Published in final edited form as:

Biochem Pharmacol. 2010 April 1; 79(7): 948–954. doi:10.1016/j.bcp.2009.11.004.

Induction and intracellular localization of Nur77 dictate fenretinide-induced apoptosis of human liver cancer cells

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Abstract

Fenretinide, a synthetic retinoid, is known to induce apoptosis in various cancer cells. However, the mechanism by which fenretinide induces apoptosis remains unclear. The current study examines the mechanisms of fenretinide-induced apoptosis in human hepatoma cells. The induction of Nur77 and the cytoplasmic distribution of Nur77 induced by fenretinide were positively correlated with the apoptotic effect of fenretinide in HCC cells. The sensitivity of Huh-7 cells was related to Nur77 translocation and targeting mitochondria, whereas the mechanism of resistance for HepG2 cells seemed due to Nur77 accumulating in the nucleus. The intracellular location of Nur77 was also associated with the differential capability of fenretinide-induced ROS generation in these two cell lines. In addition, the knockdown of Nur77 expression by siRNA greatly reduced fenretinide-induced apoptosis and cleaved caspase3 in Huh7 cells. Therefore, our findings demonstrate that fenretinide-induced apoptosis of HCC cells is Nur77 dependent and that the intracellular localization of Nur77 dictates the sensitivity of the HCC cells to fenretinide-induced apoptosis.

Keywords

Fenretinide; Nur77; apoptosis; ROS; nuclear receptor; retinoids

1. Introduction

Retinoids, natural and synthetic derivatives of vitamin A, have a profound effect on cellular homeostasis including growth, differentiation, and apoptosis [1,2]. Clinically, retinoids have displayed therapeutic activity against a number of proliferative diseases. Experimental and clinical studies have demonstrated that retinoids can inhibit or reverse the carcinogenic process in certain organs, including premalignant and malignant lesions in the oral cavity, head and neck, breast, skin, and liver [3].

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Hepatocellular carcinoma (HCC), the primary liver malignancy, is the fifth most common neoplasm in the world and the third most common cause of cancer-related mortality [4]. HCC is highly resistant to available chemotherapy, thus resulting in a 5-year relative survival rate of less than 7% [5]. Therefore, discovery of new and effective therapies against HCC is greatly needed.

Fenretinide (N-(4-hydroxyphenyl) retinamide; 4HPR) is a structure analogue of all-trans retinoic acid that was first synthesized by R. W. Johnson Pharmaceuticals in the late 1960's. Many laboratory and clinical studies have demonstrated that fenretinide may hold great potential in cancer chemoprevention and therapy. Data from *in vitro* models demonstrate that fenretinide not only inhibited cell proliferation, but also induced apoptosis in human cancer cell types derived from a variety of tumors including head and neck, lung, melanoma, prostate, bladder carcinoma, neuroblastoma, and leukemia [6–13]. Furthermore, fenretinide is effective against carcinogenesis of the breast, prostate, pancreas, and skin in animal models [14–16]. In clinical trials, fenretinide slowed the progression of prostate cancer in men and protected against the development of ovarian cancer and a second breast malignancy in premenopausal women [17]. Therefore, fenretinide offers great promise as a therapeutic agent in cancer treatment and prevention.

The different signaling pathways involved in fenretinide-induced apoptosis in cancer cells including reactive oxygen species (ROS) generation, ceramide, and ganglioside GD3 and the intrinsic or mitochondrial-mediated pathways seem to play a central role in cancer cells elimination [17]. The most commonly observed property of fenretinide-induced apoptosis in cancer cells is its inhibition by antioxidants such as vitamin C, vitamin E, and N-acetylcysteine, and pyrrolidine dithiocarbamate, thus suggesting an essential role of ROS and oxidative stress in fenretinide's cytotoxicity [18–20].

Nur77 (NR4A1, TR3, NGFI-B) belongs to nuclear receptor superfamily NR4A subfamily. Nur77 is one of the orphan nuclear receptors with no identified physiological ligands. Nur77 is highly expressed in various tissues including liver [21]. Nur77 was initially classified as an immediate-early response gene as it can be rapidly induced by growth factors, phorbol esters, calcium ionophores and other stimuli acting via cyclic AMP-dependent synthesis pathways [22]. Most importantly, a number of studies have indicated that Nur77 plays an important role in chemotherapeutic agent-induced apoptosis. One retinoid-related compound, 6-(3-(1-adamantyl)-4-hydroxyphenyl)-2-naphthalenecarboxylic acid, also known as AHPN/CD437, was shown to trigger Nur77 nuclear export and mitochondria targeting, which is the key mechanism responsible for CD437-induced apoptosis of cancer cells [23,24]. It is unknown whether Nur77 plays a role in fenretinide-induced apoptosis.

In the present study, we provide direct evidence that Nur77 is involved in mediating the apoptotic effect of fenretinide in HCC cells. Furthermore, our findings establish the distinct modes of action of Nur77 between the sensitive and resistant cells in response to fenretinide. Our data show the intracellular localization of Nur77 determines the susceptibility of HCC cells to the apoptotic effect of fenretinide.

2. Materials and methods

2.1. Reagents

All reagents and chemicals used were from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. Fenretinide (10 mM) dissolved in DMSO was stored at -80°C . MitoSOXTM Red mitochondrial superoxide indicator, Hank's Balanced Salt Solution (HBSS) with calcium and magnesium, TRIzol reagent and LipofectamineTM RNAiMAX Transfection Reagent were purchased from Invitrogen. (Carlsbad, CA). VECTASHIELD Mounting Medium with DAPI

was purchased from Vector Laboratories (Burlingame, CA). Rabbit polyclonal antibodies for Nur77, goat polyclonal cleaved caspase-3, Poly ADP-ribose polymerase (PARP) and goat anti-rabbit IgG-Texas Red were purchased from Santa Cruz (Santa Cruz, CA). Protease and phosphatase inhibitors and In Situ Cell Death Detection Kit were purchased from Roche Applied Science (Indianapolis, IN).

2.2. Cell culture and treatment

Huh-7 cells were maintained in Dulbecco's Modification of Eagle's Medium. HepG2 cells were maintained in Minimum Essential Medium (Mediatech, Herndon, VA). The media were supplemented with 10% fetal calf serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). Cells were cultured at 37°C in 5% CO₂ atmosphere with a relative humidity of 95%. Cells were plated with approximately 1×10⁶ cells per T-25 flask or 5×10⁴ per well of 24-well plates/4-well chamber slides 12–16 hours prior to the treatments and cultured overnight. Cells were incubated with DMSO or fenretinide (10 μM) in serum-free media for indicated time. The final concentration of DMSO in the culture medium was 0.1% in all treatments. Fresh medium containing corresponding compounds was provided every 24 hours.

2.3. Apoptosis assay

Apoptosis was evaluated by caspase 3/7 activity and cell survival. Caspase 3/7 activity and cell viability were determined by CellTiter-Glo® Luminescent Cell Viability and Caspase-Glo® 3/7 kit following the protocol supplied in the kit (Promega, Madison, WI).

2.4. Total RNA preparation

Total RNA was extracted with TRIzol reagent according to the manufacturer's instruction. RNA was quantified and assessed for purity on a UV spectrophotometer.

2.5. Reverse transcription and quantitative real-time PCR

Total RNA (1 μg) was reverse-transcribed with oligo (dT) primer and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) for first strand cDNA synthesis. cDNA corresponding to 32 ng total RNA was used as the template in a 20 μL real-time PCR reaction with the ABI TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the appropriate primer pair and Taqman probe. The primer pairs and Taqman probes for Nur77 were designed with Primer Express software v2.0 (Nur77 forward primer: AGCATTATGGTGTCCGCACAT; reverse primer: TTGGCGTTTTTCTGCACTGT; probe: TGAGGGGCTGCAAGGGCTTCTTCAA. β-actin forward primer: CCTGGCACCCAGCACAAT; reverse primer: GCCGATCCACACGGAGTACT; probe: ATCAAGATCATTGCTCCTCCTGAGCGC). Real-time PCR was conducted using the ABI Prism 7300 real-time PCR system (Applied Biosystems, Foster City, CA). The quantification analysis for target gene expression was performed using the relative quantification comparative CT method.

2.7. Confocal microscopy

Huh-7 and HepG2 cells were grown in Chamber BD Falcon™ Cultureslides (BD Biosciences, Bedford MA, USA). Following treatment, cells were fixed for 15 minutes at room temperature with freshly prepared 1% paraformaldehyde (Mallinckrodt Baker Inc, Phillipsburg, NJ) in PBS. After fixation, cells were rinsed with PBS containing 0.2% Triton X100; cells were then incubated with PBS containing 0.2% Triton X100 and 5% normal goat serum (Abcam, Cambridge, MA) for 30 minutes at room temperature. Primary antibody specific for Nur77 (1:100 dilution) was applied to cells overnight at 4°C or 1 hour at 37°C in a humidified chamber. After washed with PBS containing 0.2% Triton X100 and 1% normal goat serum, cells were incubated with FITC-conjugated secondary antibody (diluted at 1:400, in PBS containing 0.2%

Triton X100 and 1% normal goat serum) for overnight at 4°C or 1 hour at 37°C. After washing with PBS, cells were mounted with VECTASHIELD Mounting Medium with 4', 6-diamidino-2-phenylindole (DAPI) and analyzed under confocal microscope (Nikon, EZ-C1, Japan).

2.8. Subcellular fraction isolation

Trypsinized cells (1×10^7) were collected by centrifugation at 1000 rpm for 5 minutes. Cell pellets were resuspended with 5.5 mL of cold RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, supplemented with protease and phosphatase inhibitors) and incubate on ice for 90 minutes. Cells were then lysed in a dounce homogenizer and mixed with 4 mL 2.5x MSB buffer (525 mM Mannitol, 175 mM Sucrose, 12.5 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, pH 7.5). The lysate was centrifuged at 1300g for 5 minutes at 4°C for two times and the combined pellets were the nuclear fraction. The supernatant was centrifuged at 17000g for 15 minutes at 4°C and the subsequent supernatant represented cytosol fraction and the pellets represented crude mitochondria fraction.

2.9. Western blotting and antibodies

Cells from indicated treatments were collected and lysed with lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% (V/V) NP-40 with protease and phosphatase inhibitors). Equal amounts of lysates (50 μ g total protein) were electrophoresed on SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad, Hercules, CA). The membranes were first incubated with PBS supplemented with 0.1% Tween 20 and 5% nonfat dry milk (PBST-milk) for 1 hour at room temperature to block nonspecific binding sites. Immunostaining was performed by incubating the membranes with primary antibodies for Nur77, PARP, and Porin in PBST-milk overnight at 4°C. After three washes, membranes were incubated with the appropriate secondary antibodies for 1 hour in PBST-milk. The signal was detected using the ECL system SuperSignal West Pico Chemiluminescent Substrates (Pierce, Rockford, IL).

2.10. Mitochondrial superoxide staining

MitoSOX™ Red mitochondrial superoxide indicator stock solution (5 mM in DMSO, stored at -20°C in dark) was further diluted into 2.5 μ M working solution with HBSS right before use. Huh-7 and HepG2 cells were incubated with or without fenretinide (10 μ M) in serum-free media in T-75 flasks or 6-well plates followed by MitoSOX™ Red mitochondrial superoxide indicator staining according to the manufacturer's protocol. In brief, medium was replaced with 2.5 μ M MitoSOX™ reagent working solution. The cells were incubated for 15 minutes at 37°C, protected from light. After the staining, the cells treated for 3 hours were collected by trypsin and analyzed for MitoSOX™ Red according to the manufacturer's instruction on a Fluorescence Activated Cell Sorter Calibur (FACSCalibur) (BD Biosciences, San Jose, CA). The cells treated for 8 hours were washed gently three times with warm HBSS and examined under fluorescence microscope (Zeiss, Germany) in serum-free medium.

2.11. siRNA transfection

Scramble siRNA and pre-designed siRNA for human Nur77 gene were purchased from Ambion (Austin, TX). Huh-7 cells were transfected with siRNA (25 and 50 nM per 5×10^4 cells per well in a 24-well plate or a chamber slide) using Lipofectamine™ RNAiMAX Transfection Reagent following the manufacturer's instruction. Cells were harvested 48 hours post-transfection for evaluation of Nur77 knockdown efficiency by quantitative real-time PCR.

2.12. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

After 48 hours of siRNA transfection, cells were incubated with or without fenretinide (10 μ M) in serum-free medium for 24 hours followed by TUNEL staining using an *in situ* cell

death detection kit according to the manufacturer's instruction. TUNEL positive cells were examined under a light microscope.

2.13. Double staining Nur77 and cleaved caspase-3

After 48 hours of siRNA transfection, cells were incubated with 0.1% DMSO or with fenretinide (10 μ M) in medium for 16 hours followed by immunofluorescence staining. After treatment, Huh-7 cells were fixed and incubated with cleaved caspase 3 antibody (1:50), followed by goat anti-rabbit IgG- Texas Red (1:400), and then incubated with Nur77 antibody (1:100) followed by FITC-conjugated goat anti-rabbit IgG (1:400). At last, cells were mounted with VECTASHIELD Mounting Medium with 4', 6-diamidino-2-phenylindole (DAPI) and analyzed under confocal microscope.

2.14. Statistical analysis

Data are presented as mean $\pm$ S.E. Statistical analysis was performed using Student's *t*-test for two-group comparison. Significance was defined by $p < 0.05$.

3. Results

3.1. Fenretinide differentially induced Apoptosis and Nur77 mRNA expression in Huh-7 and HepG2 cells

To study the effect of fenretinide on cell death of Huh-7 and HepG2 cells, Huh-7 and HepG2 cells were treated with fenretinide (1, 5, 10, 20 μ M) for 24 hrs and cell survival was studied by CellTiter-Glo® Luminescent Cell Viability assay. Fenretinide significantly reduced the survival of Huh-7 cells and the effect was dose-dependent (Fig. 1A). The effect of fenretinide on caspase 3/7 activity of Huh-7 and HepG2 cells was studied by Caspase-Glo® 3/7 Assay. Fenretinide treatment (10 μ M) resulted in 4 folds increase of caspase 3/7 activity in Huh-7 cells and about 1.5-fold increases in HepG2 cells (Fig. 1B). In addition, the mRNA level of Nur77 was quantified in Huh-7 and HepG2 cells by realtime PCR. Fenretinide induced the mRNA level of Nur77 in Huh-7 cells; the induction occurred 12 hrs after the treatment and was time dependent (Fig. 1C). Fenretinide also induced the mRNA level of Nur77 in HepG2 cells within 6 hrs, but the effect was transient. Thus, fenretinide differentially induced apoptosis in Huh-7 and HepG2 cells and the susceptibility of the HCC cells to fenretinide-induced apoptosis was associated with differentially induction of Nur77.

3.2. Fenretinide induced mitochondrial enrichment of Nur77 in Huh-7 cells, but not in HepG2 cells

Nur77 has opposing biological activities, and the intracellular localization of Nur77 determines its role in cell survival and death [25]. We examined the subcellular localization of Nur77 in response to fenretinide. In the sensitive Huh-7 cells, Nur77 protein level was induced after 24 hrs of fenretinide treatment and the up-regulated Nur77 protein was mainly in the cytosol (Fig. 2A). This is consistent with Nur77 mRNA levels as shown in Figure 1C. After 36 hrs of treatment, at which time most Huh-7 cells were dying, Nur77 protein was distributed in cytosol as well as nucleus (Fig. 2A). In contrast to Huh-7 cells, a distinct subcellular localization pattern of Nur77 protein in response to fenretinide was observed in the resistant HepG2 cells. Nur77 was distributed in the cytosol and the nucleus of HepG2 cells after 24 hrs of treatment, however, Nur77 protein was mainly accumulated in the nucleus as the treatment prolonged to 48 hrs (Fig. 2B). The data were confirmed by western blot using subcellular fractions isolated from fenretinide-treated and untreated HCC cells. Porin and PARP (Poly (ADP-ribose) polymerase) were used as mitochondrial and nuclear markers, respectively. Western blot data showed that fenretinide induced the levels of Nur77 in Huh-7 cells 12 hrs after the treatment; and the induced Nur77 was mainly located in the mitochondria-enriched fraction (Fig. 2C). After 24 hrs

treatment, the induction of Nur77 further increased. In HepG2 cells, fenretinide induced Nur77 was mainly located in the nucleus-enriched fractions (Fig. 2D).

3.3. Fenretinide differentially generated ROS between Huh-7 and HepG2 cells

The best known action of fenretinide is ROS generation, which has been demonstrated in various types of cancer cells. However, its action in HCC cells has not been extensively studied. After Huh-7 cells were exposed to fenretinide (10 μ M) for 3 hrs, mitochondrial ROS was increased only in Huh-7 cells (1.7-fold), but not in HepG2 cells (Fig 3A). As shown in Fig. 3B, eight hrs after the fenretinide treatment, the number of mitochondrial ROS positive cells was more in Huh-7 than HepG2 cells by a ratio of about 5 to 1. Thus, fenretinide-induced Nur77 induction in mitochondria-enriched fraction was positively associated with ROS generation in HCC cells.

3.4. Nur77 is essential for fenretinide-induced apoptosis of HCC cells

In order to determine whether Nur77 is required for fenretinide-induced apoptosis of Huh-7 cells, the expression of Nur77 was knocked down by siRNA transfection followed by TUNEL assay. Two concentrations of siRNA silenced Nur77 to different levels (Fig. 4A). Nur77 siRNA at 50 nM achieved a marked knockdown effect (83.5%). After 24 hrs of fenretinide treatment, robust DNA double-strand breaks as revealed by TUNEL positive staining were detected in the cells transfected with scramble siRNA; in contrast, only very few DNA double-strand breaks were seen in the cells transfected with Nur77 siRNA (Fig. 4B). In addition, fenretinide-induced Nur77 expression in cytosol was associated with the fenretinide-induced caspase-3 cleavage within the same cells (Fig. 4C). Furthermore, the fenretinide-induced caspase-3 in Huh-7 cell was Nur77 dependent. Cleaved caspase-3 was no longer detectable when Nur77 expression was knocked down by specific siRNA (Fig. 4C).

4. Discussion

Fenretinide is emerging as a promising anticancer agent as indicated by numerous *in vitro*, animal, and chemoprevention clinical trial studies. The apoptotic effect of fenretinide has been demonstrated in more than 20 different types of tumors and cancer cells. The recent review article summarizes the possible underlying mechanisms [17], but the exact mechanism remains largely unknown. The general consensus is that fenretinide is a ROS generator and that generation of ROS plays a central role in the apoptosis process induced by fenretinide. ROS affects the mitochondrial membrane permeability (MMP) and releases cytochrome *c* and other apoptosis inducers to activate the intrinsic apoptosis pathway. Whether the action of fenretinide is dependent on retinoic acid receptor β (RAR β) is an issue under debate. Evidence for both receptor-dependent and -independent mechanisms exists. Our previous study demonstrated that fenretinide-induced apoptosis of Huh-7 cells is RAR β -dependent [26]. In the present study, for the first time, we have identified Nur77 is another mediator for fenretinide-induced apoptosis of HCC cells. We documented the differential induction of Nur77 in Huh-7 and HepG2 cells, which positively correlated with susceptibility of HCC cells to fenretinide. More importantly, the distinct intracellular distribution pattern of Nur77 protein as well as differential ROS generation in Huh-7 and HepG2 cells in response to fenretinide suggest the crucial role of Nur77 in mediating the cell death induced by fenretinide. The knockdown experiment firmly established the role of Nur77 in mediating fenretinide-induced cell death of HCC cells.

By interacting with other nuclear receptor, Nur77 can exert opposing biological activities in cell survival and death. Nur77 confers growth advantage on cancer cells by suppressing the negative growth regulatory effect of retinoids [27,28]. In lung cancer cells, overexpression of Nur77 correlates with their resistance to RA treatment. Nur77 inhibits RAR β signaling by interacting with COUP-TF [28,29]. The ligand for the Nur77 monomer and homodimer has

not been identified and might not be present. When Nur77 forms a dimer with RXR α , it confers 9-cis RA-dependent transcription to a reporter containing a direct repeat with a spacer of five nucleotides (DR-5) and Nur77 response element (TR3RE) sequence [29]. RXR α is also involved in the apoptotic effect of Nur77. It has been shown that nuclear export of Nur77 requires dimerization with RXR α in response to apoptotic stimuli [30,31]. Nuclear export of Nur77/RXR α during apoptosis is mediated through CRM1 receptors by a nuclear export signal located in the ligand binding domain of RXR α [30]. Once Nur77 is in the cytoplasm, Nur77 targets mitochondria via interaction with mitochondrial Bcl-2 [32]. Thus, there is a close interaction between Nur77 with other nuclear receptors in mediating its survival and death effects. Our study, for the first time, demonstrated that Nur77 and RAR β are both essential for mediating the apoptotic effect of fenretinide. The relationship between Nur77 and RAR β remains to be explored. By time course experiments, our unpublished data showed fenretinide induced RAR β mRNA level within 60 min. The current study shows induction of Nur77 mRNA level within 24 hrs. Thus, it is possible that RAR β is upstream of Nur77. It is also possible that RAR β and Nur77 are mediated through independent pathway to exert the apoptotic effect of fenretinide. It is essential to identify the immediate downstream of RAR β and to establish the relationship between RAR β and Nur77 in fenretinide-mediated cell death.

In summary, the current study establishes the role of Nur77 in fenretinide-mediated apoptosis. Nur77 may present a potential therapeutic target in HCC treatment. The intracellular localization of Nur77 dictates the effectiveness of fenretinide in inducing apoptosis of HCC cells.

Acknowledgments

This work was supported by NIH grants CA 53596, AA14147, and P20RR021940 Molecular Biology Core.

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Fig. 1.

Fenretinide differentially induced apoptosis and Nur77 mRNA expression in Huh-7 and HepG2 cells. (A) HCC cells were treated with DMSO or fenretinide (1–20 μ M) for 24 hrs. Cell survival was studied by CellTiter-Glo® Luminescent Cell Viability assay. (B) HCC cells were treated with and without fenretinide (10 μ M) for 24 hrs and then Caspase-Glo® 3/7 Assay was performed. (C) Nur77 mRNA level was quantified by real-time PCR and β -actin mRNA level was used as an internal control. The data shown were relative induction fold (fenretinide vs. DMSO treatment) at each time point. This experiment was repeated three times.

Fig. 2.

(A and B) Fenretinide induced mitochondria enrichment of Nur77 in Huh-7 cells, but not in HepG2 cells. Huh-7 and HepG2 cells were treated with DMSO or fenretinide (Fen, 10 μ M) for the indicated time followed by immunofluorescence staining of Nur77 and DAPI for nuclear counter stain. The images were viewed under confocal microscopy (400X). (C and D) Mitochondria and nucleus fractions were isolated from DMSO- or fenretinide-treated Huh-7 and HepG2 cells and subjected to western blot using specific antibodies. The purity of intracellular fraction was confirmed by using antibody specific to PARP and Porin. The ratio of cytosol to nuclear Nur77 expression was quantified by BanScan 5. The numbers indicate the average ratios of nuclear to mitochondria Nur77 expression level.

Fig. 3.

Fenretinide differentially generated ROS in Huh-7 and HepG2 cells. Huh-7 and HepG2 cells were treated with or without fenretinide (10 μ M) for 3 or 8 hrs followed by staining with MitoSOXTM Red mitochondrial superoxide indicator. MitoSOXTM Red reagent is highly selective for detection of mitochondrial superoxide in live cells. Once the compound is oxidized by superoxide in the mitochondria, red fluorescence is easily visible. (A) In 3 hrs treatments, analysis was performed by flow cytometry after addition of MitoSOX reagent. (B) In 8 hrs treatments, the positive cells were shown in red with MitoSOX Red mitochondrial superoxide indicator staining and four random fields of more than 200 cells each were counted under a fluorescence microscope. The results were generated from three independent experiments.

Fig. 4.

Knockdown the expression of Nur77 inhibited fenretinide-induced DNA double-strand breaks and caspase3 cleavage in Huh-7 cells. (A) Huh-7 cells were transfected with either scramble or Nur77 siRNA (25 and 50 nM) for 48 hrs. Then, Nur77 mRNA level was determined by real-time PCR. (B) Scramble or Nur77 siRNA (50 nM) transfected Huh-7 cells were treated with DMSO or fenretinide for 24 hrs followed by TUNEL assay. (C) The expression of Nur77 was knocked down by siRNA (50 nM) in Huh-7 cells, then the cells were treated with fenretinide (10 μ M) for 17 hrs followed by immunofluorescence staining of Nur77, cleaved caspase3, and DAPI. The staining was viewed under confocal microscope. Figures show representative results from duplicated experiments.