3-O-[N-(p-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid, a semisynthetic analog of oleanolic acid, induces apoptosis in breast Cancer cells.

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3-*O*-[*N*-(*p*-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid, a semisynthetic analog of oleanolic acid, induces apoptosis in breast cancer cells

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ABSTRACT

Oleanolic acid (OA), a pentacyclic triterpene acid widely distributed in food and traditional herbal remedies, exhibits diverse therapeutic effects. OA has been subjected to various chemical modifications to optimize its anticancer effect. Among other analogs, 3-*O*-[*N*-(*p*-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid (PFOA) was semisynthesized from OA. This study evaluates the cytotoxic effects of PFOA on MDA-MB-231, MCF-7, BT-474, and T-47D human breast cancer cells. Acute treatment of PFOA inhibited breast cancer cell viability in a dose-dependent manner. Treatment of PFOA at cytotoxic doses significantly induced apoptosis in cancer cells as shown by flow cytometry analysis. Activation of apoptosis in MCF-7 and BT-474 cells seemed to be initiated through induction of Fas ligand, which resulted in activation of caspase-8 and PARP-1, whereas apoptosis in MDA-MB-231 cells was initiated by the activation of caspase-9, caspase-3 and PARP-1. The mechanism of apoptosis induction in T-47D involves activation of PARP-1. PFOA decreased the expression of EGFR, HER-2, MET and ERα in human breast cancer cell lines. These findings suggest that PFOA inhibits cell growth, activates apoptosis, and decreases the expression of key proteins involved in progression of breast cancer.

1. Introduction

Breast cancer is considered to be the most frequently diagnosed cancer among the female population (Yi et al., 2013). It represents a major public health burden as it ranks the second to cancer related mortalities after lung cancer (Yi et al., 2013). Being heterogeneous in nature, the mechanisms below breast cancer development remain controversial (Hedenfalk et al., 2001). Chemotherapy, surgery or radiation showed disadvantages and little impact that should be considered (Hedenfalk et al., 2001). On the other hand, targeted therapies can selectively interfere with different cancer cell processes in diverse ways (Imai and Takaoka, 2006). Thus, targeting molecular signaling pathways to control breast malignancies is among the promising strategies.

For centuries, nature has proven as a rich source of lead compounds for treatment of various ailments (Cragg et al., 2006). Among naturally-occurring sources, plants offer the advantage of abundance, with chemically diverse secondary metabolites, qualifying them as promising resource for new anticancer drug entities, witnessed by such potent anticancer paclitaxel (Taxol) from the yew tree (Cragg et al., 2006). Other advantage for the use of anticancer plant metabolite is their possible use as co-drug dietary supplement to synergize or reduce the doses of standard chemotherapy.

Oleanolic acid (OA), a pentacyclic triterpene acid, is widely distributed in food and traditional medicinal herbs. Recently it has attracted considerable attention due to its different therapeutic activities including its anti-inflammatory, antioxidant, immunomodulatory, antiviral, and anticancer properties (Sultana and Ata, 2008).

OA has been shown to mediate its anticancer effect through modulation of multiple molecular targets. OA reduced the glioma U-87 MG cells' invasion and migration through inhibition of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway (Guo et al., 2013). Furthermore, its antiproliferative activity was reported in MCF-7 breast cancer cells through induction of cell cycle arrest (Allouche et al., 2010). OA exerted its apoptotic effect through activating the p53-induced caspase-mediated proapoptotic pathway and suppression of nuclear factor-κB-induced Bcl-2-mediated antiapoptotic pathway in melanoma cells B16F-10 (Pratheeshkumar and Kuttan, 2011). However, it has been reported that OA protected normal liver QZG cells against cytotoxicity induced by *tert*-Butyl hydroperoxide through

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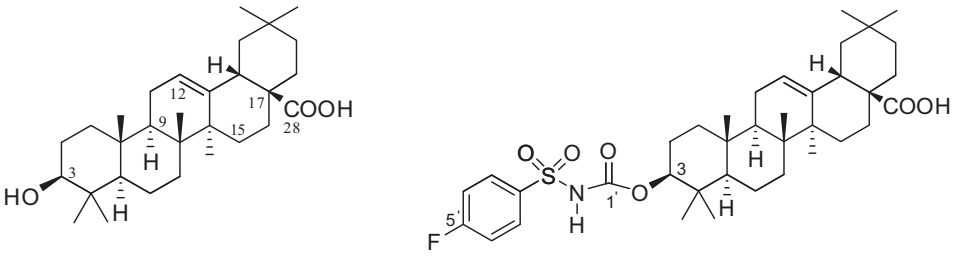


Oleanolic acid
(**OA**)

3-*O*-[*N*-(*p*-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid
(**PFOA**)

Fig. 1. Chemical structures of oleanolic acid (OA) and its semisynthetic analog 3-*O*-[*N*-(*p*-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid (PFOA).

increasing the expression of oxidative stress sensitive transcription factor-Nrf2, and MAP kinases, mainly JNK and ERK (Ma et al., 2002). OA was used as a starting scaffold to semisynthetically design different hybrids to optimize its anticancer effect. Bardoxolone methyl (2-Cyano-3,12-dioxoolean-1,9(11)-dien-28-oate) was a semisynthetic OA derivative with potent antitumorigenic activity against different cancer cell lines (Gao et al., 2011). Bardoxolone methyl was reported to inhibit the development and progression of prostate cancer in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model (Gao et al., 2011). HIMOXOL (ethyl 3-hydroxyimino-11-oxoolean-12-en-28-oate) is another OA semisynthetic derivative reported to exert its cytotoxic action in MDA-MB-231 breast cancer cells by activating apoptosis and autophagy pathways (Lisiak et al., 2014). Importantly, incorporating an azaheterocyclic ring to OA afforded hybrids with promising antiproliferative and apoptosis-inducing effects on hepatic carcinoma cells (BEL-7404) (Kang et al., 2012).

In order to discover new hits inspired by bioactive natural products for the control of human breast cancer cells, a structure-activity relationship study identified the OA semisynthetic analog 3-*O*-[*N*-(*p*-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid (PFOA) as a promising antimigratory, anti-invasive, and antiproliferative hit through suppression of Brk/Paxillin/Rac1 signaling in breast cancer cells (Elsayed et al., 2014) (Fig. 1). The aim of this study was to investigate the molecular mechanisms by which PFOA exerts its potent cytotoxic and proapoptotic effects in breast cancer cell lines.

2. Materials and methods

2.1. Chemicals, reagents, and antibodies

All materials were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. 3-*O*-[*N*-(*p*-fluorobenzenesulfonyl)-carbamoyl]-oleanolic acid (PFOA) was semisynthesized from oleanolic acid according to the previously described method (Elsayed et al., 2014). All antibodies were purchased from Cell Signaling Technology (Beverly, MA), unless otherwise stated. Goat anti-rabbit secondary antibody was purchased from PerkinElmer Biosciences (Boston, MA).

2.2. Cell lines and culture conditions

The human breast cancer cell lines MDA-MB-231, MCF-7, BT-474, and T-47D cells were purchased from American Type Culture Collection (Rockville, MD). The cell lines were maintained in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin G, 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. OA and PFOA were first dissolved in a volume of DMSO to provide final 25 mM stock solutions which were used to prepare various concentrations of treatment media.

2.3. Measurement of viable cell number

Viable cell count was determined using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Akl et al., 2012). The optical density of each sample was measured at 570 nm on a microplate reader (BioTek, VT). The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined using a hemocytometer at the start of each experiment.

2.4. Cell proliferation and cytotoxicity assays

In the proliferation assay, MDA-MB-231 cells were plated at a density of 1×10^4 cells per well (6 wells/group) in 96-well culture plates and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 0.5% FBS. Viable cell number after 24, 48 and 72 h treatment was determined using the MTT assay.

The cytotoxic effects of PFOA on MDA-MB-231, MCF-7, BT-474 or T-47D cells were assayed by MTT colorimetric assay. Cells were plated at a density $\sim 4 \times 10^4$ cells per well (6 wells/group) in 96-well culture plates and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 0.5% FBS for 24 h. Viable cell number was determined using the MTT assay.

2.5. DAPI fluorescent staining

MDA-MB-231 cells were seeded on 8-chamber culture slides (Becton Dickinson and Company, NJ, USA) at a density of 3×10^5 cells/chamber (3 replicates/group) and allowed to attach in complete growth medium supplemented with 10% FBS overnight. Cells were then washed with PBS and incubated with vehicle control or treatment media containing 5 or 10 μM PFOA for 24 h. At the end of treatments, cells were washed with pre-cooled PBS, fixed with 4% formaldehyde/PBS for 6 min at room temperature. Fixed cells were washed with PBS and embedded in Vectashield mounting medium with DAPI (Vector Laboratories IN., Burlingame, CA, USA) for 10 min. Changes in the nuclei of cells after staining with DAPI were observed using a Nikon ECLIPSE TE200-U fluorescence microscope (Nikon Instruments Inc., Melville, NY). Digital images were captured using Nikon NIS Elements software (Nikon Instruments Inc., Melville, NY).

2.6. Apoptosis analysis using annexin V/propidium iodide staining by flow cytometry

Induction of apoptosis was assessed by the binding of annexin V to phosphatidylserine, which is externalized to the outer leaflet of plasma membrane early during induction of apoptosis. Analysis of Annexin V was determined using Annexin V-FITC Early Apoptosis Detection Kit (Cell Signaling Technology, Beverly, MA). Cells were plated at a density of 5×10^6 cells/100 mm culture plates, allowed to attach overnight. Afterwards, cells were incubated in the respective control or PFOA-treated RPMI-1640 medium containing 0.5% FBS for 24 h. At the end of the experiment, cells in each treatment group were isolated with trypsin and then washed twice with ice cold PBS. Cells were then resuspended in 96 μL of ice-cold 1X Annexin V Binding Buffer. Afterwards, 1 μL Annexin V-FITC Conjugate and 12.5 μL propidium iodide (PI) solution were added to each 96 μL cell suspension. The cells were then incubated for 10 min on ice in the dark. The cell suspension was then diluted to a final volume of 250 μL per assay with ice-cold, 1X Annexin V Binding Buffer. Scatter plots were generated using CellQuest software (BD Biosciences, San Jose, CA). In the scatter plot of double variable flow cytometry, LL quadrant (FITC −/ PI −) shows living cells; UR quadrant (FITC +/PI +) stands for late apoptotic cells; and LR quadrant (FITC +/PI −) represents early apoptotic cells. All experiments were repeated at least thrice and a representative image from each experiment is shown in Fig. 5.

2.7. Western blot analysis

To study the apoptotic effects of treatments, cells were plated at a density of 4×10^6 cells/100 mm culture plate and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 0.5% FBS for 24 h (Akl et al., 2013, 2014).

At the end of treatment period, cells were lysed in RIPA buffer (Qiagen Sciences Inc., Valencia, CA) and protein concentration was determined by the BCA assay (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of protein (30 μg) were electrophoresed on SDS–polyacrylamide gels. The gels were then electroblotted onto PVDF membranes. These PVDF membranes were then blocked with 2% BSA in 10 mM Tris–HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) and then, incubated with specific primary antibodies overnight at 4 °C. At the end of incubation period, membranes were washed 5 times with TBST and then incubated with respective horseradish peroxide-conjugated secondary antibody in 2% BSA in TBST for 1 h at room temperature followed by rinsing with TBST for 5 times. Blots were then visualized by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL). Images of protein bands from all treatment groups within a given experiment and scanning densitometric analysis were acquired using Kodak Gel Logic 1500 Imaging System (Carestream Health Inc., New Haven, CT). The visualization of β-tubulin was used to ensure equal sample loading in each lane. All experiments were repeated at least 3 times and a representative Western blot image from each experiment is shown in the Figs. 6 and 7.

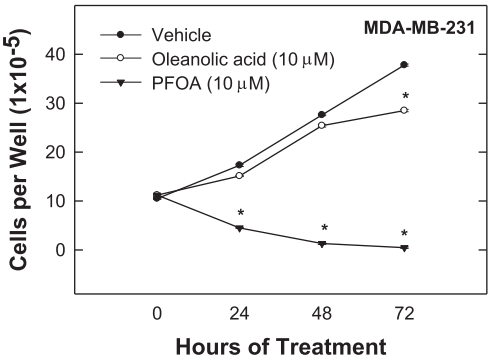


Fig. 2. Effects of OA and PFOA treatment on growth of MDA-MB-231 cancer cells after 24, 48 and 72 h treatment periods. MDA-MB-231 cells were plated at a density of 1×10^4 cells per well in 96-well culture plates and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 0.5% FBS. Viable cell number after 24, 48 and 72 h treatment was determined using the MTT assay. $^*P < 0.05$ as compared with vehicle-treated controls.

2.8. Statistics

The results are presented as mean ± S.E.M of at least three independent experiments. Differences among various treatment groups were determined by the analysis of variance (ANOVA) followed by Dunnett's test using PASW statistics® version 18. A difference of $P < 0.05$ was considered statistically significant as compared to the vehicle-treated control group. The IC_{50} values (concentrations that induce 50% cell growth inhibition) were determined using non-linear regression curve fit analysis using GraphPad Prism software version 6.

3. Results

3.1. The effect of OA and PFOA on MDA-MB-231 cell viability

The antiproliferative activity of OA and PFOA on MDA-MB-231 human breast cancer cells was evaluated by MTT assay (Fig. 2). Treatment with 10 μM OA showed a significant inhibition (24.3%) of cell viability after 72 h as compared to the vehicle control group with no significant effect on cell viability after 24 or 48 h. However, treatment with 10 μM PFOA caused a marked decline (74%) in MDA-MB-231 cell viability after 24 h (Fig. 2). The findings support the fact that PFOA may exert a significant cytotoxic influence on breast cancer cells.

3.2. Detection of morphological apoptosis with DAPI staining

Cell morphological changes and a reduction of viable cells showed that PFOA induced cytotoxic effects on MDA-MB-231 cells. To confirm whether or not PFOA induced apoptosis, cells were stained by DAPI after exposure to PFOA and the results are shown in Fig. 3. The results indicated that control untreated cells were round and had homogeneous nuclei, whereas PFOA-treated cells showed condensed nuclei and apoptotic bodies which took the form of crescents around the periphery of the nuclei, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads (Fig. 3).

3.3. Cytotoxic effects of PFOA on human breast cancer cells after acute treatment

In order to characterize the cytotoxic effects of PFOA on human breast cancer cell viability, we have utilized four human breast cancer cell lines, MDA-MB-231, MCF-7, BT-474 and T-47D cells (Fig. 4). Acute exposure of breast cancer cells to PFOA for 24 h caused a reduction of MDA-MB-231, MCF-7, BT-474 and T-47D cell viability in a dose-dependent manner with IC_{50} of 5.5, 8.1, 11.5 and 16.4 μM, respectively (Fig. 4).

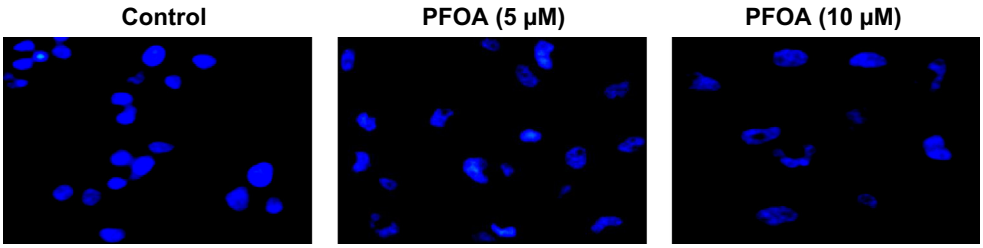


Fig. 3. PFOA-induced apoptotic body formation in MDA-MB-231 cells. Cells were treated with 5 or 10 μM PFOA for 24 h and apoptotic body formation as an indicator of apoptosis was determined by DAPI staining then photographing cells under fluorescence microscopy as described in Section 2.

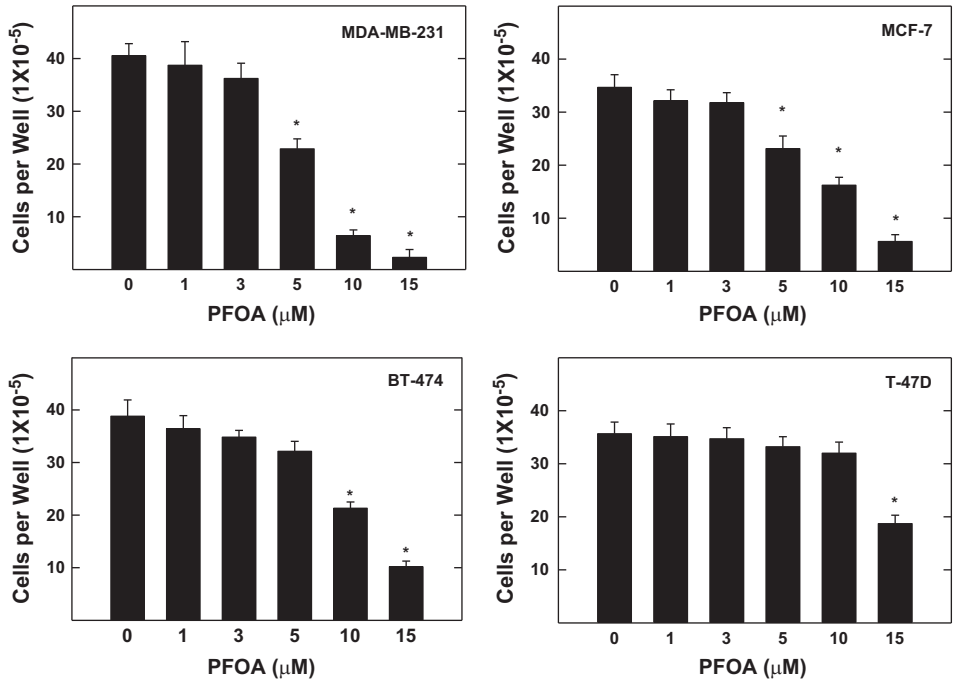


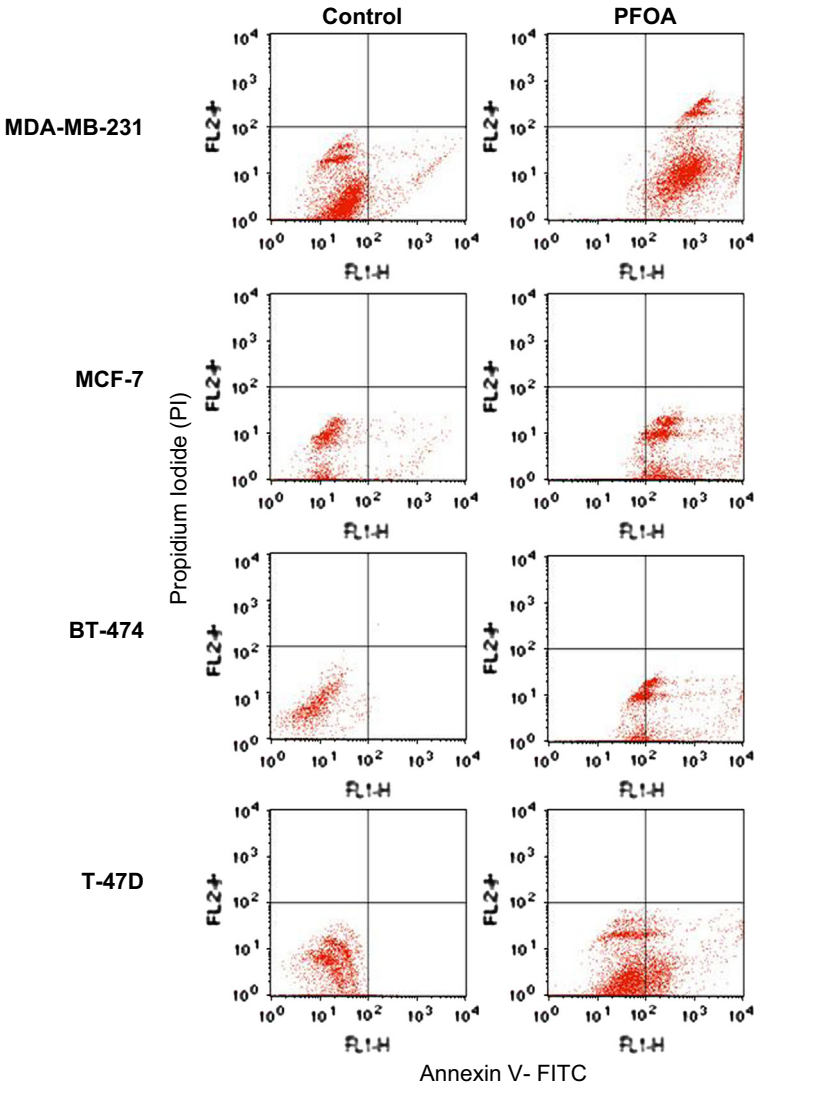
Fig. 4. Effect of PFOA on the viability of MDA-MB-231, MCF-7, BT-474, and T-47D human breast cancer cells after 24 h treatment period. Cells were plated at a density $\sim 4 \times 10^4$ cells per well in 96-well culture plates and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 0.5% FBS for 24 h. Viable cell number was determined using the MTT assay. Vertical bars indicate the mean cell count ± S.E.M in each treatment group. $^*P < 0.05$ as compared with vehicle-treated controls.

3.4. Quantification of PFOA-induced apoptosis in breast cancer cells using flow cytometry analysis

The effects of PFOA on apoptosis in breast cancer cells was evaluated by using annexin V-FITC and propidium iodide (PI) staining (Fig. 5). In normal live cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane (Emoto et al., 1997; Koopman et al., 1994; Martin et al., 1995). However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, annexin-V, is a 35–36 kDa Ca^{2+}-dependent phospholipid binding protein that has a high affinity for PS. Annexin-V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet. In addition, the red-fluorescent propidium iodide (PI) nucleic acid binding dye is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with annexin V and PI, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer. In the scatter plot of double variable flow cytometry, lower left (LL) quadrant (FITC −/ PI −) shows living cells; upper right (UR) quadrant (FITC+/PI +) stands for late apoptotic cells; and lower right (LR) quadrant (FITC +/PI −) represents early apoptotic cells (Emoto et al., 1997; Koopman et al., 1994; Martin et al., 1995). Assessed by flow cytometry and shown in Fig. 5, a marked increase in both early (80.58%) and late (15.68%) stages of apoptosis was obvious in MDA-MB-231 cells after PFOA treatment compared with control cells. In addition, a 10 μM treatment of PFOA significantly augmented early apoptosis in MCF-7 (71.94%) and BT-474 (80.58%) cells when compared to vehicle-treated control cells. However, initiation of cell death by 15 μM PFOA in T-47D cells was less pronounced. The apoptotic cell fraction of T-47D cells increased to 16.7% in PFOA-treated samples (Fig. 5).

3.5. Effects of PFOA treatment on the levels of apoptosis proteins in breast cancer cells

In order to confirm that PFOA-induced apoptosis was achieved through the changes of associated proteins, Western blotting analysis was performed (Fig. 6). MDA- MB-231, MCF-7 and BT-474 cells were exposed to 0, 5 or 10 μM PFOA for 24 h, while T-47D cells were exposed to 0, 10 or 15 μM PFOA for 24 h. In MDA-MB-231 cells, treatment with PFOA increased the cleavage of PARP-1, caspase-3, caspase-9 and RIP in a dose-dependent manner. In addition, it caused a little increase of caspase-8 and BID cleavage,



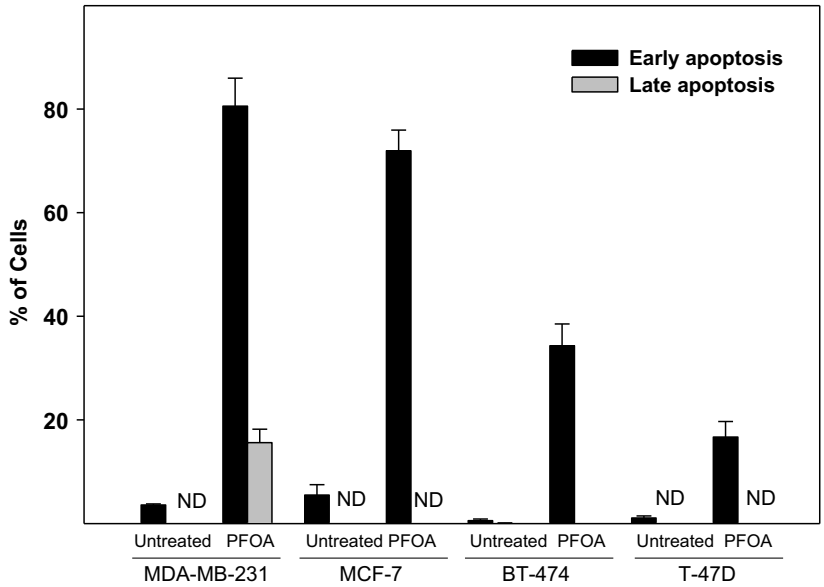


Fig. 5. Pro-apoptotic effect of PFOA treatment in MDA-MB-231, MCF-7, BT-474, and T-47D human breast cancer cells after 24 h treatment period using flow cytometry analysis. Cells were plated at a density of 5×10^6 cells/100 mm culture plates, allowed to attach overnight. Afterwards, cells were incubated in the respective control or PFOA-treated RPMI-1640 medium containing 0.5% FBS for 24 h. At the end of the experiment, cells in each treatment group were trypsinized, washed then resuspended in ice-cold 1X Annexin V Binding Buffer. Afterwards, the cells were treated as described in Section 2. In the scatter plot of double variable flow cytometry, LL quadrant (FITC −/ PI −) shows living cells; UR quadrant (FITC +/PI +) stands for late apoptotic cells; and LR quadrant (FITC +/PI −) represents early apoptotic cells. *Lower panel* shows percentage of cells undergoing early or late apoptosis upon treatment on breast cancer cells with PFOA. Vertical bars show the average of 3 independent experiments ± S.E.M.

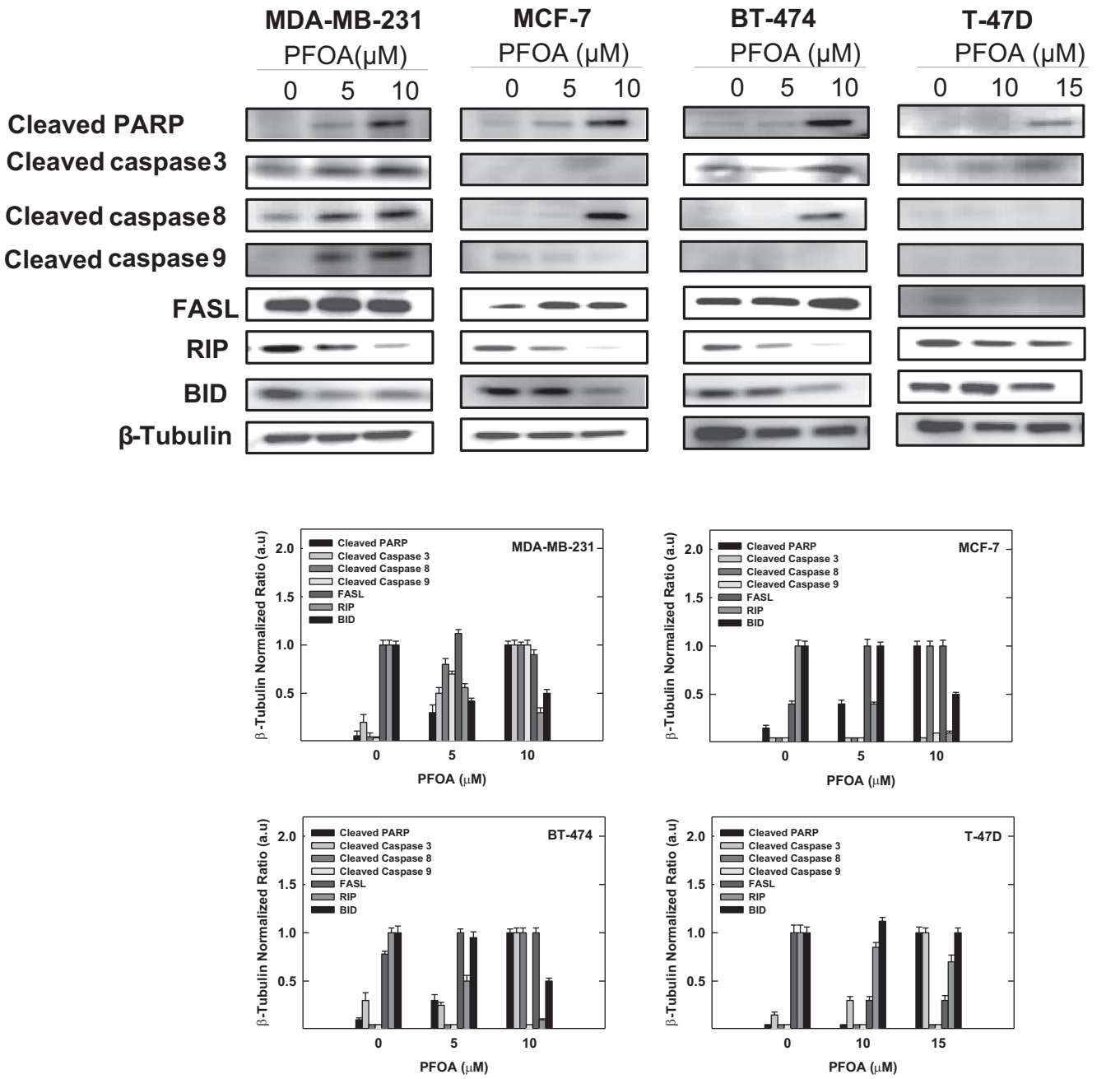


Fig. 6. Western blot analysis of relative levels of apoptotic proteins after PFOA treatment for 24 h in breast cancer cells. Cells were plated at a density of 4×10^6 cells/100 mm culture plate and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 0.5% FBS for 24 h. At the end of treatment period, cells were lysed and equal amounts of whole cell extracts were fractionated on SDS-PAGE gels and immunoblotted as described in Section 2. Scanning densitometric analysis was performed on all blots done in triplicate and the integrated optical density of each band was normalized with corresponding β-tubulin, as shown in bar graphs below their respective Western blot images. Vertical bars in the graph indicate the normalized integrated optical density of bands visualized in each lane ± S.E.M.

while it had no effect on Fas ligand (FASL) expression (Fig. 6). The pattern of apoptotic protein expression seen in PFOA-treated MDA-MB-231 cells is markedly different from changes induced by PFOA in the other three breast cancer cell lines. In BT-474 cells, PFOA treatment activated cleavage of PARP-1, caspase-3, caspase-8, BID and RIP, but not caspase-9, and increased the expression of FASL. Identical changes were seen in MCF-7 cells except for a lack of expression of caspase-3, which has been previously reported (Leist et al., 1998). In T-47D cells, PFOA treatment activated cleavage of PARP-1 and to a little extent caspase-3, while it had no effect on the cleavage of caspase-8, caspase-9, RIP, BID or on the expression of FASL (Fig. 6).

3.6. Effect of PFOA treatment on EGFR, HER-2, MET and ER protein expression

The effects of PFOA treatment for 24 h at apoptotic doses on EGFR, HER-2, MET and ER protein expression in breast cancer cells were evaluated by Western blotting (Fig. 7). PFOA treatment down-regulated EGFR and Met in MDA-MB-231 cells. PFOA treatment caused downregulation of HER-2, Met and ERα in both MCF-7 and BT-474 cells. Moreover, it reduced EGFR protein expression in BT-474 cells. PFOA treatment caused a dose-dependent reduction of EGFR, HER-2 and ERα protein expression in T-47D cells (Fig. 7).

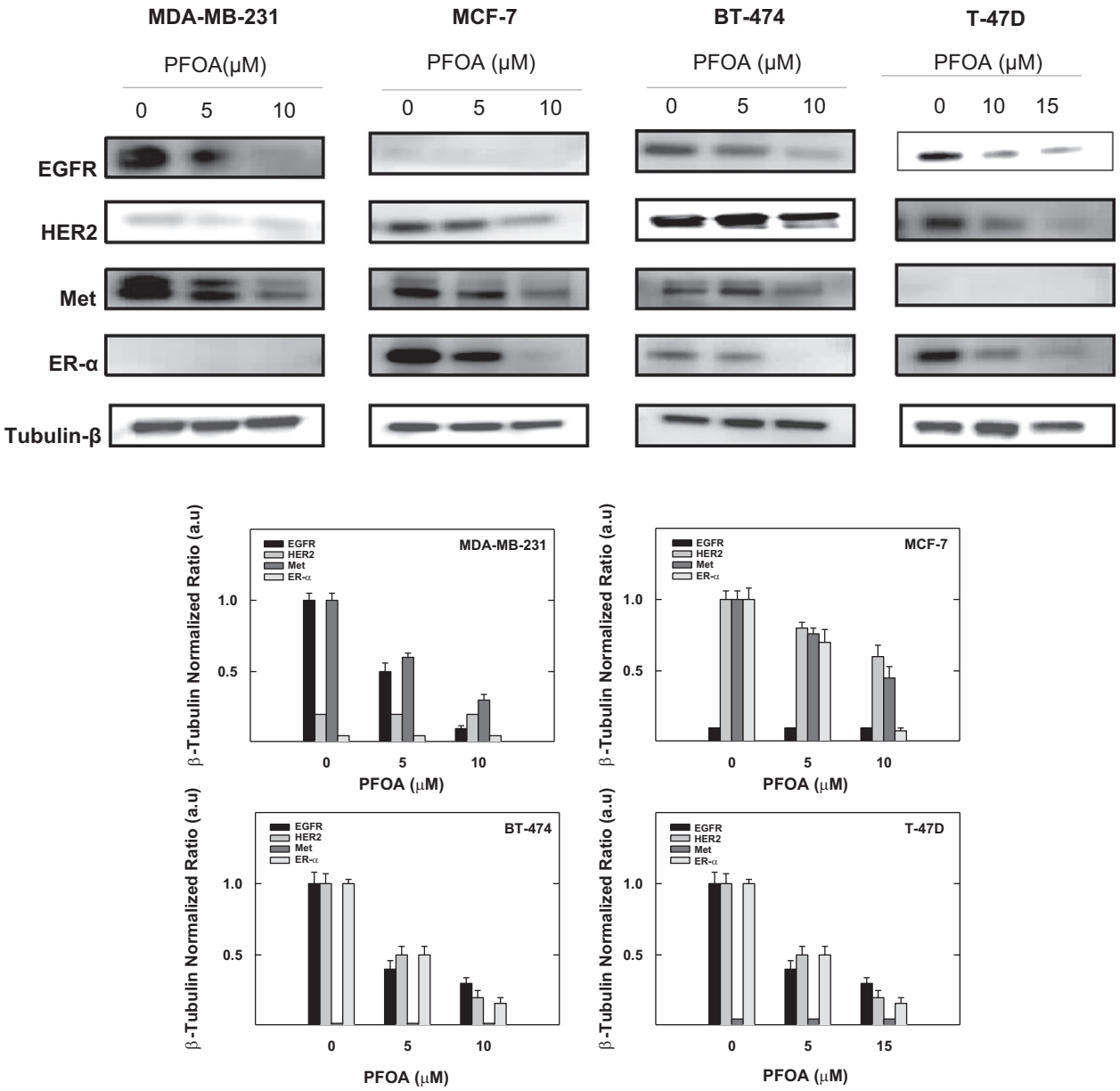


Fig. 7. Western blot analysis of relative levels of key breast cancer proteins after PFOA treatment for 24 h in breast cancer cells. Cells were plated at a density of 4×10^6 cells/100 mm culture plate and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight. The next day, cells were divided into different treatment groups and then given various treatments in RPMI-1640 medium containing 0.5% FBS for 24 h. At the end of treatment period, cells were lysed and equal amounts of whole cell extracts were fractionated on SDS-PAGE gels and immunoblotted as described in Section 2. Scanning densitometric analysis was performed on all blots done in triplicate and the integrated optical density of each band was normalized with corresponding β-tubulin, as shown in bar graphs below their respective Western blot images. Vertical bars in the graph indicate the normalized integrated optical density of bands visualized in each lane ± S.E.M.

4. Discussion

Results of the present study clearly demonstrate that PFOA, a structurally optimized derivative of the natural product OA (Fig. 1), has shown enhanced cytotoxicity and proapoptotic activity compared to OA against human breast cancer cells. Acute treatment with PFOA caused a significant reduction of breast cancer cell viability which was associated with morphological changes, chromatin condensation and formation of apoptotic bodies. The cell lines in this study were chosen as they represent a wide range of breast cancer phenotypes. ERα is expressed in MCF-7, BT-474 and T-47D cells, whereas MDA-MB-231 cells lack expression of ERα due to epigenetic silencing (Holliday and Speirs, 2011; Pledgie-Tracy et al., 2007). In addition, EGFR is expressed in T-47D, BT-474 and MDA-MB-231 cells while HER-2 is expressed at lower levels in MDA-MB-231 cells and expressed at higher levels in MCF-7, BT-474 and T-47D cells. Met is expressed in MDA-MB-231, MCF-7 and BT-474, while it is absent in T-47D cells.

Our previous studies suggested that PFOA significantly blocked proliferation, migration and invasion of the highly invasive MDA-MB-231 breast cancer cells (Elsayed et al., 2014). This effect might be related, at least in part, to the suppression of Brk/Paxillin/Rac1 signaling pathway. In addition, treatment with PFOA showed marked inhibition of phosphorylation (activation) of Akt and ERK1/2 which are important signaling molecules for survival and proliferation, respectively, without affecting their total levels (Elsayed et al., 2014).

Results of the present study revealed that triple negative MDA-MB-231 human breast cancer cells are the most sensitive to the cytotoxic effects of PFOA compared to other cell lines as 10 μM was

required to cause 96% of MDA-MB-231 breast cancer cells to undergo apoptosis after 24 h. Triple negative breast cancer is characterized by a lack of the expression of the ER, progesterone receptor and HER-2 (Carey et al., 2007; Kaplan and Malmgren, 2008; Sørlie et al., 2001). It accounts about 17% of all breast cancers, represents an aggressive clinical behavior and is generally associated with poor prognosis; thus chemotherapy remains the only systemic treatment option available for these patients (Carey et al., 2007; Kaplan and Malmgren, 2008; Sørlie et al., 2001). In addition to triple negative cell line, PFOA showed potent cytotoxicity in ER-positive, HER-2-positive and Met-expressing breast cancer cells. Treatment with 10 μM PFOA downregulated EGFR, HER-2, Met and ERα expression in breast cancer cells.

Apoptosis, the prevalent form of programmed cell death, plays a fundamental role in the development and homeostasis of all multi-cellular organisms (Danial and Korsmeyer, 2004; Horvitz, 2003; Rathmell and Thompson, 2002). There are two well-identified apoptotic pathways in cells (Wang, 2001). The extrinsic pathway is initiated by ligand-induced activation of death receptors at the plasma membrane (Nagata, 1999; Peter and Krammer, 2003). The intrinsic cell death pathway, on the other hand, is triggered by cellular stress signals such as DNA damage (Wang, 2001). The mechanism of apoptosis is remarkably conserved across species, involving a cascade of sequential activation of initiator and effector caspases, cysteine proteases with aspartate substrate specificity (Riedl and Shi, 2004; Thornberry and Lazebnik, 1998). Apoptotic signals are generally mediated through caspase activation controlled by one of two distinct pathways that are associated with either caspase-8 (death receptors) or caspase-9 (mitochondria) (Nagata, 1999; Peter and Krammer, 2003). Many chemotherapeutic agents have been shown to cause apoptosis through caspase-dependent pathway. Caspase-3 is an executioner caspase, which upon activation can systematically demolish cells through cleaving PARP-1 then lead to DNA fragmentation (Nagata, 1999; Peter and Krammer, 2003). It has been reported that BID, a proapoptotic Bcl_2 family member, is a specific substrate of caspase 8 in the Fas apoptotic signaling pathway (Li et al., 1998). While full-length BID is localized in cytosol, truncated BID translocates to mitochondria and leads to loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation in a caspase-dependent fashion (Li et al., 1998). Besides BID and downstream caspases, RIP is another critical substrate of caspase-8 (Lin et al., 1999). Whereas cleavage of BID and downstream caspases amplifies apoptotic signal, cleavage of RIP shuts off the protective pathway and enhances killing (Lin et al., 1999).

Although PFOA inhibits cell viability in a similar manner in the breast cancer cell lines evaluated, the activation of apoptosis occurs in a cell type-specific manner. In MCF-7 and BT-474 cells, apoptosis seems to be initiated with an upregulation of Fas ligand (FASL), which results in activation of caspase-8, caspase-3 (in BT-474 cells only as caspase-3 is not expressed in MCF-7), RIP and BID, leading to the proteolytic cleavage of PARP-1. In MDA-MB-231 cells, apoptosis seems to be initiated through an internal, mitochondrial pathway. In this cell line, PFOA activates caspase-9, caspase-3, BID, RIP and PARP-1 cleavage in a dose-dependent manner. The mechanism of induction of apoptosis in T-47D cells by PFOA is not yet fully understood. Although treatment of cells with 15 μM PFOA showed significant cytotoxicity, changed the morphology of cells and increased PARP-1 and caspase-3 cleavage, it did not induce caspase-8 or caspase-9. It also had no effect on RIP and BID protein expressions. It is worth mentioning that cells that express c-Met (MDA-MB-231, MCF-7, and BT-474 cells) are more susceptible to apoptosis by PFOA compared to cells which lack c-Met RT kinase (T-47 D cells). It is believed that PFOA might affect the expression of c-Met at the gene level or increase its proteasomal degradation (Akl et al., 2014). These findings may indicate that PFOA sensitizes human breast cancer cells to induce apoptosis, at least in part, by affecting c-Met expression.

5. Conclusion

Natural products have documented success in the area of anticancer chemotherapy either intact or as semisynthetic parents. This study shows that PFOA, a structurally-close analog of the common dietary supplement oleanolic acid, inhibits the growth of breast cancer cells, regulates the expression of apoptosis-related proteins, and induces apoptosis in breast cancer cells. Thus, PFOA may be a potential candidate therapeutic agent for the control of breast cancer.

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