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Methyl jasmonate decreases membrane fluidity and induces apoptosis via tumor necrosis factor receptor 1 in breast cancer cells

Laxmi Yeruva[a,b], **J. Abiodun Elegbede**[a], and **Stephen W. Carper**[a]

[a]Chemistry Department, University of Nevada, Las Vegas 4505 Maryland Parkway, Las Vegas, NV 89154-4003

Abstract

In recent years, studies with plant compounds have shown both chemotherapeutic and chemopreventive properties. The current study with plant stress hormones (jasmonates) showed growth inhibitory effects in breast cancer cells. Cis-jasmone (CJ) and methyl jasmonate (MJ) inhibited the long-term proliferation of MDA-MB-435 and MCF-7 cells. Cell cycle analysis showed G0/G1 and S-phase arrest with increasing apoptotic population. Cellular signaling studies with MJ showed decreased membrane fluidity and activation of extrinsic and intrinsic apoptotic pathways. Specifically in extrinsic apoptotic pathway increased expression of TNFR1, activation of MAPK and caspase-8 was observed. MJ also decreased the mitochondrial membrane potential and activated caspase-3 in breast cancer cells. In conclusion our results revealed novel signaling mechanism of MJ in breast cancer cells, indicating that MJ could have potential applications for chemotherapeutic purposes.

Keywords

Plant compounds; caspase-8; MAPK; Membrane fluidity; Breast Cancer

Introduction

Breast cancer is the second leading cause of cancer deaths among women in the US [1]. An estimated 40,910 breast cancer deaths and 180, 510 new cases are anticipated among women in 2007 [1]. Chemotherapy, radiation, surgery and immunotherapy are among the current treatment options for breast cancer. Chemotherapy using synthetic compounds, although shown to be effective in cancer treatment, also induces severe side effects due to their toxicity in noncancerous tissues. In recent years, studies have shown that plant compounds have chemotherapeutic and pharmacological activities against many types of cancers and could be utilized as alternative chemotherapeutic agents with low toxicity [2-7].

Jasmonates consists of cyclopentenone moiety, are synthesized in plants in response to injury, insect attack and wounding [8]. Several reports have demonstrated that cyclopentenone moiety containing molecules such as prostaglandins and clavulones induce apoptosis in different signaling mechanisms [9]. Cis-jasmone (CJ) and methyljasmonate (MJ) belong to the jasmonte family of compounds. Jasmonates have been reported to inhibit the growth of leukemia cells, lung, breast and prostate cancer cells [10]. To date, three separate mechanisms have been

bFor Correspondence: Venkat Laxmi Yeruva, Biochemistry Department University of Arkansas for Medical Sciences Little Rock AR 72205, Telephone:501-686-7254 FAX: 501-686-8169, Email: vlyeruva@uams.edu, lyeruva@gmail.com.

proposed to explain the anticancer effects of jasmonates. These include: (i) the ability of MJ to open the mitochondrial permeability transition pore complex, resulting in the release of cytochrome c, and induction of apoptosis; (ii) the ability of MJ to induce the re-differentiation of leukemia cells; and finally (iii) the ability of MJ to induce the expression of reactive oxygen species [10-12].

Apoptosis is a cellular suicide mechanism that occurs by extrinsic or intrinsic mechanisms [13]. The extrinsic apoptotic pathway involves a superfamily of death receptor ligands such as tumor necrosis factor alpha (TNF- α, TNF-related apoptosis inducing ligand (TRAIL) and FAS (CD95/APO-1) (14). In breast cancer cells, TNF-α plays an important role in cellular responses, including inflammation and apoptosis [15-17]. TNF–α exerts its biological functionality by binding two membrane receptors, tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2) [16]. The majority of TNF signaling pathways are attributable to TNFR1, which can bind both membrane bound and soluble TNF whereas TNFR2 can only be activated by membrane bound TNF [16]. Activation of these receptors recruits FADD (Fas associated death domain) and TRADD (TNF receptor-associated death domain) which results in the activation of caspase-8, mitogen activated protein kinase (MAPK) and ultimately cell death [16]. Several studies have reported that both caspase-8 and MAPK can either directly activate caspase-3 via Bid or activate the intrinsic apoptotic pathway [16,18].

The MAPK pathway is part of the extrinsic apoptotic mechanism and plays an important role in regulating a number of downstream molecules including kinases and scaffold proteins. The balance between these molecules exerts cellular responses including cell proliferation, cell cycle arrest, migration, differentiation and apoptosis. Studies conducted by Takenaka et al. indicated that the p38-MAPK pathway was activated during mitotic arrest in mammalian cultured cells [19-27]. Bulavin et al. also reported that p38α-MAPK was activated in response to UV radiation and induced G2/M cell cycle arrest by decreasing CDC2 activity [22]. CDC2 can phosphorylate BAD and induce neuronal apoptosis [23]. Additionally, it has been reported that ERK1 and ERK2 kinases are activated by phoshorylation in reponse to cell death induced by external stressors [24-27]. Activation of MAPK results in signal transduction to intrinsic apoptotic proteins which ultimately results in cell death.

Activation of the intrinsic apoptotic pathway can occur either by caspase-8/caspase-2 mediated Bid cleavage or by direct stress to the mitochondria. Once the intrinsic apoptotic pathway has been activated the mitochondrial membrane potential decreases and cytochrome c is released [28-30]. Cytochrome c can form an apoptosome complex with apaf-1 (apoptotic protease activating factor-1) and procaspase-9. The apoptosome complex then activates caspase-3, caspase-6 and caspase-7 which results cell death [31].

The effects of CJ and MJ on breast cancer cells were studied using cytotoxicity, proliferation, DNA content analyses, apoptotic assays, ELISA and western blotting techniques. This study showed that both CJ and MJ inhibited the growth of breast cancer cells. Cell cycle analysis and apoptotic assays showed that CJ and MJ induced cell cycle arrest and apoptosis in breast cancer cell lines. Studies on membrane fluidity demonstrated that MJ decreased membrane fluidity, probably activates apoptotic pathways. Specifically, our study revealed that MJ increased the expression of TNFR1 and further activated extrinsic apoptotic proteins, caspase-8, and MAPK. Intrinsic apoptotic mechanism showed that MJ decreased mitochondrial membrane potential and activated caspase-3 in breast cancer cells. The results indicated that MJ induced apoptosis was associated with a decrease in membrane fluidity and intracellular signaling via TNFR1, MAPK, caspases-8, and caspase-3.

Materials and methods

Chemicals and reagents

Cell culture media (MEM), fetal bovine serum (FBS) and penicillin (1000 units/ml) and treptomycin (1000 μg/ml) (P/S) were purchased from GIBCO (Grand Island, NY). Cis-jasmone (CJ), methyl jasmonate (MJ), protease inhibitors, propidium iodide (PI), deoxycholic acid (DCA), 1, 6-biphenyl-1, 2, 6-trihydrazine (DPH) and ribonuclease were obtained from Sigma Chemical Company (St. Louis, MO). Phosphate buffered saline (PBS, lacking Ca^{2+} and Mg^{2+}) was purchased from Invitrogen Corporation (Grand Island, NY). Sodium dodecylsulfate, bisacrylamide, N, N, N, N^1 – tetra-methylethylenediamine (TEMED) and ammonium persulfate were obtained from Bio-Rad Laboratories (Hercules, CA). Primary antibody to caspase-3 was obtained from Pharmingen (Franklin Lakes, NJ) and caspase-8 was from EMD Biosciences (San Diego, CA). Enzyme conjugated horse raddish peroxidase (HRP) secondary antibody and standards were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ECL+ western blotting detection system was obtained from Amersham Biosciences (Piscataway, NJ). All other reagents and chemicals were of the purest grade and were obtained from reputable vendors.

Cell culture

Breast cancer cell lines (MDA-MB-435 and MCF7) were obtained from ATCC. All cultures were maintained in Minimum Essential Media (MEM) supplemented with 10% FBS, 1% pencillin and streptomycin (1000units/ml) and HEPES buffer. All the cultures were maintained at 37°C in humidified 95% O_2 /5% CO_2 atmosphere. During exposure to the jasmonate compounds, the medium was replaced with treatment medium and control cells received medium containing DMSO (0.1%-0.3%).

Cytotoxicity assay

Cells (~5000) were plated in 96-well plate and incubated overnight to allow adherence to the plate surface. Cells were exposed to varying concentrations of CJ or MJ for 24 hr. At termination, the medium was replaced with 100μl of fresh medium; 10μl of alamarBlue was added and incubated for 4 hr. After 4 hr fluorescence was measured at 530 nm of excitation and 590 nm of emission wavelengths with Genios fluorescence plate reader (PHENIX Research Products, Hayward, CA). Cell viability was calculated as percentage of control (100%).

Colony formation assay

The long-term (delayed) cytotoxic effects of agents on the breast cancer cells were determined using the colony formation assay (CFA). The cells were exposed to 0.5, 1.0 and 2.0 mM concentrations of the compounds for 24 hr. The treatment medium was poured off and the cells were washed with 1X PBS, harvested with trypsin EDTA, counted and replated into 60mm dishes. Cells were incubated in humidified 95% O_2 /5% CO_2 atmosphere at 37°C for 11 days. The medium was decanted and cells were stained with crystal violet (0.5g/100ml in 95% ethanol). Colonies (>50 cells/colony) were counted and the percentage survival (% viability) was calculated relative to the control group (100%).

Cell cycle analysis with flow cytometry

Cells (~0.25×10^6) were plated in 100mm tissue culture plates, incubated overnight to adhere to the plate. Cells were exposed to 0.5mM and 3mM CJ or MJ for 24hr and washed with 1X PBS. Fresh and complete medium was added and the cells were incubated for 24 hr. MDA-MB-435 cells were also exposed to 500 μM of DCA for 4 hr, 8 hr and 24 hr. Cells were harvested with trypsin EDTA, washed with PBS, counted, fixed with 100μl of PBS and 900μl of absolute ethanol and stored at 4°C prior to DNA content analyses. Samples were stained with DNA

staining solution containing 150μg/ml of propidium iodide (PI), 0.1% Triton x-100 and 1mg/ml of RNase-A (DNase free) (1:1:1 by volume) and incubated in the absence of light for one hour. Acquisition of the data was carried out with FACSCalibur flow cytometer (Becton Dickinson, Sanjose, CA) and analyzed with ModfitLT 3.0 software (Verity Software House, Topsham, ME).

Detection of apoptosis by Hoechst and DNA fragmentation

Cells (~0.01×10^6) were plated in 4-well slides and incubated for 24 hr prior to treatment. Cells were exposed to 3.0mM CJ or MJ for 24 hr, at termination the medium was removed by aspiration and the cells were then stained with Hoechst (2μg/ml Hoechst) for 15 min. Morphological changes indicative of apoptosis were observed and the images acquired with fluorescence microscopy (Nikon Instruments Inc. Melville, NY). The number of cells in the bright field and Hoechst stained cells were counted and the percentage of apoptotic cells was calculated as:

$$\%\ \text{apoptosis} = (\text{Number of Hoechst positive cells}/\text{Number of cells in bright field}) \times 100$$

Cells (~1×10^6) were seeded in 75mm tissue culture flasks and treated with 1, 2 or 3mM MJ for 24 hr. Cells treated with DCA (500 μM) for 4 hr and 8 hr were used as positive controls. At termination cells were harvested with trypsin EDTA, washed with 1X PBS and the DNA was extracted as described in an earlier study (32). Briefly, cells (~1×10^6) were suspended in 100μl of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X- 100 for 10 min at 4° C. Cells were centrifuged at 13,000 rpm for 5 min and RNase A (20μg/ml) was added to the supernatant and incubated for 1 hr at 37° C. Following incubation, Proteinase K was added to a final concentration of 20μg/ml and incubated for 30 min at 50° C. DNA was precipitated with 42mM NaCl and 50% isoproponal overnight at -20° C. The DNA pellet was obtained by centrifugation for 15 min at 13, 000 rpm. The DNA pellet was resuspended in 20μl TE buffer (10mM Tris-HCl and 1mM EDTA pH 8.0) and 10μl of the DNA solution was electrophoresed on a 2% agarose gel in TBE buffer (89 mM Tris base, 89mM boric acid and 2.6 mM EDTA). The gel was stained with 1μg/ml ethidium bromide and photographed.

Membrane fluidity studies

Cells (~0.5×10^6) were plated in 75mm tissue culture flasks and incubated for two days. Cells were exposed to 3mM MJ, 10μM cholesterol, 500uM DCA for 4 hr. At termination cells were harvested and washed with 1X PBS. The fluorescent dye, DPH (2μM), was added and polarization was measured with LS 55 Luminescence Spectrometer (Perkin Elmer). The P value for the study was calculated as follows:

$$P=\frac{Ivv\ (GF\times Ivh)}{Ivv+(GF\times Ivh)} GF=Ihv/Ihh$$

TNFR1 Expression

TNFR1 expression was measured by flow cytometry as described previously [33]. MDA-MB-435 and MCF-7 cells were plated in 75mm tissue culture flasks, incubated for two days then exposed to 0, 1mM, 2mM or 3mM MJ for 24 hr. Following exposure, the cells were harvested with trypsin-EDTA (1mM), counted and ~1×10^6 cells were washed in 50% FBS at 4°C for 15 min. Cells were washed with PBS-FBS (PBS with 1% FBS added) and incubated

with 25μg/ml anti-human TNFR1 antibodies (R&D Systems) at 4°C for 60 min. Subsequently, cells were washed three times in PBS-FBS and incubated with FITC- conjugated goat anti-rat IgG (1:40 dilution) at 4°C for 2 hr. Cells were washed in PBS-FBS three times and analyzed with FACS Calibur flow cytometer. Fluorescence data was collected and background fluorescence was determined using unstained cells.

TNFR1 activation was confirmed by treating the cells with MJ in the presence of antibody. MDA-MB-435 cells (~5000/well) were plated in each well of a 96-well plate. After overnight incubation, the cells were exposed to 0, 3mM MJ, 3mM MJ with TNFR1 antibody, TNFR1 antibody alone, or a nonspecific antibody for 24 hr. At termination the medium was removed by aspiration. Fresh medium and alamarBlue were then added and the cells were incubated for 4 hr. Fluorescence was measured using Genios plate reader and the percent viability was calculated via comparison to the control (100%).

MAPK assay by ELISA

The p38 and ERK1/2 activity was determined by an ELISA assay kit (Super array, Frederick MD). Cells (~1.5×10^4) were plated in a 96-well plate and incubated overnight in humidified 95% O_2 / 5% CO_2 atmosphere at 37°C. The following day, cells were serum starved for 18-24 hr, exposed to MJ and assayed. Briefly, adherent cells were fixed to the plate, washed, blocked and incubated with a primary antibody followed by a secondary antibody. Developing solution was then added and the samples were allowed to incubate for 10 min at room temperature before the stop solution was added. Absorbance was measured at 450 nm. Relative phosphorylation was normalized to cell number according to manufacturer's instructions.

Caspase-8, 3, and membrane potential studies

The expression of caspase-3 and caspase-8 was determined using western blot analysis. Control or treated cells (~1.0×10^6) were harvested with trypsin EDTA, pelleted in microfuge tubes at 1000 rpm for 10 min and stored at -80°C and protein extraction was carried out according to the manfacturer's instructions (Biosource Intl. Inc., Camarillo, CA). The cell pellet was thawed on ice for 2 hr prior to protein extraction. PMSF (1mM) and 100μl of extraction buffer containing protienase inhibitor (250μl/5ml) was added to ~1×10^6 cells, while mixing every 10 min for 30 min. The cell lysate was centrifuged at 13,000 rpm at 4°C for 10 min; supernatant was collected, aliquoted into tubes and stored at -80°C. Total protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL). Equal amount of protein was loaded, subjected to SDS-PAGE and transferred to PVDF membrane. The membrane was blocked for 1 hr at room temperature with blocking solution (10% nonfat dry milk solution + 0.05% tween 20%) and washed twice with 1X western wash (12.4 mM trizma, 37.4 mM NaCl, 0.5% TritonX-100, pH 7.4),. The blot was then incubated with primary antibodies (1μg/ml) to caspase-3 or caspase-8 for 1 hr at room temperature followed by washing three times with 1X western wash. Primary antibody was detected with 1:1000 diluted horse radish peroxidase (HRP) or fluorescenct secondary antibodies. HRP antibody signal was developed with ECL+ detection kit (Amersham Biosciences, Piscataway, NJ) and visualized with TYPHOON 9410 (Amersham Biosciences, Piscataway, NJ).

Caspase-3 activity was measured by flow cytometry as described in manufacturer's protocol (Biovision, CA). Briefly, cells were exposed to 3mM MJ for 24 hr, harvested, counted and washed with PBS. Caspase-3 substrate was added and washed with wash buffer (provided by the manufacturer) and activity was measured by flow cytometry. Caspase-8 activity was measured by fluoremetric assay as described in manfacturer's protocol (Sigma Chemical Company St. Louis, MO). Cells were exposed to 3mM MJ for 0, 2 or 4 hr, harvested, and the cell pellet was collected. Caspase-8 substrate was added and fluorescence was measured at excitation wavelength of 360 nm and emission wavelength of 440 nm.

Mitochondrial membrane potential was measured as described in the manufacturer's protocol (Cell technology, CA). Mitochondrial membrane potential detection kit, uses a fluorescent cationic dye, JC-1 (5,5', 6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbo-cyanine iodide) to signal the loss of mitochondrial membrane potential. In non-apoptotic cells, the dye accumulates in the mitochondrial matrix stains the mitochondria bright red and in apoptotic cells, due to mitochondrial membrane potential collapse JC-1 cannot accumulate with in the mitochondria and hence it remains in the cytoplasm in a green fluorescent monomeric form. MDA-MB-435 and MCF-7 cells were plated in 25mm flasks and incubated overnight. Cells were exposed to 0, IC50MJ (concentration that inhibits growth of 50% of cells) or 3mM MJ for 24 hr. At termination cells were harvested, counted, and washed with PBS. Cells (0.5×10^6) were stained with JC-1 reagent and incubated for 15 min at 37° C. Cells were washed twice with assay buffer (provided by manufacturer) and membrane potential was measured by flow cytometry.

Statistical analyses

The results were presented as Mean ± SEM of replicate analyses accompanied by the number of independent experiments. Statistical analyses were performed using one or two tailed t-test (Graphpad Software Inc., San Diego CA and Microsoft Excel). Differences at $p<0.05$ or better were considered statistically significant.

Results

CJ and MJ inhibited the growth of breast cancer cells

Proliferation inhibition of breast cancer cells was determined by cytotoxicity assays using almarBlue dye. Plant compounds CJ and MJ inhibited the growth of breast cancer cell lines *in-vitro*. In MDA-MB-435 cells, the concentration of CJ at which 50% of growth (IC50) was inhibited after a 24 hr treatment was 1.7 mM. The IC50 concentration was 2.5 mM for MCF-7 cells (Figure 1A). The IC50 concentration of MJ for MDA-MB-435 cells was 1.9 mM and 2.0 mM for MCF-7 cells (Figure 1B). CJ and MJ were not cytotoxic to CDC-1070-SK and MCF-10A normal cells (Figure 1C)

Long-term proliferation inhibition of breast cancer cells by CJ or MJ was evaluated by clonogenic survival assays. The concentration of CJ at which 50% of long-term proliferation of MDA-MB-435 and MCF-7 cells was inhibited (IC50) after 24 hr treatment was 1.2mM (Figure 2A). The IC50 of MJ for MDA-MB-435 cells was 1.2mM and 1.45mM for MCF-7 cells (Figure 2B). A time course with MDA-MB-435 and MCF-7 cells at 3mM CJ or MJ revealed a time dependent decrease in survival (Figure 2C and 2D). At 2 hr, CJ inhibited the long-term proliferation of 70% of MDA-MB-435 cells and 60% of MCF-7 cells (Figure 2C). MJ inhibited the long-term proliferation of 70% of both MDA-MB-435 and MCF-7 cells *in-vitro* (Figure 2D).

Cell cycle analysis

To investigate the ability of the jasmonate compounds to induce cell cycle arrest, DNA content analyses was carried out by propidium iodide staining using flow cytometry. Representative histograms of MDA-MB-435 and MCF-7 cells treated with CJ and MJ showed an increase in the apoptotic peak compared to the control (Figure 3A and 3C). CJ and MJ induced cell cycle arrest and apoptosis (Figure 3A-3D) as well as G0/G1 arrest in MDA-MB-435 and MCF-7 cell lines (Figure 3B and 3D). At 3mM MJ, MDA-MB-435 cells showed a block at S-phase while 3mM CJ showed an S-phase block in MCF-7 cells (Figure 3B and 3D). Apoptosis was observed only with 3mM MJ in both MDA-MB-435 and MCF-7 cells (Figure 3B and 3D).

Apoptotic studies have demonstrated that CJ and MJ induced apoptosis in breast cancer cells (Figure 4A and 4B). To confirm apoptosis induction, cells were stained with Hoechst and images were acquired with a fluorescenct microscope. The percentage of apoptotic cells were calculated as explained previously in the materials and methods section (Figure 4A). Cells treated with CJ or MJ revealed morphological changes in the cell shape from a full circle to an elongated oval, a change that is consistent with cells undergoing apoptosis , as well as a significant increase in the amount of apoptosis in breast cancer cells (Supplemental Figure 1). MDA-MB-435 and MCF-7 cells showed 35.0% and 37.2% apoptosis upon treatment with MJ and CJ induced 6.6% and 43.5% apoptosis in MDA-MB-435 and MCF-7 cells, respectively (Figure 4A). DCA induced G2/M arrest and apoptosis in MDA-MB-435 cells (Figure 4B).

To further characterize the levels of apoptosis induced in breast cancer cells by MJ and CJ, DNA fragmentation studies were conducted. After a 24 hr treatment with MJ, DNA laddering was not observed in MCF-7 cells, but a pronounced DNA smear was observed in MDA-MB-435 cells (Figure 4C). The DNA smear was observed with 3mM MJ, but not with the 1 or 2mM concentrations of MJ (Figure 4C). DNA laddering was also observed in MDA-MB-435 cells treated with DCA for 4 and 8 hr which is an indicative of apoptosis (Figure 4C).

Membrane fluidity

MJ is a hydrophobic molecule and therefore expected to decrease the membrane fluidity of cells. Cells treated with cholesterol and DCA were used as positive controls. MDA-MB-435 and MCF-7 cells were treated with 3mM MJ, DCA and cholesterol for 4 hr and fluidity decrease was determined statistically where an increase in P value was indicative of decrease in membrane fluidity. In both the cell lines decrease in membrane fluidity was observed compared to the control (Table 1).

Cellular signaling

Studies on the death receptors revealed that TNFR1 expression was increased and death signals were initiated in breast cancer cells. MJ Induced TNFR1 expression was dose dependent in both MD-MB-435 and MCF-7 cells (Figure 5A and 5B). Exposure of MDA-MB-435 cells to MJ in the presence of TNFR1 antibody showed increased cell viability which confirmed TNFR1 activation (Figure 5B). To confirm that MJ induced cell signaling through TNFR1 binding, caspase-8 and MAPK activity were measured. Increased p38 and ERK1,2 activity was observed after exposure to MJ treatment in MDA-MB-435 cells but not in MCF-7 cells (Figure 6). Activation of p38 was observed 2.0 hr after treatment and ERK1,2 activity was observed after 1.0 hr treatment with 3mM MJ in MDA-MB-435 but not in MCF-7 cells (Figure 6A, 6B, 6C and 6D). Caspase-8 was activated following 2 hr treatment in MDA-MB-435 cells treated with 3mM MJ (Figure 7A). Procaspase-8 was cleaved and activated until 8 hr in MDA-MB-435 cells but not in MCF-7 cells (Figure 7A, MCF-7 data not shown). Further confirmatory studies indicated 5 fold increase in caspase-8 activity in MDA-MB-435 cells compared to the control (Figure 7B)

To elucidate the involvement of mitochondria in response to MJ induced apoptosis, mitochondrial membrane potential and caspase-3 activity was measured. Cells treated with MJ showed a decrease in membrane potential and procaspase-3 activity compared to the control except in caspase-3 mutant cell line MCF-7 (Figure 8A, 8B, and 8C). Flow cytometry analysis demonstrated that caspase-3 activity was increased in MDA-MB-435 cell line, however, no change in MCF-7 cells was observed (Figure 8B).

Discussion and conclusion

Breast cancer is the second leading cause of cancer related deaths among women in the US. Alternative strategies are required to combat the deaths caused by this disease. Jasmonates are plant compounds, which have been shown to inhibit growth and promote apoptosis in lung and prostate cancer and breast cancer cells. However, apoptotic mechanism of these compounds has not been investigated in breast cancer cells. Our study demonstrated that jasmonates inhibit growth, induce cell cycle arrest and apoptosis in breast cancer cells. Apoptotic signaling mechanisms revealed that MJ increased the expression of TNFR1, activated caspase-8, p38, ERK1/2, and caspase-3 as well as decreased mitochondrial membrane potential in breast cancer cells. To the best of our knowledge, this is the first report to demonstrate death receptor TNFR1 expression, membrane fluidity decrease, caspase-8 activation in breast cancer cells.

Recent reports with MJ showed growth inhibition of lung cancer, breast cancer and leukemia cells [10]. It was reported that MJ induced apoptosis in human breast cancer cell line, MCF-7, and this study showed that CJ and MJ induced apoptosis in MDA-MB-435 and MCF-7 cell lines. Cell cycle analysis revealed that CJ and MJ induced a block at G0/G1 in MDA-MB-435 at all concentrations studied. In MCF-7 cells, CJ and MJ also induced a block at G0/G1 at all concentrations studied above 0.5mM. At 0.5 mM, MJ and CJ induced a block in S-phase. In MCF-7 cells though DNA smear was not observed with 3mM MJ treatment, apoptosis was observed. These results indicate that the observed cell death was due to cell cycle arrest and apoptosis.

Earlier studies reported that the hydrophobic molecule DCA changes membrane fluidity. DCA decreases membrane fluidity by rearranging cholesterol in the membrane which reduced membrane permeability [34]. It has been reported that DCA induced apoptosis involves activation of caspase-2, caspase-3, caspase-7 and caspase-8 in the HT-29 colon cancer cell line [35,36]. In HCT-116 colon cancer cells, DCA activated p38 and ERK1/2. Studies using pharmacological inhibitors specific to ERK1/2 revealed that elevated ERK1/2 activity suppressed DCA induced apoptosis. Taken together, these data suggest that DCA can activate different apoptotic proteins within different cell lines [35,36]. The mechanism for activation of apoptosis has not been proposed, however, activation of these molecules are likely the result of the activation of receptor molecules (i. e., TNFR1) due to changes in membrane fluidity in the presence of DCA. Given that MJ is a hydrophobic molecule like DCA, the plant compound may work similarly, by decreasing membrane fluidity which assembles receptors and leads to autophosphorylation and activation of death receptors. This study determined that MJ decreased membrane fluidity might have resulted in apoptotic signaling in cancer cells.

Previous report demonstrated that jasmonates affect the mitochondria, release cytochrome-c via permeability transition pore complex in leukemia cells with out effecting normal cells [10,11]. Compared to normal cells, several cancer cells have shown high mitochondrial membrane potential contributing to apoptosis resistance. Mitochondrial membrane potential decrease by dichloroacetate treatment induced apoptosis in cancer cells [37]. Similarly, jasmonate treatment showed a decrease in mitochondrial membrane potential and induced apoptosis in breast cancer cells. In non cancerous cells jasmonates treatment showed no cytotoxic effect (mitochondrial assay) on normal cells (Figure 1C) and does not increase TNFR1 expression (unpublished data). This indicates that jasmonates specifically act on cancerous cells and the observation is consistent with earlier report [10,11]. Our findings on breast cancer cells extend the apoptotic mechanism of jasmonates observed.

Previous studies have demonstrated that jasmonates induce growth arrest and re-differentiation of leukemia cells in mitogen-activated kinase dependent pathway [11]. The results presented here showed activation of p38 and ERK1/2 in MDA-MB-435 cells but not in MCF-7 cells. MJ

induced DNA fragmentation, hallmark of apoptosis in callus plant cells and we observed DNA fragmentation in MDA-MB-435 cells but not in MCF-7 cells. In MCF-7 cells lack of DNA fragmentation is possibly due to mutant caspase-3 and a different research group also demonstrated caspase-3 requirement in DNA fragmentation [38]. Hence in conjunction with the data provided from previous studies suggest that jasmonates have a chemotherapeutic effect by inducing multiple apoptotic signaling pathways in cancer cells which involveTNFR1, caspase-8, capase-3, MAPK, bax/bclx/s, and cytochrome-c [9-12].

In conclusion, plant compounds induced apoptosis is probably associated with TNFR1 expression. This study lends further insight into the apoptotic signaling mechanisms that are activated by MJ which can be exploited for therapeutic purposes. MJ has shown to act on three different types of cancer cells; hence it could be used for a wide variety of cancer treatments. To reduce the toxicity of conventional chemotherapy experienced by patients and to improve the efficacy of treatments, future studies using a combination of MJ with other chemotherapeutic agents need to be conducted.

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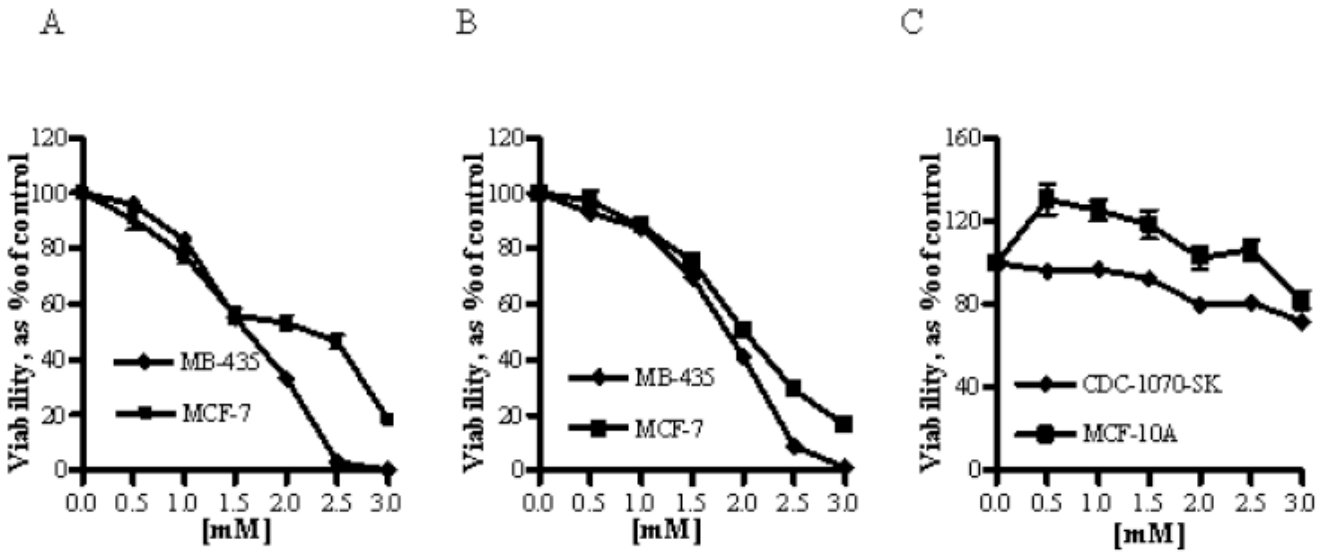

Figure 1.

Cytotoxicity of CJ or MJ on human breast cancer cells (MDA-MB-435 and MCF-7) as well as on normal human cells (CDC-1070-SK and MCF-10A) in culture. Panel A: MDA-MB-435 and MCF-7 cells were exposed to varying concentrations of CJ for 24 hr. Panel B: MDA-MB-435 and MCF-7 cells were exposed to MJ for 24 hr. Panel C: CDC-1070-SK and MCF-10A were exposed to different concentrations of MJ for 24 hr. The effects on cell viability were determined by mitochondrial dehydrogenase assay using alamarBlue dye as described in the methods section. Cell viability was calculated as a percentage of untreated cells (100%). Values were Mean±SD (n=6); results are representative of three independent experiments. At concentrations 1.5mM and higher there was a significant decrease in the viability of breast cancer cells compared to the control. One tailed t-test was carried out and at concentrations of 1 mM and above, a statistically significant ($p < 0.0001$) decrease in viability was observed. MJ was not cytotoxic to CDC-1070-SK and MCF-10A cells (Panel C). Results are representative of three different experiments.

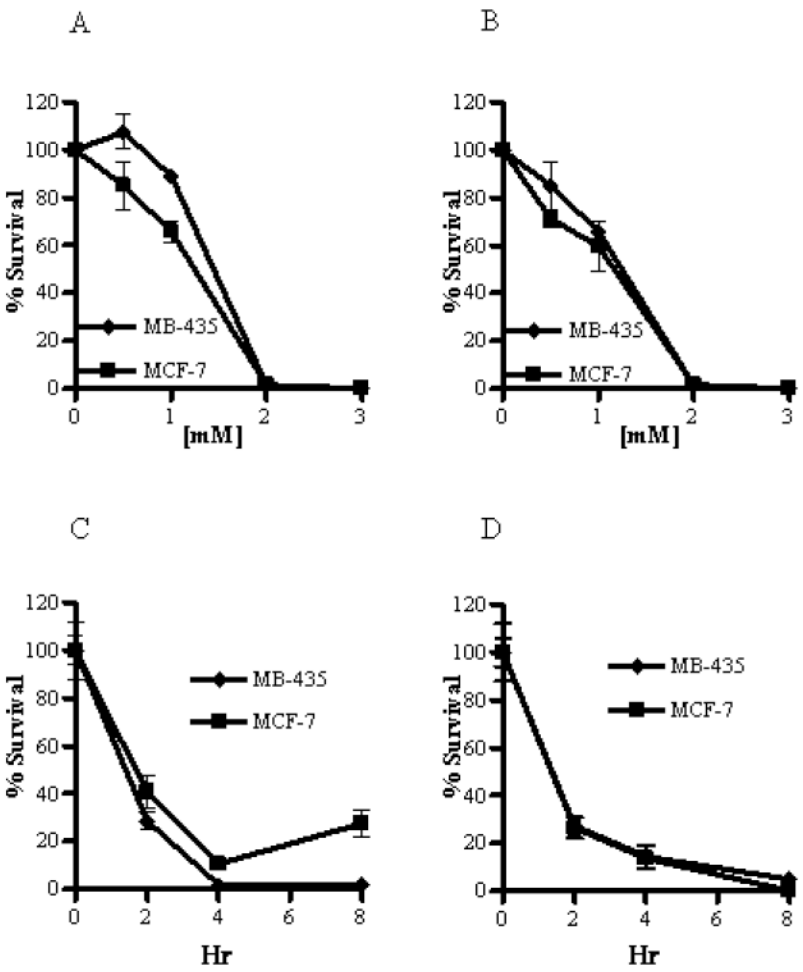

Figure 2.

Panel A-D: CJ and MJ inhibited the long-term proliferation of breast cancer cells. Panel A: MDA-MB-435 cells were exposed to varying concentrations of CJ or MJ for 24hr. Panel B: MCF-7 cells were exposed to varying concentrations CJ or MJ for 24 hr. Panel C: MDA-MB-435 and MCF-7 cells were exposed to 3 mM CJ for 0, 2, 4 and 8 hr. Panel D: MDA-MB-435 and MCF-7 cells were exposed to 3 mM MJ for 0, 2, 4 and 8 hr. At termination the drugs were washed off and the effects on long term cell proliferation were determined. Colonies (≥50 cells/colony) were counted and survival was calculated as percentage of control cells (100%). Values are Mean±SEM; results are representative of duplicate analyses of two separate experiments. CJ and MJ significantly inhibited the long-term proliferation of all two cell lines in dose and time dependent manner ($p<0.001$; one tailed t-test).

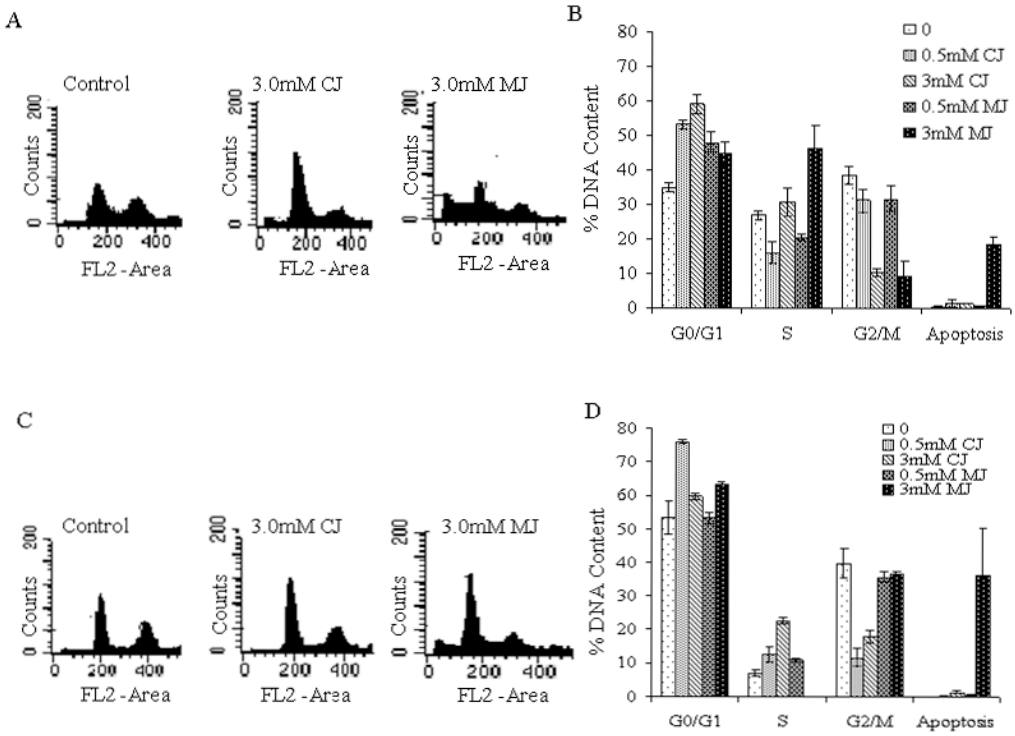

Figure 3.

Panel A: Representative histograms of MDA-MB-435 cells treated with 3mM CJ or MJ for 24 hr. Panel B: Quantitative graph of MDA-MB-435 cells treated with 0.5mM and 3mM CJ or MJ for 24 hr. Both CJ and MJ induced a significant G0/G1 block in MDA-MB-435 cells and 3mM MJ also induces S-block with increasing sub-diploid population. Panel C: Representative histograms of MCF-7 cells treated with 3mM CJ or MJ. Panel D: CJ and MJ induced G0/G1 block in MCF-7 cells and 3mM CJ also induced S-block while 3mM MJ increased sub-diploid population. (Two-tailed t-test, $p<0.05$). Results are representative of two different experiments.

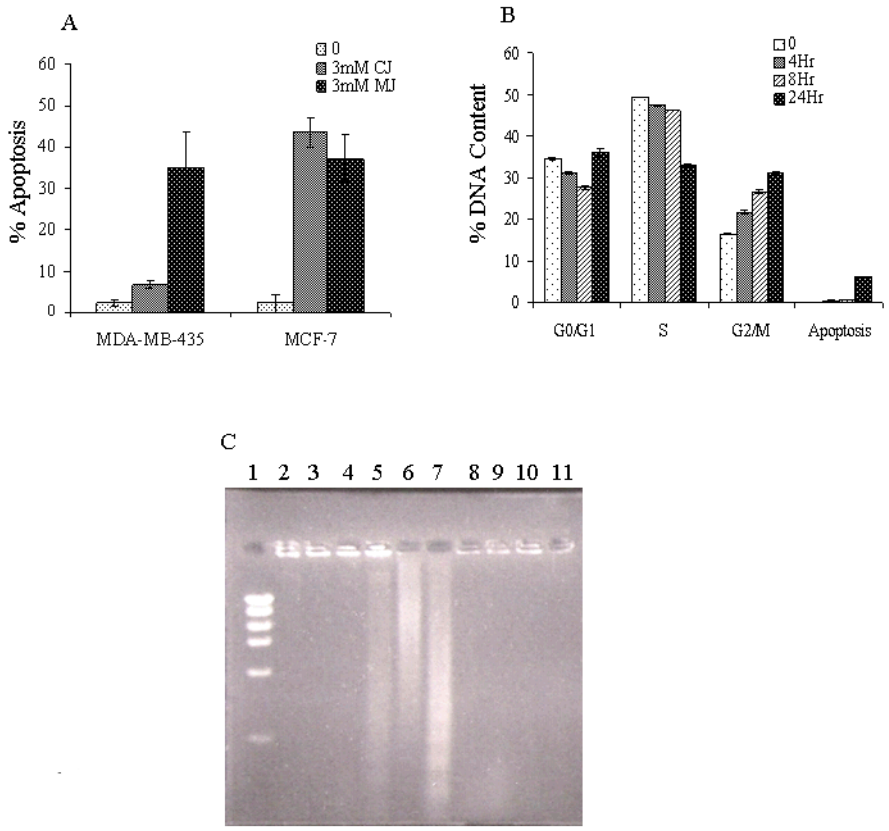

Figure 4.

Panel A: To confirm apoptosis induction breast cancer cells were stained cells with Hoechst and fluorescence microscopy images were acquired. Percent apoptosis was calculated as explained in the methods section. Both CJ and MJ induced apoptosis in breast cancer cells. Results are representative of two different experiments ($p<0.01$). Panel B: MDA-MB-435 cells were treated with DCA (500μM) for 4 hr, 8 hr and 24 hr. DCA induced G2/M arrest at all three time points measured and apoptosis at 24 hr. Panel C: DNA gel photograph of MDA-MB-435 and MCF-7 cells treated with MJ for 24 hr. Lane 1: High DNA Mass™ Ladder shows 1000, 2000, 3000, 4000, 6000, 10000 bp bands. Lane 2: MDA-MB-435 cells treated with 0.03% DMSO (Control). Lane 3: MDA-MB-435 cells treated with 1mM MJ. Lane 4: MDA-MB-435 cells treated with 2mM MJ. Lane 5: MDA-MB-435 cells treated with 3mM MJ. Lane 6: MDA-MB-435 cells were treated with DCA for 4hr. Lane 7: MDA-MB-435 cells were treated with DCA for 8 hr. Lane 8: MCF-7 cells treated with 0.03% DMSO (Control). Lane 9: MCF-7 cells treated with 1mM MJ. Lane 10: MCF-7 cells treated with 2mM MJ. Lane 11: MCF-7 cells treated with 3mM MJ. DNA smear was observed only in MDA-MB-435 cells treated with 3mM MJ and DCA treated cells were used as positive controls. Results are representative of three different experiments.

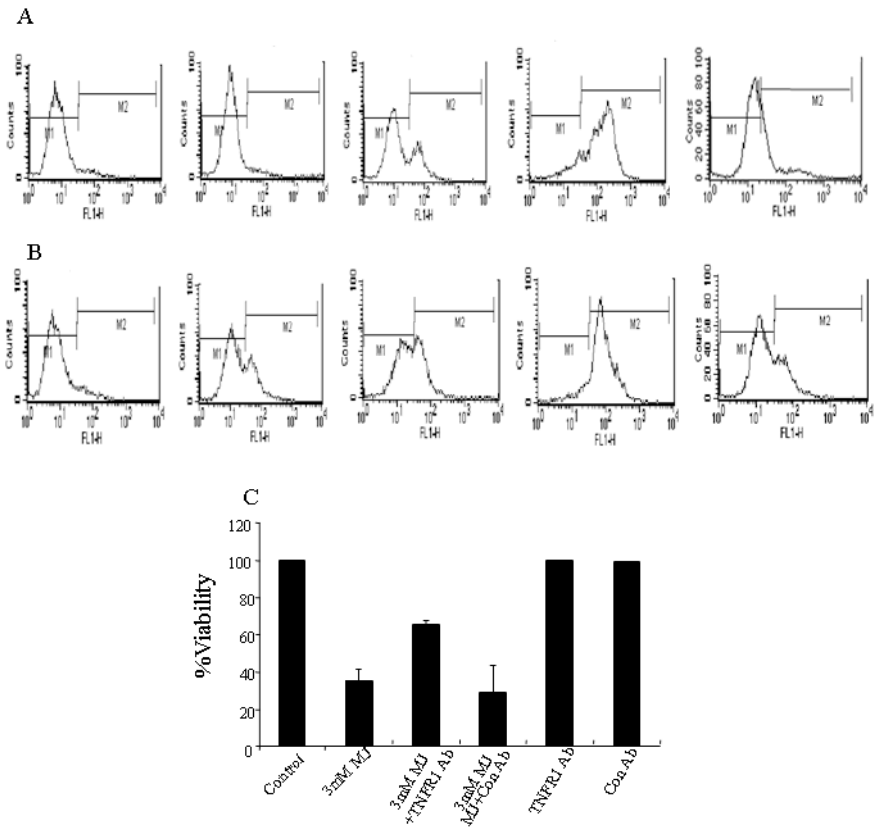

Figure 5.

Increase in TNFR1 expression and MAPK activity was measured as explained in the methods section. Panel A: From left, first histogram represents TNFR1 expression in MDA-MB-435 cells treated with DMSO (0.3%). From left, second histogram represents TNFR1 expression in MDA-MB-435 cells treated with 1mM MJ for 24 hr. From left, third histogram represents TNFR1 expression in MDA-MB-435 cells treated with 2mM MJ. From left, fourth histogram represents TNFR1 expression in MDA-MB-435 cells treated with 3mM MJ. Fifth histogram represents TNFR1 expression in MDA-MB-435 cells treated with 500 μM DCA, which showed increase in TNFR1 expression. Increase in TNFR1 expression was dose dependent and at 3mM MJ a complete shift in fluorescence intensity was observed. Panel B: From left, first histogram represents TNFR1 expression in MCF-7 cells treated with DMSO (0.3%). From left, second histogram represents TNFR1 expression in MCF-7 cells treated with 1mM MJ for 24 hr. From left, third histogram represents TNFR1 expression in MCF-7 cells treated with 2mM MJ. From left, fourth histogram represents TNFR1 expression in MCF-7 cells treated with 3mM MJ. Fifth histogram represents TNFR1 expression in MCF-7 cells treated with 500 μM DCA, which showed increase in TNFR1 expression. Increase in TNFR1 expression was dose dependent and at 3mM MJ a complete shift in fluorescence intensity was observed. Panel C: MDA-MB-435 cells were exposed to 3 mM MJ in the presence of TNFR1 antibody. Percent viability was increased which indicated TNFR1 expression in breast cancer cells. Cells treated with TNFR1 antibody and control antibody served as negative controls.

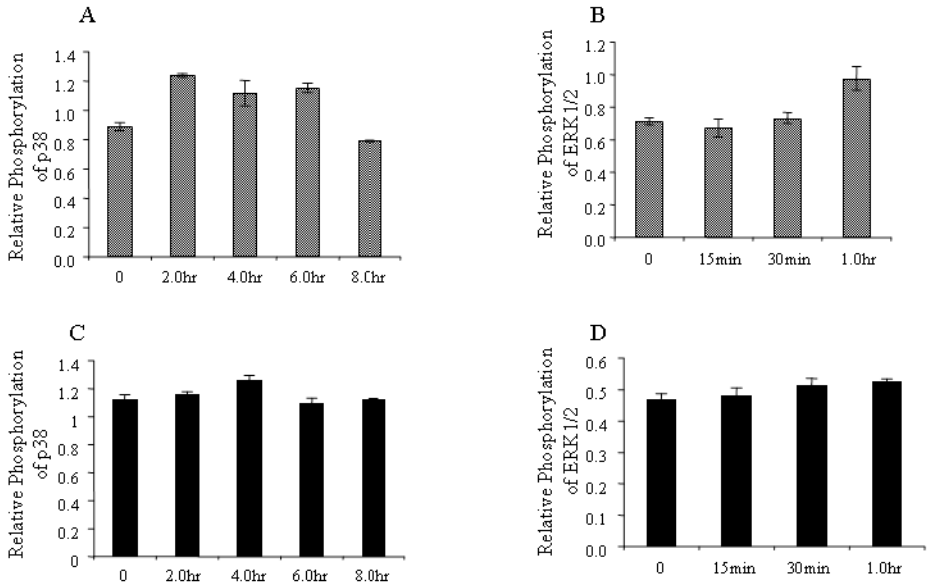

Figure 6.

MAPK activity was measured as explained in methods. Panel A: In MDA-MB-435 cells increase in p38 phosphorylation was significant at 2hr ($p<0.04$) and starts decreasing from 4 hr till 8 hr. Panel B: In MDA-MB-435 cells significant increase in ERK1/2 phosphorylation was observed at 1hr ($p<0.04$). Panel C: In MCF-7 cells p38 phosphorylation was not observed at all time points studied. Panel D: In MCF-7 cells ERK1/2 phosphorylation was not observed at all time points studied. Results are representative of two independent experiments.

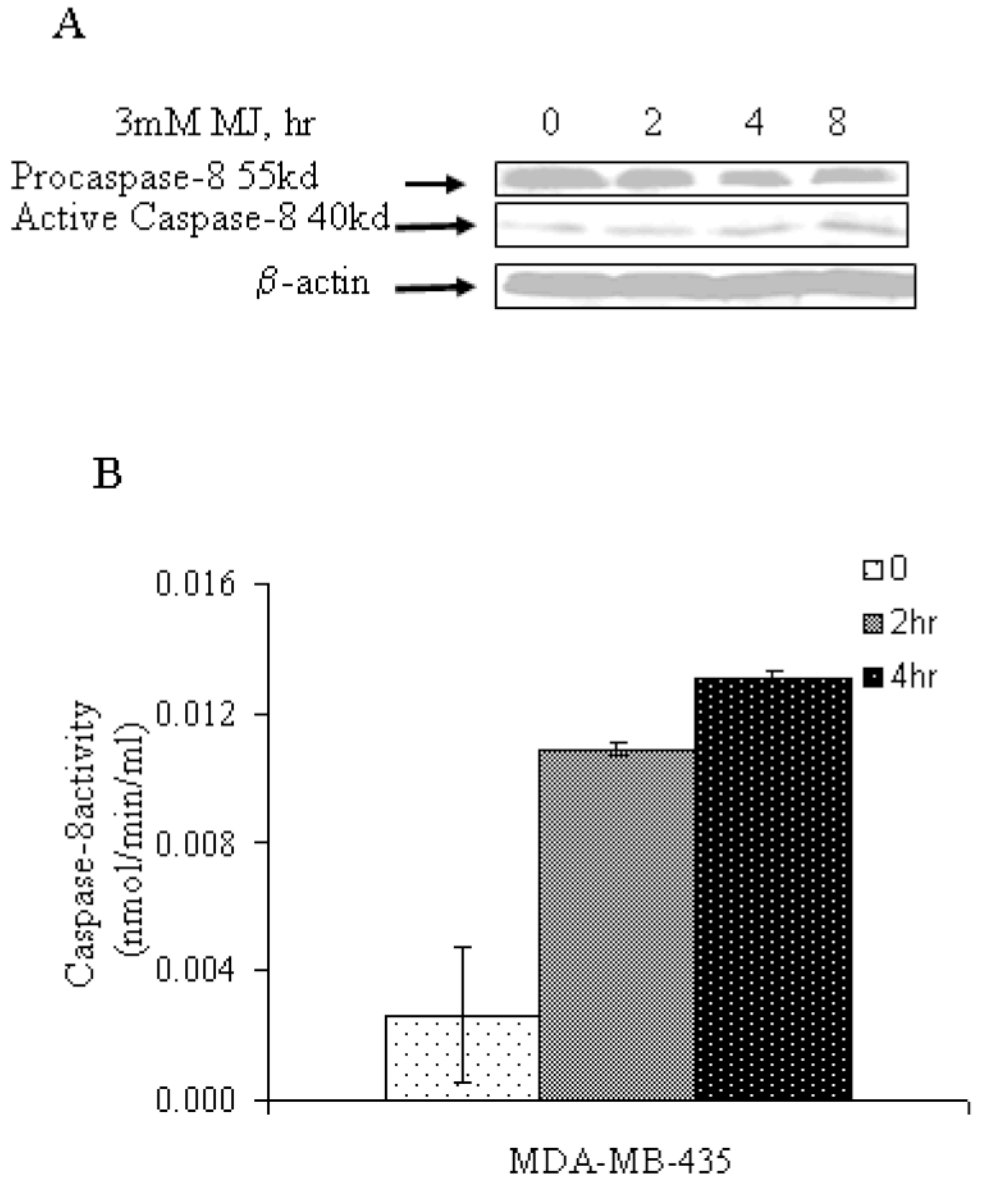

Figure 7.

Panel A: Western blot of caspase-8 in MDA-MB-435 cells treated with 3mM MJ for 2 hr, 4 hr and 8 hr. Procaspase-8 was processed and cleaved to active caspase-8 at all the time points studied. Caspase-8 activity was increased with 3mM MJ treatment in MDA-MB-435 cells compared to the control. β-actin was a loading control. Panel B: Fluoremetric assay showed increased caspase-8 activity with MJ treatment at 2 and 4 hr time points ($p<0.01$).

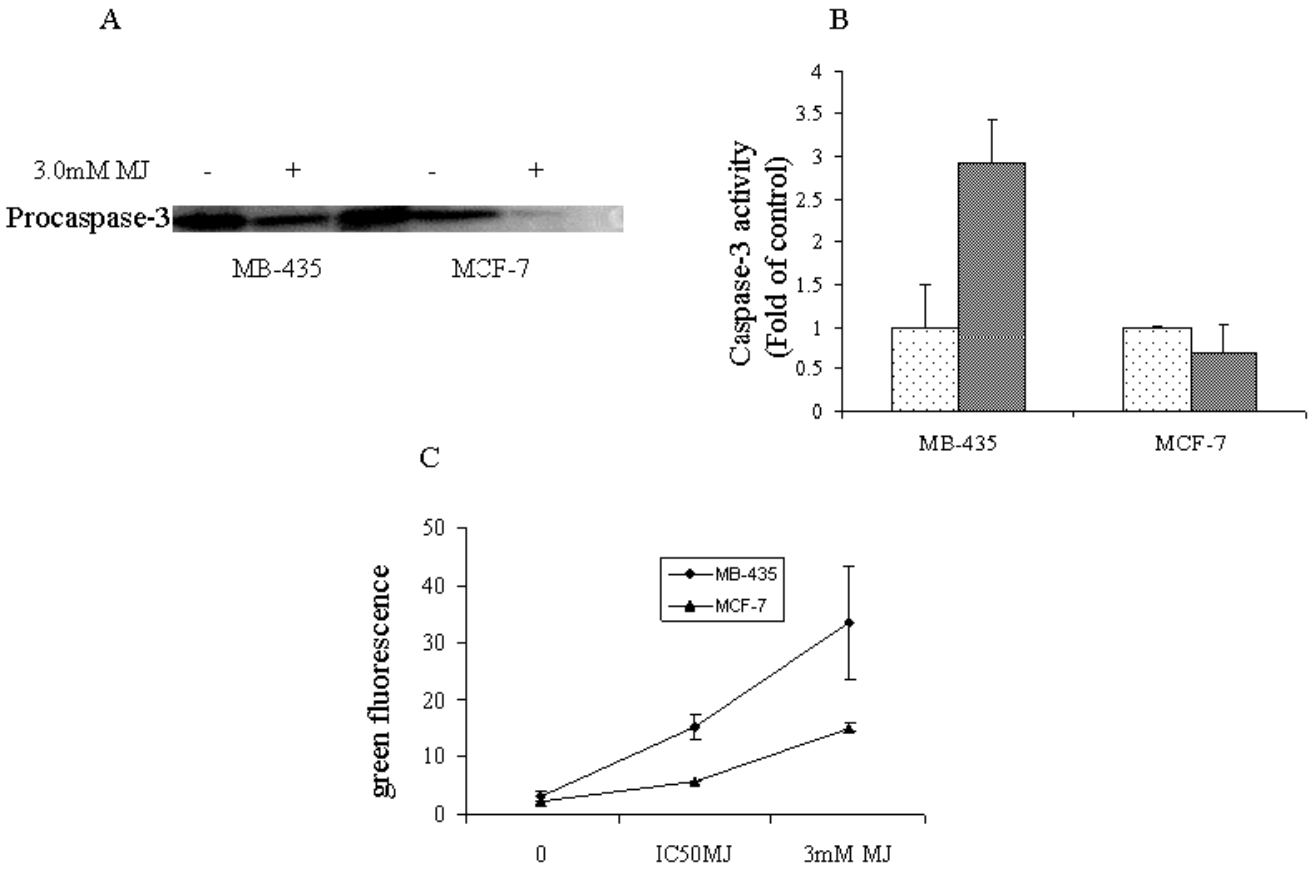

Figure 8.

MJ activated caspase-3 in MDA-MB-435 cells. Panel A: Western blot of procaspase-3. Decreased levels of procaspase-3 were observed in MDA-MB-435 cells treated with 3mM MJ. Panel B: Caspase-3 activity measurement by flow cytometry showed activation of caspase-3 in MDA-MB-435 cells but not in MCF-7 cells. Fold increase was calculated with respect to the control (1). Results are representative of two different experiments. Panel C: Mitochondrial membrane potential was determined in breast cancer cells as explained in methods. In both MDA-MB-435 and MCF-7 cells IC50 MJ and 3mM MJ decreased the membrane potential as observed by an increase in green fluorescence compared to the control ($p<0.001$).

Table 1

MJ decreased membrane fluidity in Breast cancer cells

Treatment (4hr)	MB-435	MCF-7
Control	0.29 ±0.05	0.32±0.005
Cholesterol	0.50 ±0.05	0.41±0.01
DCA	0.44 ±0.04	0.40±0.01
3mM MJ	0.40 ±0.05	0.41±0.01

MDA-MB-435 cells were treated with 3mM MJ for 4hr and polarization was measured and P value was calculated as explained in methods. There is significant decrease in membrane fluidity. ($p<0.05$, one tailed t-test)