data collectively suggest that at 10–30 μM, CMH induced inhibition of cell survival by a mechanism(s) independent of c-FLIP variants expression.

To our knowledge, this study is the first to show that (a) malignant breast cancer cells can undergo apoptosis when expression of c-FLIP$_L$ and c-FLIP$_S$ variants is decreased by the small molecule inhibitor CMH and (b) CMH downregulates c-FLIP$_L$ and c-FLIP$_S$ at the transcriptional level and by decreasing the stability of RNA, respectively. In summary, our data suggest that downregulation of c-FLIP$_L$ and c-FLIP$_S$ by the small molecule inhibitor CMH causes inhibition of cell survival and triggers cell death. Since c-FLIP variants are master regulators of cell survival and apoptosis, as well contributing to cell proliferation and metastasis, inhibiting the expression of c-FLIP variants by small molecule inhibitors such as CMH may provide a useful strategy to treat malignancies.

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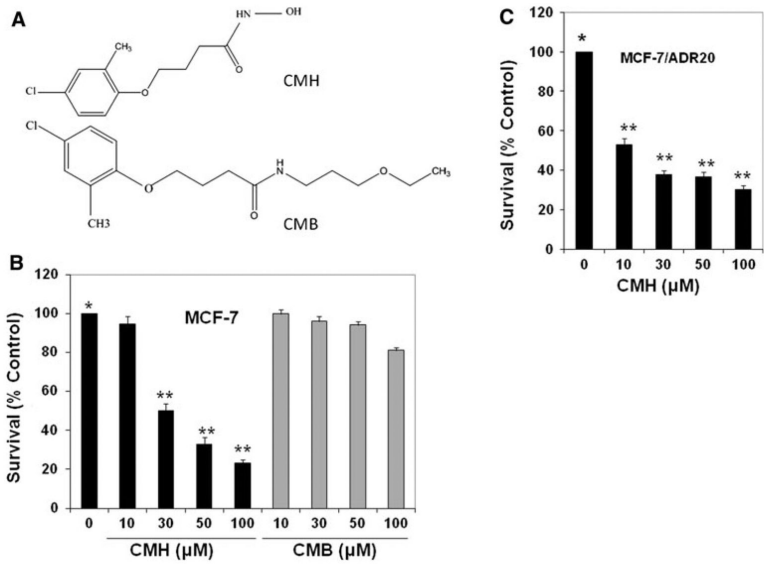

Fig. 1.

CMH but not its inactive analog (CMB) robustly inhibited cell survival in MCF-7 breast cancer cell line. **a** Chemical structures of 4-(4-chloro-2-methylphenoxy) *N*-hydroxybutanamide (CMH) and its inactive analog 4-(4-chloro-2-methylphenoxy) *N*-(3-ethoxypropyl) butanamide (CMB). **b** MCF-7 or **c** MCF-7/ADR20 cells were treated with 10–100 μM of CMH or its inactive analog CMB for 72 h. After harvesting the cells, survival was determined by cell survival assay. For each treatment, 1×10^4 cells were seeded in a 96-well plate and treated with or without CMH. Cells were stained with 1% methylene blue in 50% methanol. After the plates were washed by immersing in dH_2O, and excess of water was removed the dye was dissolved in 100 μl of 0.5 M HCl. Absorbance was determined by an automated scanning photometer at a wavelength of 630 nm. The results were obtained from triplicate experiments. Percent inhibitions of cell survival by various CMH concentrations compared to the control untreated cells (0) ± SD are shown ($P < 0.001$)

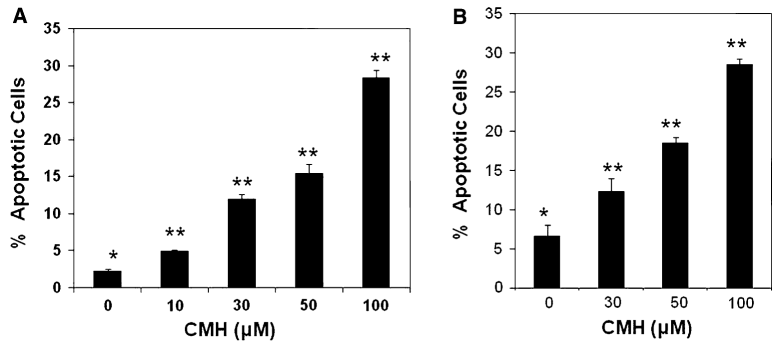

Fig. 2.

CMH triggered dose-dependent apoptosis in MCF-7 breast cancer cells. **a** MCF-7 cells were treated with 10–100 μM of CMH for 48 h and apoptotic cell death was determined by DAPI staining as previously described [42]. Apoptotic cells were identified by condensation and fragmentation of nuclei. A minimum of 300 cells were counted for each treatment, and the percentage of apoptotic cells was calculated. **b** Apoptosis was determined by FACScan analysis as described under “Materials and methods” section. Compensation was executed for each experiment using untreated cells stained with Annexin V and propidium iodide. Experiments were performed in triplicate. Error bars show SD from triplicate measurements ($P < 0.005$)

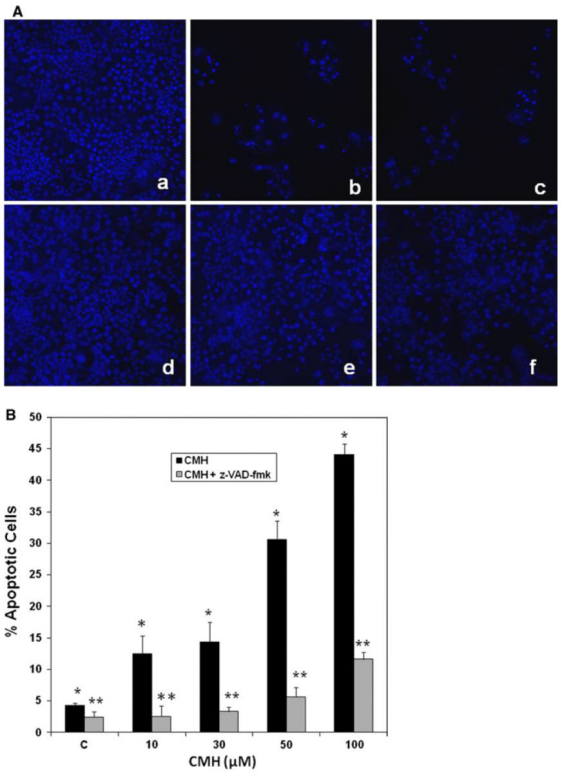

Fig. 3.

CMH triggered caspase-dependent apoptosis in MCF-7 breast cancer cells. DAPI staining was carried out as described in “Materials and methods” section. **A** fluorescence microscopy of untreated (a), 50 and 100 μM CMH treated (b, c), MCF-7 cells compared with z-VAD-fmk pre-treated (d), plus 50 and 100 μM CMH treated (e, f) cells for 48 h. **B** Apoptosis was quantified by counting fragmented nuclei and condensed stained among 300 cells from the control, z-VAD-fmk, and cells treated with 10–100 μM CMH. Data are average of triplicate experiments (** $P < 0.01$)

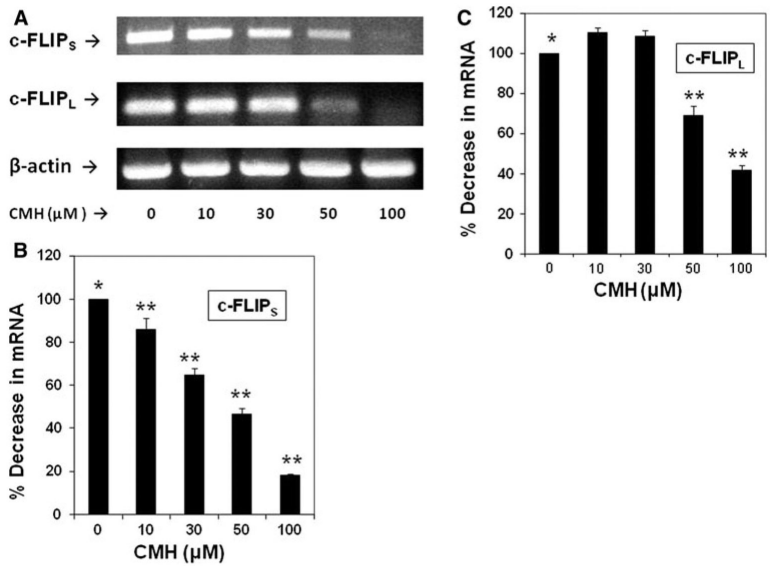

Fig. 4.

CMH-induced decrease of c-FLIP$_S$ and c-FLIP$_L$ levels occurs at the mRNA level. a MCF-7 cells were treated without (0) or with 10–100 μM CMH for 72 h, and the total RNA was isolated for semiquantitative RT-PCR as we previously described (2). Equivalent addition of RNA was confirmed by PCR analysis of *β*-actin. (**b, c**) c-FLIP$_S$ and c-FLIP$_L$ mRNA expression relative to *β*-actin expression shown in panel (**a**) was quantified using Image J 1.37c software. Data are average of triplicate measurements (** $P < 0.05$)

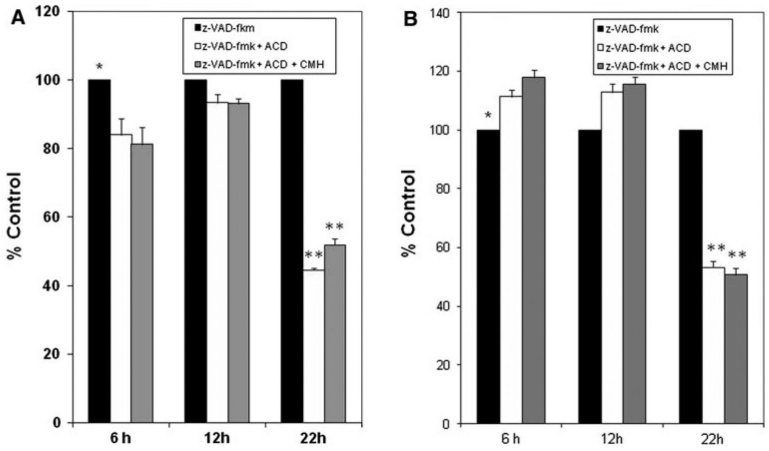

Fig. 5.

Densitometric analysis of semiquantitative RT PCR of c-FLIP$_L$ (a) and c-FLIP$_S$ mRNA (b) expression following treatment with actinomycin D or actinomycin D plus CMH for 22 h. *c-FLIP$_L$* and *c-FLIP$_S$* gene expression were measured in MCF-7 cells treated with 5 μg/ml actinomycin D for the times indicated in the presence or absence of 50 μM CMH. In order to prevent apoptosis, cells were pretreated for 2 h with the 100 μM of the general caspase inhibitor z-VAD-fmk. Results show that while the stability of c-FLIP$_L$ mRNA is not affected by CMH, it decreases the stability of c-FLIP$_S$ mRNA. mRNA expression was normalized to *β*-actin mRNA levels. Data are average of triplicate measurements (** $P <$ 0.005)

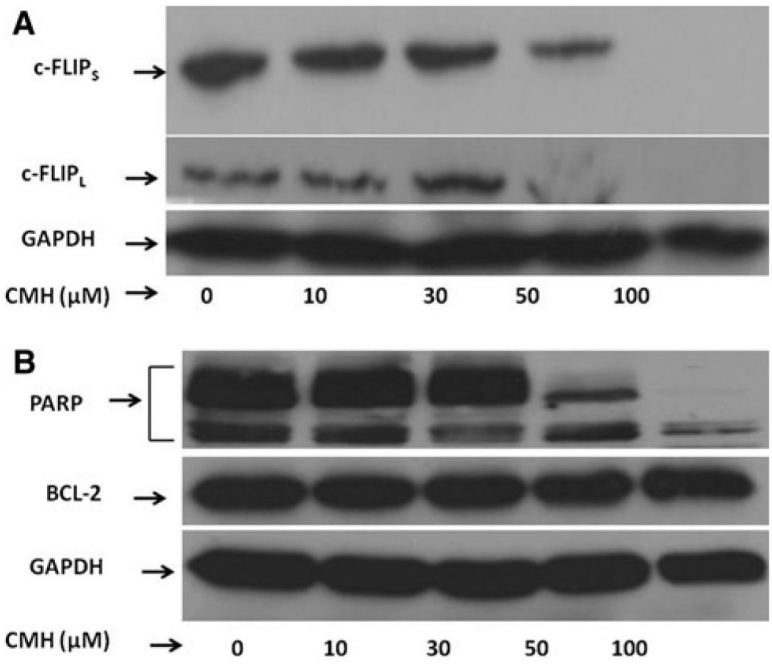

Fig. 6.

Western blot analysis of c-FLIP$_S$, c-FLIP$_L$, PARP, and BCL-2 in MCF-7 cells treated with CMH. Cell lysates (70 βg protein/lane) from MCF-7 cells treated without (0) or with 10, 30, 50, and 100 βM CMH were subjected to SDS polyacrylamide gel electrophoresis (PAGE) and Western blot analysis using **a** an anti-c-FLIP antibody (Cell Signaling Technology, Danvers, MA) and **b** anti-PARP antibody, anti-BCL 2 antibody, as well as anti-GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA)