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

Laxmi Yeruva, John Abiodun Elegbede and Stephen W. Carper

In recent years, studies with plant compounds have shown both chemotherapeutic and chemopreventive properties. This study with plant stress hormones (jasmonates) showed growth inhibitory effects in breast cancer cells. cis-Jasmone and methyl jasmonate (MJ) inhibited the long-term proliferation of MDA-MB-435 and MCF-7 cells. Cell cycle analysis showed G_0/G_1 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 TNF receptor 1, activation of mitogen-activated protein kinase 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. *Anti-Cancer Drugs* 19:766–776 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Chemistry Department, University of Nevada, Las Vegas, Las Vegas, Nevada, USA

Correspondence to Venkat Laxmi Yeruva, Biochemistry Department, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA
Tel: + 1 501 686 7254; fax: + 1 501 686 8169;
e-mail: vlyeruva@uams.edu; lyeruva@gmail.com

Introduction
Breast cancer is the second leading cause of cancer deaths among women in the United States [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 because of 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 methyl jasmonate (MJ) belong to the jasmonate 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 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 redifferentiation 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 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 is 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 Fas-associated death domain and 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 through 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.* [22] also reported that p38α-MAPK was activated in response to UV radiation and induced G_2/M cell cycle arrest by decreasing CDC2 activity. CDC2 can phosphorylate BAD and induce neuronal apoptosis [23]. Additionally, it has been reported that extracellular signal-related kinase (ERK) 1and ERK2 are activated by phosphorylation in response 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 or 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 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, enzyme-linked immunosorbent assay, 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 activated apoptotic pathways. Specifically, this 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 through TNFR1, MAPK, caspases-8, and caspase-3.

Materials and methods
Chemicals and reagents
Cell culture media (minimum essential media), fetal bovine serum (FBS) and penicillin (1000 U/ml) and streptomycin (1000 µg/ml) (P/S) were purchased from GIBCO (Grand Island, New York, USA). CJ, 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, Missouri, USA). Phosphate-buffered

saline (PBS, lacking Ca^{2+} and Mg^{2+}) was purchased from Invitrogen Corporation (Grand Island, New York, USA). Sodium dodecyl sulfate, bisacrylamide, *N,N,N, N′*-tetramethylethylenediamine and ammonium persulfate were obtained from Bio-Rad Laboratories (Hercules, California, USA). Primary antibody to caspase-3 was obtained from Pharmingen (Franklin Lakes, New Jersey, USA) and caspase-8 was from EMD Biosciences (San Diego, California, USA). Enzyme-conjugated horseradish peroxidase (HRP) secondary antibody and standards were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, California, USA). ECL + western blotting detection system was obtained from Amersham Biosciences (Piscataway, New Jersey, USA). 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 (University Boulevard, Manassas, Virginia, USA). All cultures were maintained in minimum essential media supplemented with 10% FBS, 1% penicillin and streptomycin (1000 U/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 dimethyl sulfoxide (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 h. At termination, the medium was replaced with 100 µl of fresh medium; 10 µl of alamarBlue was added and incubated for 4 h. After 4 h, fluorescence was measured at 530 nm of excitation and 590 nm of emission wavelengths with Genios fluorescence plate reader (PHENIX Research Products, Hayward, California, USA). 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. The cells were exposed to 0.5, 1.0, and 2.0 mmol/l concentrations of the compounds for 24 h. The treatment medium was poured off and the cells were washed with 1 × PBS, harvested with trypsin EDTA, counted and replated into 60-mm 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/100 ml 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 ($\sim 0.25 \times 10^6$) were plated in 100-mm tissue culture plates, incubated overnight to adhere to the plate. Cells were exposed to 0.5 and 3 mmol/l CJ or MJ for 24 h and washed with $1 \times$ PBS. Fresh and complete medium was added and the cells were incubated for 24 h. MDA-MB-435 cells were also exposed to 500 µmol/l of DCA for 4, 8, and 24 h. Cells were harvested with trypsin EDTA, washed with PBS, counted, fixed with 100 µl of PBS and 900 µl of absolute ethanol and stored at 4°C before DNA content analyses. Samples were stained with DNA staining solution containing 150 µg/ml of PI, 0.1% Triton X-100 and 1 mg/ml of RNase-A (DNase free) (1 : 1 : 1 by volume) and incubated in the absence of light for 1 h. Acquisition of the data was carried out with FACS Calibur flow cytometer (Becton Dickinson, San Jose, California, USA) and analyzed with ModfitLT 3.0 software (Verity Software House, Topsham, Maine, USA).

Detection of apoptosis by Hoechst and DNA fragmentation

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

$$\% \, \text{apoptosis} = \frac{(\text{number of Hoechst positive cells})}{(\text{number of cells in bright field})} \times 100$$

Cells ($\sim 1 \times 10^6$) were seeded in 75-mm tissue culture flasks and treated with 1, 2, or 3 mmol/l MJ for 24 h. Cells treated with DCA (500 µmol/l) for 4 and 8 h were used as positive controls. At termination cells were harvested with trypsin EDTA, washed with $1 \times$ PBS and the DNA was extracted as described in an earlier study [32]. In brief, cells ($\sim 1 \times 10^6$) were suspended in 100 µl of lysis buffer containing 10 mmol/l Tris–HCl (pH 7.4), 10 mmol/l 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 (20 µg/ml) was added to the supernatant and incubated for 1 h at 37°C. After incubation, proteinase K was added to a final concentration of 20 µg/ml and incubated for 30 min at 50°C. DNA was precipitated with 42 mmol/l 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 20 µl TE buffer (10 mmol/l Tris–HCl and 1 mmol/l EDTA pH 8.0) and 10 µl of the DNA solution was electrophoresed on a 2% agarose gel in Tris-borate-EDTA buffer (89 mmol/l Tris base, 89 mmol/l boric acid, and 2.6 mmol/l EDTA). The gel was stained with 1 µg/ml ethidium bromide and photographed.

Membrane fluidity studies

Cells ($\sim 0.5 \times 10^6$) were plated in 75-mm tissue culture flasks and incubated for 2 days. Cells were exposed to 3 mmol/l MJ, 10 µmol/l cholesterol, and 500 µmol/l DCA for 4 h. At termination cells were harvested and washed with $1 \times$ PBS. The fluorescent dye, DPH (2 µmol/l), was added and polarization was measured with LS 55 Luminescence Spectrometer (Perkin Elmer, Winter Street, Waltham, Massachusetts, USA). The P value for the study was calculated as follows:

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

Tumor necrosis factor receptor 1 expression

TNFR1 expression was measured by flow cytometry as described earlier [33]. MDA-MB-435 and MCF-7 cells were plated in 75-mm tissue culture flasks, incubated for 2 days and then exposed to 0, 1, 2, or 3 mmol/l MJ for 24 h. After exposure, the cells were harvested with trypsin-EDTA (1 mmol/l), counted and about 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 25 µg/ml anti-human TNFR1 antibodies (R&D Systems, McKinley Place NE, Minneapolis, Minnesota, USA) at 4°C for 60 min. Subsequently, cells were washed three times in PBS–FBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rat IgG (1 : 40 dilution) at 4°C for 2 h. Cells were washed in PBS–FBS three times and analyzed with FACS Calibur flow cytometer (Becton Dickinson). Fluorescence data were 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, 3 mmol/l MJ, 3 mmol/l MJ with TNFR1 antibody, TNFR1 antibody alone, or a nonspecific antibody for 24 h. At termination the medium was removed by aspiration. Fresh medium and alamarBlue were then added and the cells were incubated for 4 h. Fluorescence was measured using Genios plate reader (BD Biosciences, Qume Drive, San Jose, California, USA) and the percentage viability was calculated through comparison with the control (100%).

Mitogen-activated protein kinase assay by enzyme-linked immunosorbent assay

The p38 and ERK1/2 activity was determined by an enzyme-linked immunosorbent assay kit (Super array, Frederick Maryland, USA). Cells ($\sim 1.5 \times 10^4$) were plated in a 96-well plate and incubated overnight in

humidified 95% O_2/5% CO_2 atmosphere at 37°C. The next day, cells were serum starved for 18–24 h, exposed to MJ and assayed. In brief, 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 the manufacturer's instructions.

Caspase-8, caspase-3, and membrane potential studies

The expression of caspase-3 and caspase-8 was determined using western blot analysis. Control or treated cells ($\sim 1.0 \times 10^6$) 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, California, USA). The cell pellet was thawed on ice for 2 h before protein extraction. Phenyl-methylsulfonyl fluoride (1 mmol/l) and 100 μl of extraction buffer containing proteinase inhibitor (250 μl/5 ml) was added to about 1×10^6 cells, whereas 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, Illinois, USA). An equal amount of protein was loaded, subjected to SDS–PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature with blocking solution (10% nonfat dry milk solution + 0.05% tween 20%) and washed twice with $1 \times$ western wash (12.4 mmol/l trizma, 37.4 mmol/l NaCl, 0.5% Triton X-100, pH 7.4). The blot was then incubated with primary antibodies (1 μg/ml) to caspase-3 or caspase-8 for 1 h at room temperature after washing three times with $1 \times$ western wash. Primary antibody was detected with 1 : 1000 diluted HRP or fluorescent secondary antibodies. HRP antibody signal was developed with ECL +detection kit (Amersham Biosciences) and visualized with TYPHOON 9410 (Amersham Biosciences).

Caspase-3 activity was measured by flow cytometry as described in manufacturer's protocol (Biovision, California, USA). In brief, cells were exposed to 3 mmol/l MJ for 24 h, 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 fluorometric assay as described in the manfacturer's protocol (Sigma Chemical Company). Cells were exposed to 3 mmol/l MJ for 0, 2 or 4 h, 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, California, USA). 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 nonapoptotic cells, the dye accumulates in the mitochondrial matrix stains the mitochondria bright red and in apoptotic cells, because of 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 25-mm flasks and incubated overnight. Cells were exposed to 0, IC50MJ (concentration that inhibits growth of 50% of cells) or 3 mmol/l MJ for 24 h. 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 carried out using one or two-tailed *t*-test (Graphpad Software Inc., San Diego, California, USA and Microsoft Excel). Differences at $P < 0.05$ or better were considered statistically significant.

Results

cis-Jasmone and methyl jasmonate 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-h treatment was 1.7 mmol/l. The IC50 concentration was 2.5 mmol/l for MCF-7 cells (Fig. 1a). The IC50 concentration of MJ for MDA-MB-435 cells was 1.9 and 2.0 mmol/l for MCF-7 cells (Fig. 1b). CJ and MJ were not cytotoxic to CDC-1070-SK and MCF-10A normal cells (Fig. 1c).
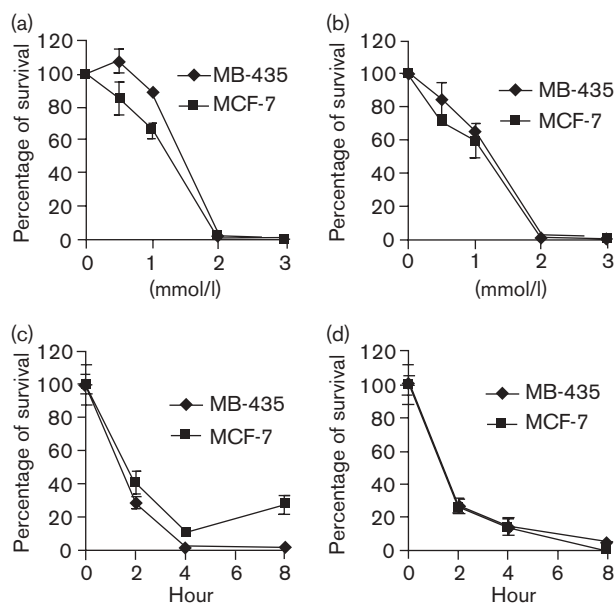
Long-term proliferation inhibition of breast cancer cells by CJ or MJ was evaluated by clonogenic survival assays. The concentration of CJ at which 50% of long-term proliferation of MDA-MB-435 and MCF-7 cells was inhibited (IC50) after 24-h treatment was 1.2 mmol/l (Fig. 2a). The IC50 of MJ for MDA-MB-435 cells was 1.2 and 1.45 mmol/l for MCF-7 cells (Fig. 2b). A time course with MDA-MB-435 and MCF-7 cells at 3 mmol/l CJ or MJ revealed a time-dependent decrease in survival (Fig. 2c and d). At 2 h, CJ inhibited the long-term proliferation of 70% of MDA-MB-435 cells and 60% of MCF-7 cells

Fig. 1
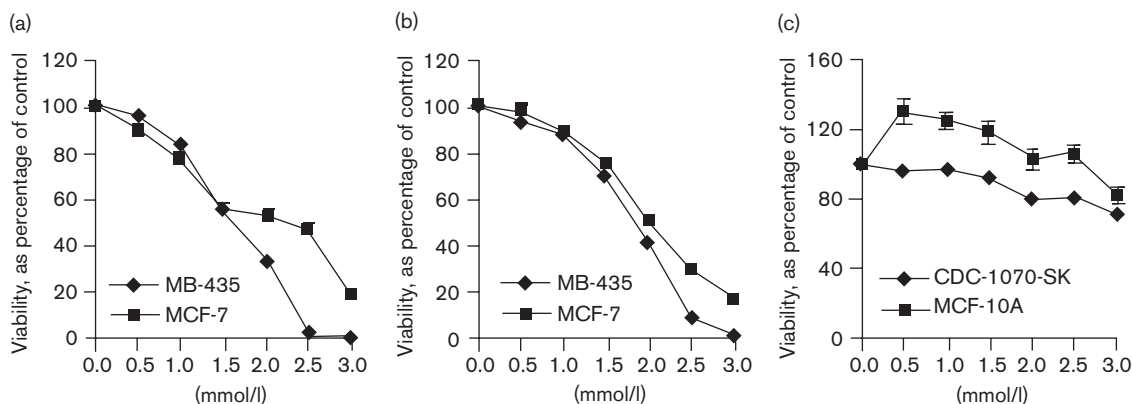
Cytotoxicity of CJ or MJ on human breast cancer cells (MDA-MB-435 and MCF-7) as well as on normal human cells (CDC-1070-SK and MCF-10A) in culture. (a) MDA-MB-435 and MCF-7 cells were exposed to varying concentrations of CJ for 24 h. (b) MDA-MB-435 and MCF-7 cells were exposed to MJ for 24 h. (c) CDC-1070-SK and MCF-10A were exposed to different concentrations of MJ for 24 h. The effects on cell viability were determined by mitochondrial dehydrogenase assay using alamarBlue dye as described in the Materials and methods. 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 1.5 mmol/l and higher there was a significant decrease in the viability of breast cancer cells compared with the control. One tailed t-test was carried out and at concentrations of 1 mmol/l 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 (c). Results are representative of three different experiments. CJ, cis-jasmone; MJ, methyl jasmonate.

Fig. 2

(a–d) CJ and MJ inhibited the long-term proliferation of breast cancer cells. (a) MDA-MB-435 cells were exposed to varying concentrations of CJ or MJ for 24 h. (b) MCF-7 cells were exposed to varying concentrations CJ or MJ for 24 h. (c) MDA-MB-435 and MCF-7 cells were exposed to 3 mmol/l CJ for 0, 2, 4, and 8 h. (d) MDA-MB-435 and MCF-7 cells were exposed to 3 mmol/l MJ for 0, 2, 4, and 8 h. 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). CJ, cis-jasmone; MJ, methyl jasmonate.

(Fig. 2c). MJ inhibited the long-term proliferation of 70% of both MDA-MB-435 and MCF-7 cells *in vitro* (Fig. 2d).

Cell cycle analysis

To investigate the ability of the jasmonate compounds to induce cell cycle arrest, DNA content analysis was carried out by PI 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 with the control (Fig. 3a and c). CJ and MJ induced cell cycle arrest and apoptosis (Fig. 3a–d) as well as G_0/G_1 arrest in MDA-MB-435 and MCF-7 cell lines (Fig. 3b and d). At 3 mmol/l MJ, MDA-MB-435 cells showed a block at S-phase, whereas 3 mmol/l CJ showed an S-phase block in MCF-7 cells (Fig. 3b and d). Apoptosis was observed only with 3 mmol/l MJ in both MDA-MB-435 and MCF-7 cells (Fig. 3b and d).

Apoptotic studies have demonstrated that CJ and MJ induced apoptosis in breast cancer cells (Fig. 4a and b). To confirm apoptosis induction, cells were stained with Hoechst and images were acquired with a fluorescence microscope. The percentage of apoptotic cells was calculated as explained in the Materials and methods section (Fig. 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 Fig. 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

Fig. 3

(a)

(b)

(c)

(d)
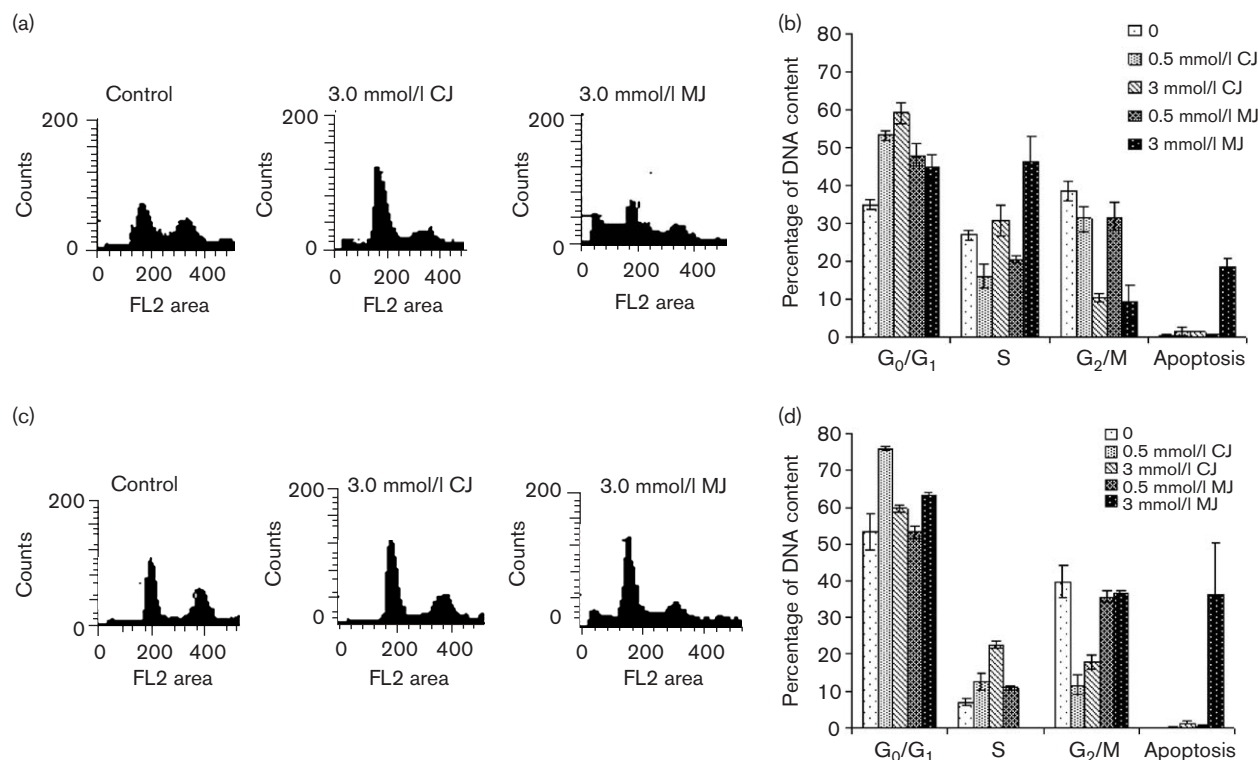
(a) Representative histograms of MDA-MB-435 cells treated with 3 mmol/l CJ or MJ for 24 h. (b) Quantitative graph of MDA-MB-435 cells treated with 0.5 and 3 mmol/l CJ or MJ for 24 h. Both CJ and MJ induced a significant G_0/G_1 block in MDA-MB-435 cells and 3 mmol/l MJ also induces S-block with increasing subdiploid population. (c) Representative histograms of MCF-7 cells treated with 3 mmol/l CJ or MJ. (d) CJ and MJ induced G_0/G_1 block in MCF-7 cells and 3 mmol/l CJ also induced S-block, whereas 3 mmol/l MJ increased subdiploid population (two-tailed *t*-test, $P < 0.05$). Results are representative of two different experiments. CJ, *cis*-jasmone; MJ, methyl jasmonate.

MCF-7 cells, respectively (Fig. 4a). DCA induced G_2/M arrest and apoptosis in MDA-MB-435 cells (Fig. 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-h treatment with MJ, DNA laddering was not observed in MCF-7 cells, but a pronounced DNA smear was observed in MDA-MB-435 cells (Fig. 4c). The DNA smear was observed with 3 mmol/l MJ, but not with the 1 or 2 mmol/l concentrations of MJ (Fig. 4c). DNA laddering was also observed in MDA-MB-435 cells treated with DCA for 4 and 8 h, which is an indicative of apoptosis (Fig. 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 3 mmol/l MJ, DCA, and cholesterol for 4 h 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 with 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 (Fig. 5a and b). Exposure of MDA-MB-435 cells to MJ in the presence of TNFR1 antibody showed increased cell viability, which confirmed TNFR1 activation (Fig. 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 (Fig. 6). Activation of p38 was observed 2.0 h after treatment and ERK1,2 activity was observed after 1.0-h treatment with 3 mmol/l MJ in MDA-MB-435 but not in MCF-7 cells (Fig. 6a–d). Caspase-8 was activated after 2-h treatment in MDA-MB-435 cells treated with 3 mmol/l MJ (Fig. 7a). Procaspase-8 was cleaved and activated until 8 h in MDA-MB-435 cells but not in MCF-7 cells (Fig. 7a, MCF-7 data not shown).

Fig. 4
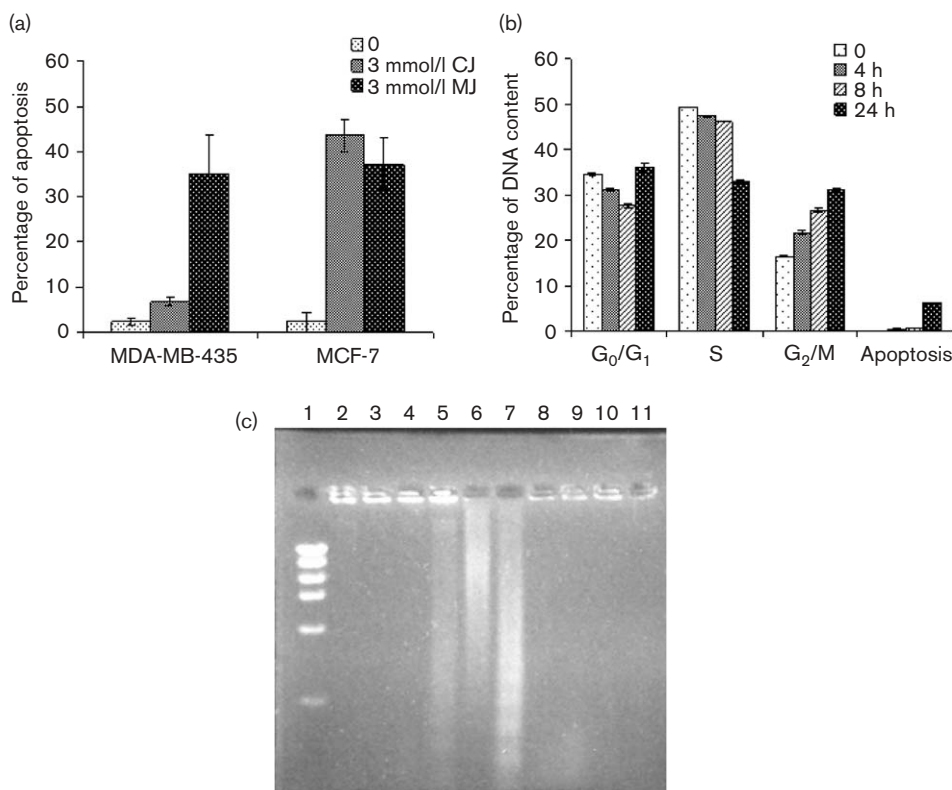
(a) To confirm apoptosis induction breast cancer cells were stained cells with Hoechst and fluorescence microscopy images were acquired. Percent apoptosis was calculated as explained in the Materials and methods. Both CJ and MJ induced apoptosis in breast cancer cells. Results are representative of two different experiments ($P < 0.01$). (b) MDA-MB-435 cells were treated with DCA (500 μmol/l) for 4, 8, and 24 h. DCA induced G_2/M arrest at all three time points measured and apoptosis at 24 h. (c) DNA gel photograph of MDA-MB-435 and MCF-7 cells treated with MJ for 24 h. Lane 1: high DNA mass ladder shows 1000, 2000, 3000, 4000, 6000, 10 000 bp bands. Lane 2: MDA-MB-435 cells treated with 0.03% DMSO (control). Lane 3: MDA-MB-435 cells treated with 1 mmol/l MJ. Lane 4: MDA-MB-435 cells treated with 2 mmol/l MJ. Lane 5: MDA-MB-435 cells treated with 3 mmol/l MJ. Lane 6: MDA-MB-435 cells were treated with DCA for 4 h. Lane 7: MDA-MB-435 cells were treated with DCA for 8 h. Lane 8: MCF-7 cells treated with 0.03% DMSO (control). Lane 9: MCF-7 cells treated with 1 mmol/l MJ. Lane 10: MCF-7 cells treated with 2 mmol/l MJ. Lane 11: MCF-7 cells treated with 3 mmol/l MJ. DNA smear was observed only in MDA-MB-435 cells treated with 3 mmol/l MJ and DCA-treated cells were used as positive controls. Results are representative of three different experiments. CJ, cis-jasmone; DCA, deoxycholic acid; MJ, methyl jasmonate; DMSO, dimethyl sulfoxide.

Further confirmatory studies indicated a five-fold increase in caspase-8 activity in MDA-MB-435 cells compared with the control (Fig. 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 with the control except in caspase-3 mutant cell line MCF-7 (Fig. 8a–c). 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 (Fig. 8b).

Discussion and conclusion
Breast cancer is the second leading cause of cancer-related deaths among women in the United States.

Alternative strategies are required to combat 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. Apoptotic mechanism of these compounds has, however, not been investigated in breast cancer cells. This 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, and 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

Table 1 MJ decreased membrane fluidity in breast cancer cells

Treatment (4 h)	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
3 mmol/l MJ	0.40 ± 0.05	0.41 ± 0.01

MDA-MB-435 cells were treated with 3 mmol/l MJ for 4 h and polarization was measured and the *P* value was calculated as explained in Materials and methods. A significant decrease in membrane fluidity is observed. ($P < 0.05$, one-tailed *t*-test).

DCA, deoxycholic acid; MJ, methyl jasmonate

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 G_0/G_1 in MDA-MB-435 at all concentrations studied. In MCF-7 cells, CJ and MJ also induced a block at G_0/G_1 at all concentrations studied above 0.5 mmol/l. At 0.5 mmol/l, MJ and CJ induced a block in S-phase. In MCF-7 cells though DNA smear was not observed with 3 mmol/l MJ treatment, apoptosis was

Fig. 5
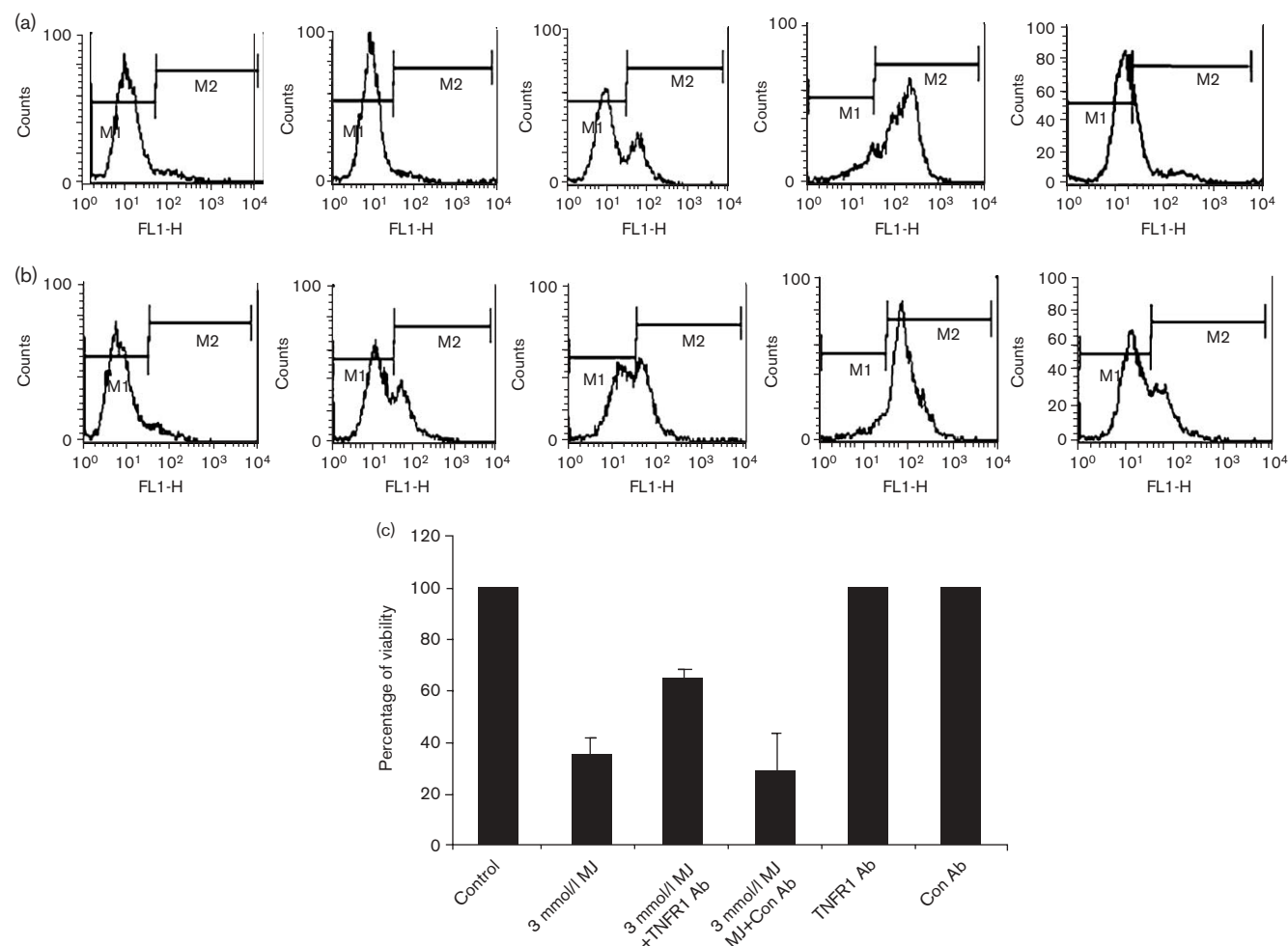
Increase in TNF receptor 1 expression and MAPK activity was measured as explained in the Materials and methods. (a) From left, first histogram represents TNFR1 expression in MDA-MB-435 cells treated with DMSO (0.3%). From left, second histogram represents TNFR1 expression in MDA-MB-435 cells treated with 1 mmol/l MJ for 24 h. From left, third histogram represents TNFR1 expression in MDA-MB-435 cells treated with 2 mmol/l MJ. From left, fourth histogram represents TNFR1 expression in MDA-MB-435 cells treated with 3 mmol/l MJ. Fifth histogram represents TNFR1 expression in MDA-MB-435 cells treated with 500 μmol/l DCA, which showed increase in TNFR1 expression. Increase in TNFR1 expression was dose dependent and at 3 mmol/l MJ a complete shift in fluorescence intensity was observed. (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 1 mmol/l MJ for 24 h. From left, third histogram represents TNFR1 expression in MCF-7 cells treated with 2 mmol/l MJ. From left, fourth histogram represents TNFR1 expression in MCF-7 cells treated with 3 mmol/l MJ. Fifth histogram represents TNFR1 expression in MCF-7 cells treated with 500 μmol/l DCA, which showed increase in TNFR1 expression. Increase in TNFR1 expression was dose dependent and at 3 mmol/l MJ a complete shift in fluorescence intensity was observed. (c) MDA-MB-435 cells were exposed to 3 mmol/l 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. DCA, deoxycholic acid; MJ, methyl jasmonate; DMSO, dimethyl sulfoxide.

Fig. 6
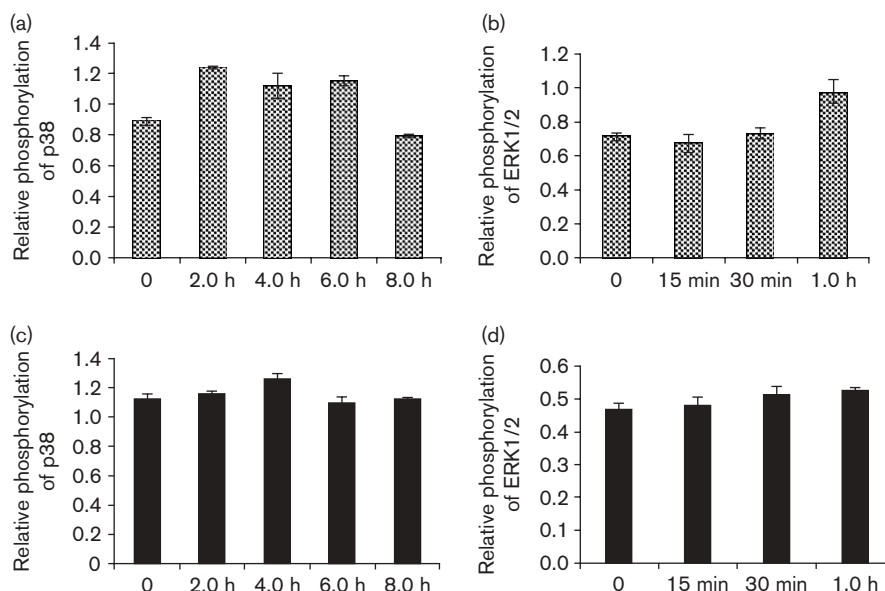
MAPK activity was measured as explained in Materials and methods. (a) In MDA-MB-435 cells, increase in p38 phosphorylation was significant at 2 h ($P < 0.04$) and starts decreasing from 4 h till 8 h. (b) In MDA-MB-435 cells, significant increase in ERK1/2 phosphorylation was observed at 1 h ($P < 0.04$). (c) In MCF-7 cells, p38 phosphorylation was not observed at all time points studied. (d) In MCF-7 cells, ERK1/2 phosphorylation was not observed at all time points studied. Results are representative of two independent experiments.

Fig. 7
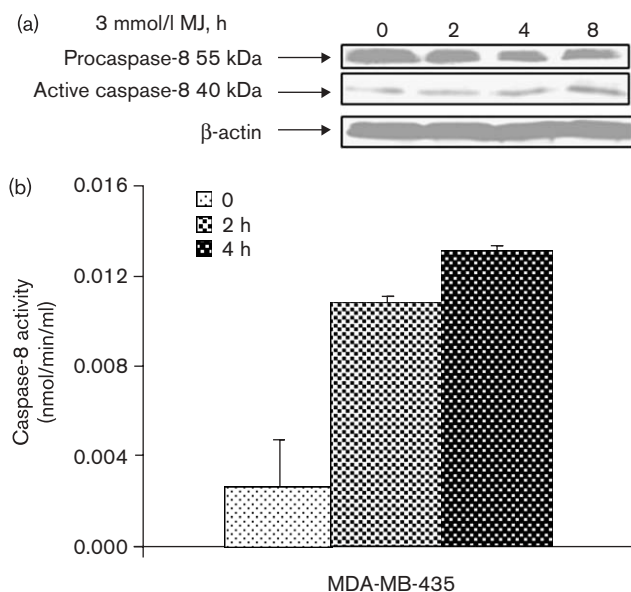
(a) Western blot of caspase-8 in MDA-MB-435 cells treated with 3 mmol/l MJ for 2, 4, and 8 h. Procaspase-8 was processed and cleaved to active caspase-8 at all the time points studied. Caspase-8 activity was increased with 3 mmol/l MJ treatment in MDA-MB-435 cells compared with the control. Beta-actin was a loading control. (b) Fluoremetric assay showed increased caspase-8 activity with MJ treatment at 2 and 4 h time points ($P < 0.01$). MJ, methyl jasmonate.

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) because of 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.

Fig. 8
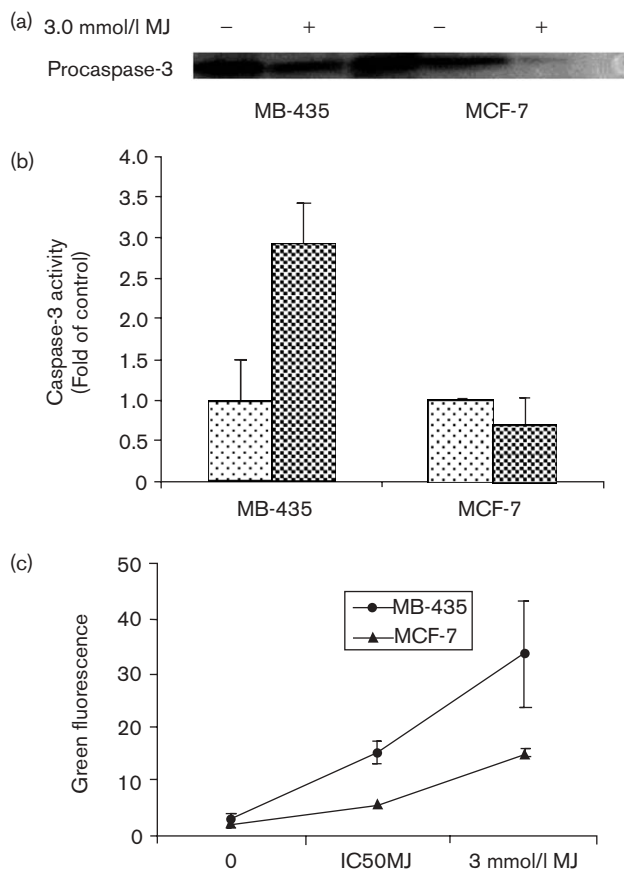
MJ activated caspase-3 in MDA-MB-435 cells. (a) Western blot of procaspase-3. Decreased levels of procaspase-3 were observed in MDA-MB-435 cells treated with 3 mmol/l MJ. (b) Caspase-3 activity measurement by flow cytometry showed activation of caspase-3 in MDA-MB-435 cells but not in MCF-7 cells. Fold increase was calculated with respect to the control (1). Results are representative of two different experiments. (c) Mitochondrial membrane potential was determined in breast cancer cells as explained in Materials and methods. In both MDA-MB-435 and MCF-7 cells, IC50 MJ and 3 mmol/l MJ decreased the membrane potential as observed by an increase in green fluorescence compared with the control ($P < 0.001$). MJ, methyl jasmonate.

Earlier report demonstrated that jasmonates affect the mitochondria release cytochrome c through permeability transition pore complex in leukemia cells without affecting normal cells [10,11]. Compared with 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 noncancerous cells, jasmonates treatment showed no cytotoxic effect (mitochondrial assay) on normal cells (Fig. 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.

Earlier studies have demonstrated that jasmonates induce growth arrest and redifferentiation 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 because of 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 earlier studies suggest that jasmonates have a chemotherapeutic effect by inducing multiple apoptotic signaling pathways in cancer cells, which involve TNFR1, caspase-8, capase-3, MAPK, bax/bcl$_{x/s}$, and cytochrome c [9–12].

In conclusion, apoptosis induced by plant compounds 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 been 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.

Acknowledgements

References
1 American Cancer Society, Cancer facts and figures. American Cancer Society Inc., Atlanta, Georgia: 2007.
2 Haag JD, Gould MN. Mammary carcinoma regression induced by perillyl alcohol, a hydroxylated analog of limonene. *Cancer Chemother Pharmacol* 1994; **34**:477–483.
3 Yuri T, Danbara N, Tsujita-Kyutoku M, Kiyozuka Y, Senzaki H, Shikata N, *et al*. Perillyl alcohol inhibits human breast cancer cell growth. *Breast Cancer Res Treatment* 2004; **84**:251–260.
4 Bardon S, Foussard V, Fournel S, Loubat A. Monoterpenes inhibit proliferation of human colon cancer cells by modulating cell cycle-related protein expression. *Cancer Lett* 2002; **181**:187–194.
5 Kallio A, Zheng A, Dahllund J, Heiskanen KM, Harkonen P. Role of mitochondria in tamoxifen-induced rapid death of MCF-7 breast cancer cells. *Apoptosis* 2005; **10**:1395–1410.
6 Yang HL, Chen CS, Chang WH, Lu FJ, Lai YC, Chen CC, *et al*. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by Antrodia camphorate. *Cancer Lett* 2006; **231**:215–227.

7   Wu CC, Chan ML, Chen WY, Tsai CY, Chang FR, Wu YC. Pristimerin induces caspase-dependent apoptosis in MDA-MB-231 cells via direct effects on mitochondria. *Mol Cancer Ther* 2005; **4**:1277–1285.

8   Farmer EE, Ryan CA. Interplant communication: air-borne methyl jasmonate induces synthesis of proteinases inhibitors in plant leaves. *Proc Natl Acad Sci U S A* 1990; **87**:7713–7716.

9   Conti M. Cyclopentenone: a special moiety for anticancer drug design. *Anticancer Drugs* 2006; **17**:1017–1022.

10 Flescher E. Jasmonates-a new family of anti-cancer agents. *Anticancer Drugs* 2005; **16**:911–916.

11 Rotem R, Heyfets A, Fingrut O, Blickstein D, Shaklai M, Flescher E. Jasmonates: novel anticancer agents acting directly and selectively on human cancer cell mitochondria. *Cancer Res* 2005; **65**:1984–1993.

12 Kim JH, Lee SY, Oh SY, Han SI, Park HG, Yoo MA, *et al.* Methyl jasmonate induces apoptosis through induction of Bax/Bcl-XS and activation of caspase-3 via ROS production in A549 cells. *Oncol Rep* 2004; **12**:1233–1238.

13 Pinton P, Ferrari D, Di Virgilio F, Pozzan T, Rizzuto R. Molecular machinery and signaling events in apoptosis. *Drug Dev Res* 2001; **52**:558–570.

14 Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998; **281**:1305–1308.

15 Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 2001; **11**:372–377.

16 Mathiasen IS, Hansen CM, Foghsgaard L, Jaatiela M. Sensitization to TNF-induced apoptosis by 1, 25-dihydroxy vitamin D_3 involves up-regulation of the TNF receptor 1 cathepsin. *B Int J Cancer* 2001; **93**:224–231.

17 Jin Z, El-diery WS. Overview of cell death signaling pathways. *Cancer Biol Ther* 2005; **4**:139–163.

18 Lowe LC, Senaratne SG, Colston KW. Induction of apoptosis in breast cancer cells by apomine is mediated by caspase and p38 mitogen activated protein kinase activation. *Biochem Biophys Res Commun* 2005; **329**:772–779.

19 Chang L, Karin M. Mammalian MAP kinase signaling cascades. *Nature* 2001; **410**:37–40.

20 Caffrey DR, O'Neill LA, Shields DC. The evolution of the MAP kinase pathways: coduplication of interacting proteins leads to new signaling cascades. *J Mol Evol* 1999; **49**:567–582.

21 Takenaka K, Moriguchi T, Nishida E. Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science* 1998; **280**:599–602.

22 Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, *et al.* Initiation of a G_2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* 2001; **411**:102–107.

23 Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. *Mol Cell* 2002; **9**:1005–1016.

24 Seger R, Krebs EG. The MAPK signaling cascade. *FASEB* 1995; **9**:726–735.

25 Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002; **298**:1911–1912.

26 Gille H, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH, *et al.* ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J* 1995; **14**:951–962.

27 Persons DL, Yazlovitskaya EM, Pelling JC. Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. *J Biol Chem* 2000; **275**:35778–35785.

28 Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, *et al.* Bid-induced conformational change of bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 1999; **144**:891–901.

29 Degli Esposti M, Dive C. Mitochondrial membrane permeabilisation by Bax/Bak. *Biochem Biophys Res Commun* 2003; **304**:455–461.

30 Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J* 1998; **17**:3878–3885.

31 Cain K, Bratton SB, Cohen GM. The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie* 2002; **84**:203–214.

32 Yui S, Saeki T, Kanamoto R, Iwami K. Charesteristics of apoptosis in HCT116 colon cancer cells induced by deoxycholic acid. *J Biochem* 2005; **28**:151–157.

33 Smolnikar K, Loffek S, Schulz T, Michna H, Diel P. Treatment with the pure antiestrogen faslodex (ICl182780) induces tumor necrosis factor receptor 1 (TNFR1) expression in MCF-7 breast cancer cells. *Breast Cancer Res Treat* 2000; **63**:249–259.

34 Jean-Louis S, Akare S, Ali MA, Mash EA Jr, Meuillet E, Martinez JD. Deoxycholic acid induces intracellular signaling through membrane perturbations. *J Biol Chem* 2006; **281**:14948–14960.

35 Qiao D, Stratagouleas ED, Martinez JD. Activation and role of mitogen-activated protein kinases in deoxycholic acid-induced apoptosis. *Carcinogenesis* 2001; **22**:35–41.

36 Schlottmann K, Wachs FP, Krieg RC, Kullmann F, Scholmerich J, Rogler G. Characterization of bile salt-induced apoptosis in colon cancer cell lines. *Cancer Res* 2000; **60**:4270–4276.

37 Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, *et al.* A mitochondria-K + channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer cell* 2007; **11**:37–51.

38 Jänicke RU, Engels IH, Dunkern T, Kaina B, Schulze-Osthoff K, Porter AG. Ionizing radiation but not anticancer drugs causes cell cycle arrest and failure to activate the mitochondrial death pathway in MCF-7 breast carcinoma cells. *Oncogene* 2001; **20**:5043–5053.