Perillyl alcohol and methyl jasmonate sensitize cancer cells to cisplatin

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Breast cancer is the second leading cause of cancer deaths among women in the United States. Several treatment options exist, with different side effects. To alleviate the side effects, several research groups have studied chemotherapeutic effects of plant compounds on cancer cells. These could be used as an alternative treatment option either alone or in combination with other chemotherapeutic drugs. The aim of this study was to evaluate the activity of a combination of perillyl alcohol (POH), methyl jasmonate (MJ) with cisplatin to define the most effective schedule and to investigate the mechanism of action in breast cancer cells. POH and MJ treatment (20% decrease in cell viability concentration) enhanced the cytotoxicity for subsequent exposure to cisplatin in MDA-MB-435 and MDA-MB-231 cells. Combination treatment of POH and MJ blocked cells at the G_0/G_1 phase of the cell cycle and the addition of cisplatin forced the cells to progress through the cell cycle and induced apoptosis. Apoptotic mechanistic studies indicated that POH and MJ treatment activated tumor necrosis factor receptor 1 and this was further increased by the addition of cisplatin. It was also found that mitochondrial membrane potential decreased with POH and MJ treatment; this effect was further enhanced by cisplatin treatment. These findings contributed to a better understanding of molecular mechanism of apoptosis in combination treatment of POH, MJ, and cisplatin. Results also showed that the combination treatment of three drugs is more effective than single drug alone or two drugs together.

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Introduction

Breast cancer patients have several treatment options including surgery, chemotherapy, radiation therapy, and a combination of these therapies. Owing to disadvantages with single-agent chemotherapy, drug combinations at tolerable doses are administered to increase efficacy [1]. New strategies for breast cancer treatment such as the use of plant compounds in combination with chemotherapeutic drugs will improve the efficacy of current therapeutic regimens.

Perillyl alcohol (POH) and methyl jasmonate (MJ) are plant compounds that can be used for combination therapy [2–11]. POH is a naturally occurring monoterpne that inhibits the growth of cancer cells and induces apoptosis [2,4,5]. A recent report indicated that POH sensitized human myeloid U937 cells to pentoxifylline. Combination treatment of POH with pentoxifylline increased Bcl-2 and Bax expression as well as induced apoptosis [6]. POH has been reported to sensitize prostate and malignant glioma cells to cisplatin/radiation through the Fas-mediated death receptor pathway [7,8]. Malignant glioma cells preincubation with POH demonstrated a dose-dependent sensitivity to cisplatin and doxorubicin [8]. MJ is a plant stress hormone that inhibits growth of lung, breast, and leukemia cells [9–11]. MJ enhances ATP depletion in the presence of glycolysis inhibitor, 2DG in CT26 (murine colon carcinoma) and D122 (murine lung carcinoma) cells [11]. MJ has been shown to affect mitochondria by disrupting hexokinase and voltage-dependent anion channel association, causing glycolysis inhibition and inducing mitochondrial outer membrane permeabilization [12]. Mitochondrial outer membrane permeabilization results in cytochrome-c release, activation of Bcl-2 family of proteins, caspases and eventually leads to cell death [13]. Combination studies of MJ with cisplatin, adriamycin, taxol, 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) showed super-additive effects with BCNU and cisplatin in human pancreas carcinoma MIA PaCa-2 cells [11]. MJ in combination with taxol showed cooperative effects in MCF-7 cells but not in combination with cisplatin [11]. These studies indicate that research on plant compounds in combination with chemotherapeutic agents will provide new avenues for breast cancer treatment.

All supplementary data are available directly from the authors.

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Cisplatin has antitumorigenic activities against a wide variety of cancers but its nephrotoxicity limits the amount that can be given to patients. Several groups have shown that cisplatin-caused nephrotoxicity is a result of tumor necrosis factor (TNF)-α release [14–16]. TNF-α is a proinflammatory cytokine, involved in diseases such as arthritis and multiple sclerosis. It binds to two receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 [14–18]. In B6 PTC (proximal tubular cells) cells [from C57BL/6 (B6) mice], cisplatin treatment increases TNF-α production and Fas expression [19]. TNF-α release in cell culture medium and Fas activation led to cell death in mice proximal tubular cells [19]. Hence, agents that sensitize cells to low dose of cisplatin will result in fewer side effects for the patient.

This study showed that POH inhibited the growth of human breast cancer cells (MDA-MB-231, MDA-MB-435, and MCF-7) and induced apoptosis. The novel finding in the study is that POH (20% decrease in cell viability, IC20), MJ (IC20), and cisplatin (1 μmol/l) in combination showed synergistic effects in growth inhibition in MDA-MB-435 and MDA-MB-231 cells. The 50% growth inhibition (IC50) for cisplatin is 600 μmol/l and in combination with POH and MJ, it decreases to 1 μmol/l, which is a 600-fold in increase in sensitivity to cisplatin. In MDA-MB-231 cells, in the presence of POH and MJ, a 1200-fold increase in sensitivity to cisplatin was observed. It was determined that combination treatment increased TNFR1 expression and decreased mitochondrial membrane potential in MDA-MB-435 and MDA-MB-231 cells.

Materials and methods

Chemicals and reagents

Cell culture media (minimum essential medium), fetal bovine serum (FBS), and penicillin (1000 U/ml) and streptomycin (1000 μg/ml) were purchased from GIBCO (Grand Island, New York, USA). Cis-jasmone, MJ, cisplatin, propidium iodide (PI), 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). All other reagents and chemicals were of the purest grade and were obtained from reputable vendors.

Cell culture

Breast cancer cell lines (MDA-MB-231, MDA-MB-435, and MCF-7) were obtained from the ATCC (Manassas, Virginia, USA). All cultures were maintained in minimum essential medium supplemented with 10% FBS, 1% of penicillin and streptomycin (1000 units/ml), and HEPES buffer. All the cultures were maintained at 37°C in humidified 5% CO_2 atmosphere. During exposure to the compounds, medium was replaced with treatment medium containing various agents while control cells received medium with dimethyl sulfoxide (0.1–0.3%).

Cytotoxicity assay

Cells (5000) were plated in a 96-well plate and incubated overnight to adhere to the plate. Cells were exposed to varying concentrations of cisplatin and POH for 24 h. For combination treatments, MDA-MB-435 and MDA-MB-231cells were exposed to either two or three compounds for 24 h. The concentration of POH or MJ was always at a dose that resulted in IC20 by the individual compound. Two agent combinations included: cisplatin and POH, cisplatin and MJ as well as POH and MJ, whereas the three agent combinations included POH, MJ, and cisplatin. At termination, the medium was replaced with 100 μl of fresh medium, 10 μl of alamarBlue (Invitrogen, Carlsbad, California, USA) was added and incubated for 4 h. After 4 h, fluorescence was measured at 530 nm excitation and 590 nm 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 for 24 h. The treatment medium was poured off and the cells were washed with 1X PBS, harvested with trypsin–EDTA, counted and replated into 60 mm dishes. Cells were incubated in humidified CO_2 atmosphere at 37°C for 11 days. The medium was decanted and cells were stained with crystal violet (0.5 g/100 ml in 95% ethanol). Colonies (> 50 cells/colony) were counted and the percentage survival was calculated relative to the control (100%) group.

Cell cycle analysis by propidium iodide

Cells (0.5×10^6) were plated in 100 mm tissue culture plates, incubated overnight to adhere to the plate. Cells were exposed to 2 mmol/l POH for 24 h and washed with 1X PBS. Fresh complete medium was added and incubated for 24 h. For combination treatments, cells were exposed to either two or three compounds for 24 h. The concentration of POH or MJ was always at a dose that resulted in IC20 by the individual compound. Two agent combinations included: cisplatin and POH, cisplatin and MJ as well as POH and MJ, whereas the three agent combinations included POH, MJ, and cisplatin. 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 dark for 1 h. Acquisition of the data was carried out with a FACS Calibur flow cytometer (Becton Dickinson, Sanjose, California, USA) and analyzed with ModfitLT 3.0 software (Verity Software House, Topsham, Maine, USA).

Detection of apoptosis by Hoechst

Cells (1×10^4) were plated in four-well slides and incubated for 24 h before treatment. Cells were exposed to 2 mmol/l POH for 24 h, at termination medium was aspirated off and stained with Hoechst (2 μg/ml; Invitrogen) for 15 min. Morphological changes indicative of apoptosis were observed and the images were 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 percentage of apoptotic cells was calculated as:

$$\%\,\text{Apoptosis} = \left(\frac{\text{Number of Hoechst positive cells}}{\text{Number of cells in bright field}}\right) \times 100.$$

Tumor necrosis factor receptor 1 expression and tumor necrosis factor-α detection

TNFR1 expression was measured by flow cytometry [17]. MDA-MB-435 and MDA-MB-231 cells were plated and incubated for 48 h. Cells were exposed to different combinations of cisplatin, POH, and MJ [IC20 POH and MJ or IC20 POH and cisplatin (1 μmol/l) or IC20 MJ and cisplatin as well as combination of IC20 POH, IC20 MJ, and cisplatin] for 24 h. After exposure, cells were harvested by scraping in PBS–EDTA, counted and 1×10^6 cells were incubated 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, Emeryville, California, 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. Fluorescence data were collected and background fluorescence was determined using unstained cells. TNF-α release was measured by enzyme-linked immunosorbent assay according to manufacturer's protocol (BD Biosciences, San Jose California, USA). MDA-MB-435 cells were plated and incubated for 48 h. Cells were exposed to different combinations of cisplatin, POH, and MJ [IC20 POH and IC20 MJ or IC20 POH and cisplatin (1 μmol/l) or IC20 MJ and cisplatin as well as combination of IC20 POH, IC20 MJ, and cisplatin] for 24 h. Medium was collected and TNF-α release was measured.

Mitochondrial membrane potential studies

Mitochondrial membrane potential was measured as described in manufacturer's protocol (Cell technology, Mountain View, 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; whereas in apoptotic cells, because of mitochondrial membrane potential collapse, JC-1 cannot accumulate within the mitochondria, and hence it remains in the cytoplasm in a green fluorescent monomeric form. MDA-MB-435 and MDA-MB-231 cells were plated and incubated for 48 h. Cells were exposed to different combinations of cisplatin, POH, and MJ. Two-drug combination included IC20 POH and MJ or IC20 POH and cisplatin (1 μmol/l) or IC20 MJ and cisplatin. Three-drug combinations included IC20 POH, IC20 MJ and cisplatin (1 μmol/l) for 24 h. At termination, cells were harvested, counted, washed with PBS, 0.5×10^6 cells 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, California, USA and Microsoft Excel). Differences at a P value of less than 0.05 or better were considered statistically significant.

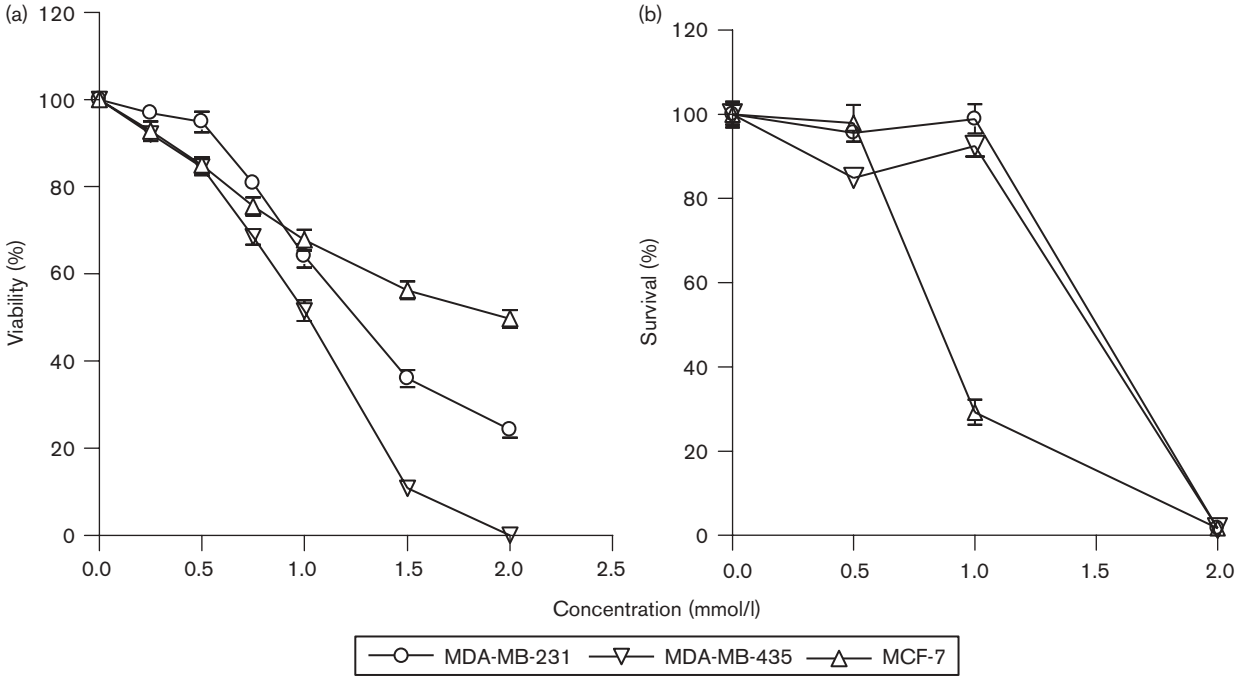
Results

Perillyl alcohol inhibited the proliferation of breast cancer cells

The proliferation inhibition of breast cancer cells was determined by cytotoxicity assay using almarBlue dye. POH inhibited breast cancer cell growth *in vitro*. MDA-MB-231 and MDA-MB-435 were the most sensitive to POH (Fig. 1a). The concentration of POH at which 20% of MDA-MB-231 cell growth (IC20) was inhibited after 24 h treatment was 0.76, 0.6 mmol/l for MDA-MB-435, and 0.8 mmol/l for MCF-7 cells (Fig. 1a). Long-term proliferation inhibition of breast cancer cells by POH was evaluated by clonogenic survival assay (Fig. 1b). The concentration of POH at which 50% of MDA-MB-231 cell proliferation was inhibited after 24 h treatment was 1.5 mmol/l, 1.5 mmol/l for MDA-MB-435, and 0.8 mmol/l for MCF-7 cells (Fig. 1b). At 1 mmol/l POH, MCF-7 cells showed 25% survival, whereas MDA-MB-231 and MDA-MB-435 cells showed 95% survival. POH at 2 mmol/l resulted in 2% survival in all three cell lines (Fig. 1b).

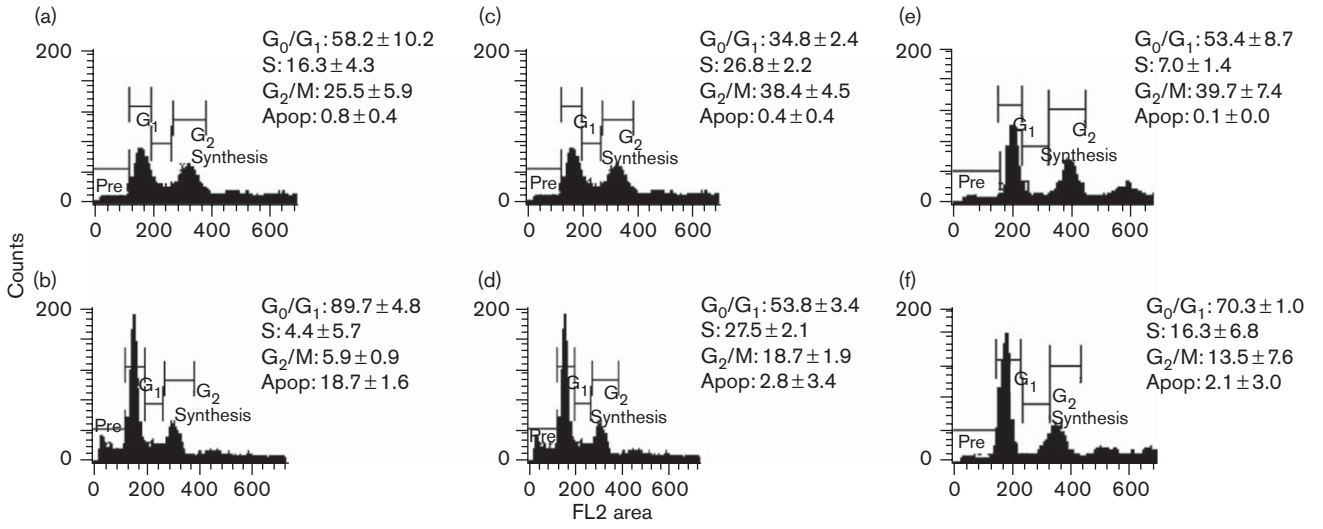
Combination studies were conducted in MDA-MB-435 and MDA-MB-231 cells. A three-drug combination (POH, MJ, and cisplatin) showed the lowest growth compared with the two-drug combination in both cell lines (Fig. 3a and b). POH and MJ together showed only 39% growth inhibition in MDA-MB-435 cells and 57% in MDA-MB-231 cells (Supplementary data). However, the three-drug combination at the lowest cisplatin concentration studied (IC20 POH, IC20 MJ, and 1 μmol/l cisplatin) showed 68% growth inhibition in MDA-MB-435 and 98% growth inhibition in MDA-MB-231 cells (Fig. 3a and b). The combination index

Fig. 1



Cytotoxicity of perillyl alcohol (POH) on human breast cancer cells MDA-MB-231, MDA-MB-435, and MCF-7. (a) MDA-MB-231, MDA-MB-435, and MCF-7 cells were exposed to varying concentrations of POH for 24 h and viability was measured by mitochondrial dehydrogenase assay as explained in the methods section. Cell viability was calculated as a percentage of untreated cells (100%). Values were mean ± SEM ($n=6$); results are representative of three independent experiments. A 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. (b) POH inhibited the long-term proliferation of breast cancer cells. Cells were exposed to varying concentrations of POH and percentage survival was determined as explained in the Materials and methods. MDA-MB-231 and MDA-MB-435 cells showed significant decrease in survival at 2 mmol/l POH ($P<0.001$), whereas in MCF-7 cells, both 1 and 2 mmol/l POH caused significant decrease in survival (one-tailed *t*-test, $P<0.001$). Values were mean ± SEM ($n=3$); results are representative of two independent experiments.

Fig. 2



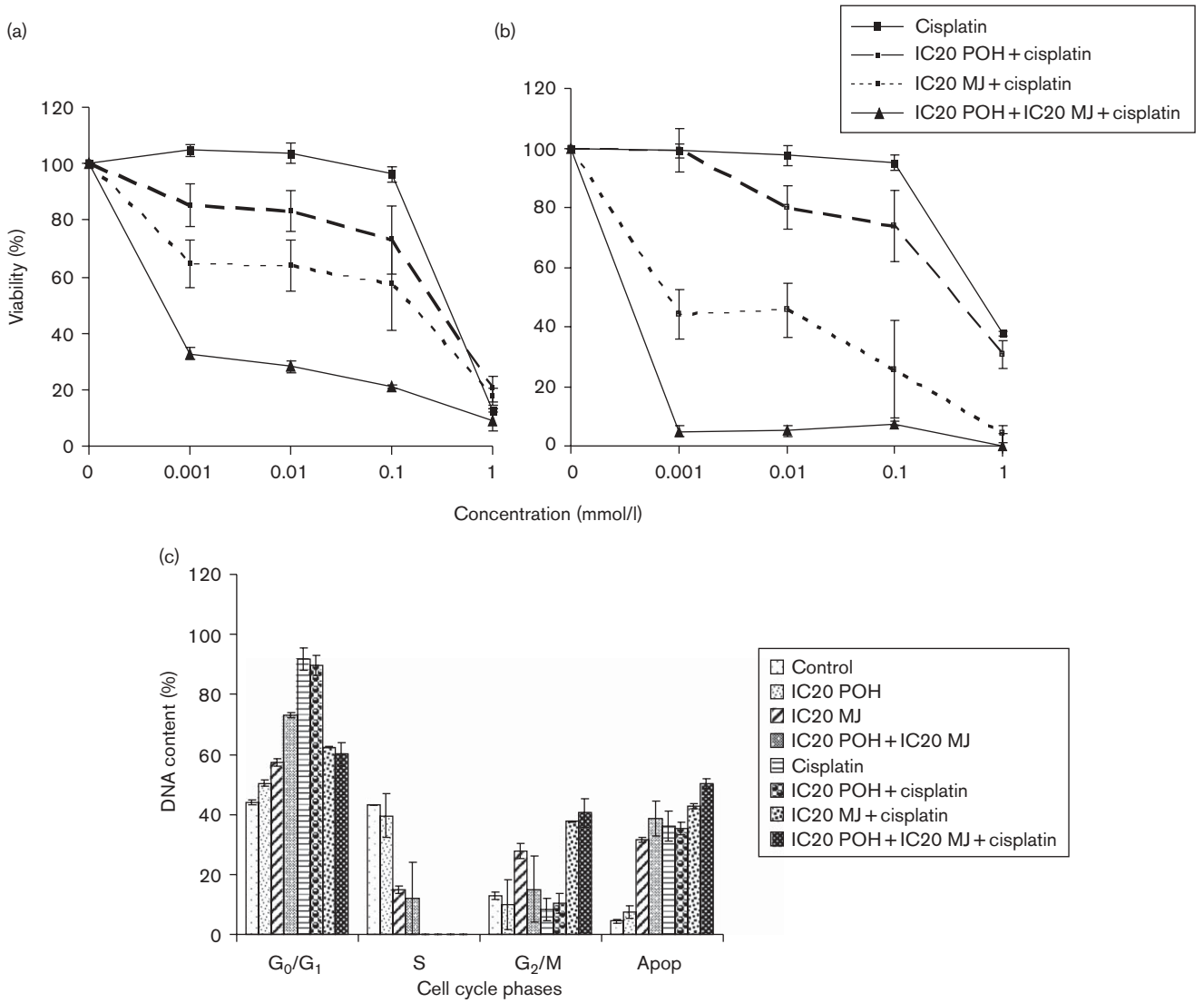
Representative histograms of breast cancer cell lines exposed to 2 mmol/l perillyl alcohol (POH). (a) MDA-MB-231 cells were exposed to 0.2% dimethyl sulfoxide (DMSO) for 24 h to compensate for the DMSO present in cisplatin-treated cells. (b) MDA-MB-231 cells were exposed to 2 mmol/l POH for 24 h. (c) MDA-MB-435 cells were exposed to 0.2% DMSO for 0, 24 h. (d) MDA-MB-435 cells were exposed to 2 mmol/l POH for 24 h. (e) MCF-7 cells were exposed to 0.2% DMSO for 24 h. (f) MCF-7 cells were exposed to 2 mmol/l POH for 24 h. POH induced significant G_0/G_1 (two-tailed *t*-test, $P<0.01$) arrest in all three cell lines studied. Values are mean ± SEM; results are representative of duplicate analyses of two separate experiments. Apop, apoptosis.

(CI) value was calculated to determine drug interactions. A CI value significantly less than 1 (CI < 1) indicates synergy, CI value greater than 1 (CI > 1) indicates antagonism, and CI value not greater than or less than 1 indicates an additive effect (CI=1). In both MDA-MB-435 and MDA-MB-231 cells, POH and cisplatin treatment resulted in CI value indicative of antagonism, whereas MJ and cisplatin treatment resulted in additive effect. In addition to that three-drug combination (POH, MJ, and cisplatin) treatment showed synergism.

Perillyl alcohol induced cell cycle arrest and apoptosis in breast cancer cells

To investigate the cell cycle arrest, DNA content analyses were carried out by PI staining using flow cytometry. POH showed a block in the cell cycle and also induced apoptosis in breast cancer cell lines (Fig. 2). POH induced G_0/G_1 arrest with increasing the subdiploid population (apoptotic population) in MDA-MB-231 (Fig. 2a and b), MDA-MB-435 (Fig. 2c and d), and MCF-7 (Fig. 2e and f) cells. Studies with combination

Fig. 3



(a) MDA-MB-435 cells were exposed to cisplatin alone, cisplatin with 20% decrease in cell viability concentration (IC20) perillyl alcohol (POH), cisplatin with IC20 methyl jasmonate (MJ), and cisplatin with IC20 POH and IC20 MJ. Combination of POH, MJ, and cisplatin enhanced growth inhibition compared with the drugs in combination of two. Values are mean ± SEM; results are representative of duplicate analyses of two separate experiments. (b) MDA-MB-231 cells were exposed to cisplatin alone, cisplatin with IC20 POH, cisplatin with IC20 MJ, and cisplatin with IC20 POH and IC20 MJ. Combination of POH, MJ, and cisplatin enhanced growth inhibition compared with the drugs in combination of two. Values are mean ± SEM; results are representative of duplicate analyses of two separate experiments. (c) Cells were exposed to POH, MJ, and cisplatin as explained in the Materials and methods. A significant G_0/G_1 block was observed in all combinations that were studied with increasing the subdiploid populations (two tailed *t*-test, $P<0.05$). Apop, apoptosis.

treatments showed a G_0/G_1 block at all combinations that were studied with increasing subdiploid population (Fig. 3c).

POH induced apoptosis in breast cancer cells (Table 1). To further confirm apoptosis induction, cells were stained with Hoechst, images were acquired with fluorescence microscopy, and the percentage of apoptotic cells was calculated as explained in the Materials and methods. POH showed significant increase in apoptosis with breast cancer cell lines (Table 1). POH induced 33.7% apoptosis in MDA-MB-231, 12.1% in MDA-MB-435, and 12.6% in MCF-7 cells, respectively (Table 1).

Table 1 POH induced apoptosis in breast cancer cells

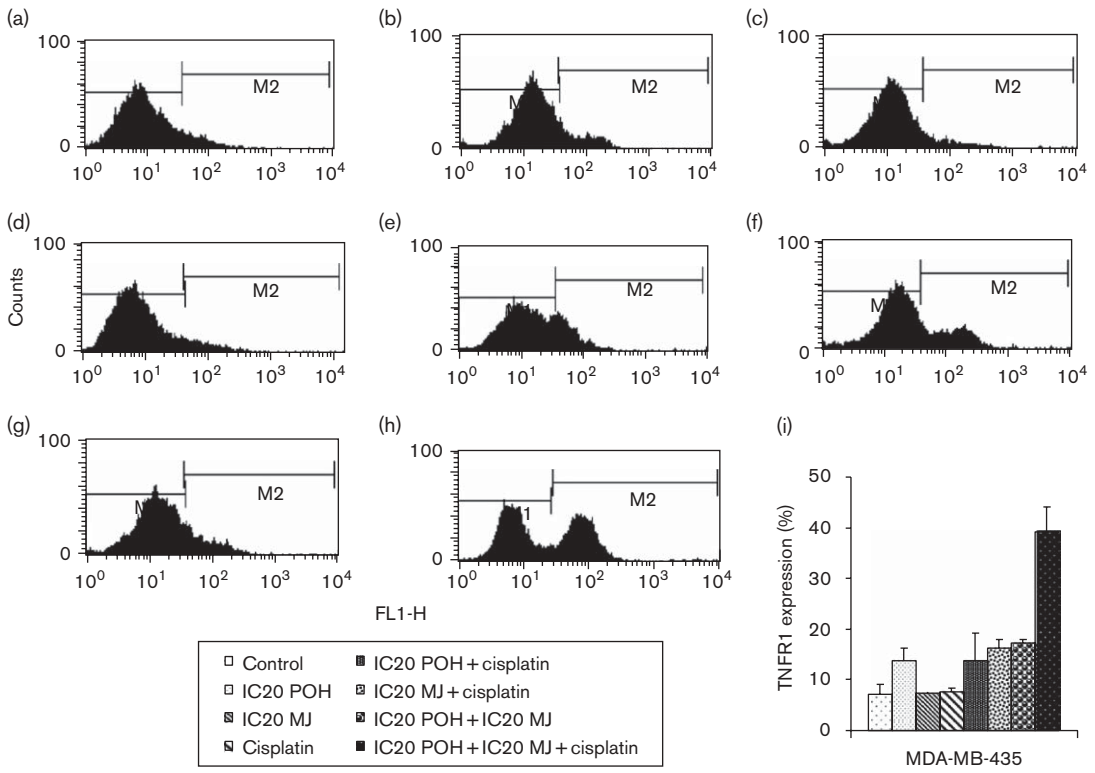
Cell line	Control	2.0 mmol/l POH
MDA-MB-231	9.84 ± 0.16	33.7 ± 5.71
MDA-MB-435	2.2 ± 0.87	12.1 ± 2.14
MCF-7	2.0 ± 2.0	12.6 ± 2.33

Perillyl alcohol (POH) showed significant increase in apoptotic population compared with the control in all three breast cancer cells (one-tailed *t*-test; $P<0.01$).

Tumor necrosis factor receptor 1 activation and tumor necrosis factor-α detection

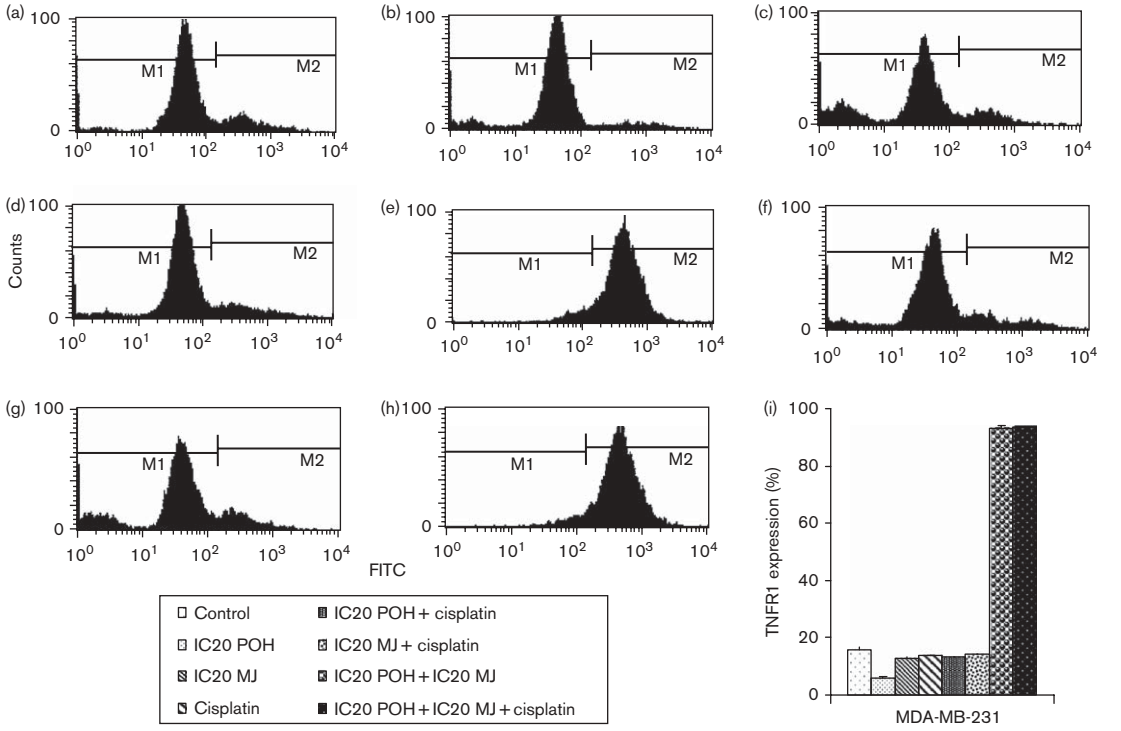
TNFR1 activation was measured by flow cytometry. TNFR1 activation was observed in combination treatments in MDA-MB-435 and MDA-MB-231 cells. A shift in fluorescence intensity of histograms was observed in POH and MJ, POH and cisplatin, MJ and cisplatin as well as in POH, MJ, and cisplatin treatments (Figs 4 and 5). The largest increase in fluorescence intensity was observed with the combination treatment of POH, MJ, and cisplatin in both cell lines (Figs 4 and 5). The results indicated that three drugs (POH, MJ, and cisplatin) in combination activated larger amounts of TNFR1 in MDA-MB-435 and MDA-MB-231 cells compared with drugs in combination of two (POH and MJ, POH and cisplatin as well as MJ and cisplatin) (Figs 4 and 5). A shift in fluorescence intensity was quantitated and shown in Figs 4i and 5i. However, in MDA-MB-231 cells, POH and MJ treatment as well as POH, MJ, and cisplatin (1 μmol/l) showed similar increase in TNFR1 expression. TNF-α release was measured by

Fig. 4



In MDA-MB-435 cells activation of tumor necrosis factor receptor 1 (TNFR1) was measured by flow cytometry. (a) Cells were exposed to 0.2% dimethyl sulfoxide (DMSO) to compensate for the DMSO present in cisplatin-treated cells. (b) Cells were exposed to 20% decrease in cell viability concentration (IC20) perillyl alcohol (POH) for 24 h. (c) Cells were exposed to IC20 methyl jasmonate (MJ) for 24 h. (d) Cells were exposed to cisplatin (1 μmol/l) for 24 h. (e) Cells were exposed to IC20 POH and IC20 MJ for 24 h. (f) Cells were exposed to IC20 POH and cisplatin (1 μmol/l). (g) Cells were exposed to IC20 MJ and cisplatin (1 μmol/l). (h) Cells were exposed to IC20 POH, IC20 MJ, and cisplatin (1 μmol/l) for 24 h. (i) FACS results quantitation of M2 peak (shift in fluorescence) from (a) to (h). Increased TNFR1 expression was observed only in combination treatments (POH and MJ, POH and cisplatin, MJ and cisplatin as well as in POH, MJ, and cisplatin-treated cells), which was determined by a shift in fluorescence intensity of histograms. Results are representative of two independent experiments.

Fig. 5



In MDA-MB-231 cells expression of tumor necrosis factor receptor 1 (TNFR1) was measured by flow cytometry. (a) Cells were exposed to 0.2% dimethyl sulfoxide (DMSO) to compensate for the DMSO present in cisplatin-treated cells. (b) Cells were exposed to 20% decrease in cell viability concentration (IC20) perillyl alcohol (POH) for 24 h. (c) Cells were exposed to IC20 methyl jasmonate (MJ) for 24 h. (d) Cells were exposed to cisplatin (1 μmol/l) for 24 h. (e) Cells were exposed to IC20 POH and IC20 MJ for 24 h. (f) Cells were exposed to IC20 POH and cisplatin (1 μmol/l). (g) Cells were exposed to IC20 MJ and cisplatin (1 μmol/l). (h) Cells were exposed to IC20 POH, IC20 MJ, and cisplatin (1 μmol/l for 24 h. (i) FACS results quantitation of M2 peak (shift in fluorescence) from (a) to (h). Increased TNFR1 expression was observed only in combination treatments (POH and MJ, as well as in POH, MJ, and cisplatin-treated cells), which was determined by a shift in fluorescence intensity of histograms. Results are representative of two independent experiments.

enzyme-linked immunosorbent assay as described in the Materials and methods. Though TNFR1 expression was increased in combination treatments (POH, MJ, and cisplatin), compared with the control TNF-α release was not significant (Fig. 5b, TNF-alpha data not shown).

Mitochondrial membrane potential detection

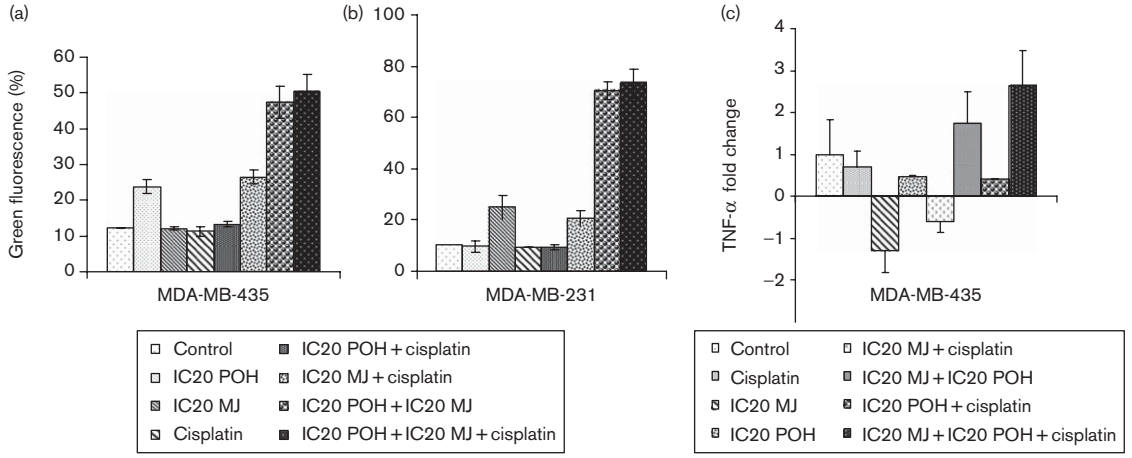
To determine the involvement of mitochondria in combination treatment of POH, MJ, and cisplatin, mitochondrial membrane potential was measured by flow cytometry. It was demonstrated that mitochondrial membrane potential was decreased in combination treatments compared with the control. The highest decrease in mitochondrial membrane potential was observed with combination treatments of POH and MJ as well as with POH, MJ, and cisplatin. This is consistent in both breast cancer cell lines (MDA-MB-435 and MDA-MB-231).

Discussion and conclusion

Combination therapies will provide an alternate treatment option to reduce the toxicity and increase the efficacy of single-agent therapy. To the best of our knowledge, this is the first report to show that POH, MJ, and cisplatin in combination resulted in growth inhibition. DNA content analysis with combination treatments showed a G_0/G_1 block with increasing hypodiploid population (apoptosis). Studies on the mitochondrial membrane potential and TNFR1 indicated decreased mitochondrial membrane potential and activation of TNFR1 in POH and MJ, POH and cisplatin, MJ and cisplatin as well as POH, MJ, and cisplatin treatments. The results are consistent in two different breast cancer cell lines studied (MDA-MB-435 and MDA-MB-231).

Earlier studies reported that POH inhibited the growth of lung, prostate, and colon cancer cells [2,4,5]. It has been reported that POH induced G_0/G_1 block in lung and colon cancer cells as well as G_2/M block in prostate cancer cells with increasing the apoptotic population [2,4,7]. Along similar lines, this study showed that POH inhibited growth, induced a G_0/G_1 block, and caused apoptosis in breast cancer cells. Earlier studies on MJ showed cytotoxic effects at 1–5 mmol/l range in lung and leukemia cells [9–11]. Cisplatin at 6.6 μmol/l inhibited 48% cell growth and combination with 50–100 μmol/l silibinin resulted in 63–80% growth inhibition of prostate

Fig. 6



(a) Mitochondrial membrane potential was determined in breast cancer cells using a fluorescent dye as explained in the methods section. In the column graph, lane 1 is MDA-MB-435 cells treated with dimethyl sulfoxide (DMSO). Lane 2: cells were exposed to 20% decrease in cell viability concentration (IC20) perillyl alcohol (POH) for 24 h. Lane 3: cells were exposed to IC20 methyl jasmonate (MJ) for 24 h. Lane 4: cells were exposed to 1 μmol/l cisplatin for 24 h. Lane 5: cells were exposed to IC20 POH and 1 μmol/l cisplatin for 24 h. Lane 6: cells were exposed to IC20 MJ and 1 μmol/l cisplatin. Lane 7: cells were exposed to IC20 POH and IC20 MJ for 24 h. Lane 8: cells were exposed to IC20 POH, IC20 MJ, and 1 μmol/l cisplatin. In MDA-MB-435 cells, an increase in green fluorescence indicated decreased membrane potential. (b) In the column graph, lane 1 is MDA-MB-231cells treated with DMSO. Lane 2: cells were exposed to IC20 POH for 24 h. Lane 3: cells were exposed to IC20 MJ for 24 h. Lane 4: cells were exposed to 1 μmol/l cisplatin for 24 h. Lane 5: cells were exposed to IC20 POH and 1 μmol/l cisplatin for 24 h. Lane 6: cells were exposed to IC20 MJ and 1 μmol/l cisplatin. Lane 7: cells were exposed to IC20 POH and IC20 MJ for 24 h. Lane 8: cells were exposed to IC20 POH, IC20 MJ, and 1 μmol/l cisplatin. In MDA-MB-231 cells, an increase in green fluorescence indicated decreased membrane potential. (c) Tumor necrosis factor (TNF)-α release was measured in MDA-MB-435 cells as described in the Materials and methods. Compared with the control, there was no significant increase in TNF-α release in combination treatments (POH + MJ, MJ + cisplatin, POH + cisplatin, and POH + MJ + cisplatin) in MDA-MB-435 cells.

cancer cells [20]. This study showed that cisplatin at 1 μmol/l concentration showed *2%* growth inhibition, whereas with IC20 POH and IC20 MJ showed 68% growth inhibition in MDA-MB-435 cells (Fig. 3a). In MDA-MB-231 cells, 1 μmol/l cisplatin did not show growth inhibition but with IC20 POH and IC20 MJ 98% growth inhibition was observed (Fig. 3b). The IC50 value of cisplatin in MDA-MB-435 cells was 0.6 mmol/l and 0.7 mmol/l in MDA-MB-231 cells (Fig. 3a and b). Cisplatin in combination with IC20 POH and IC20 MJ, the IC50 was decreased to 1 μmol/l in MDA-MB-435 cells, which is a 600-fold shift in IC50 (Fig. 3b). In MDA-MB-231 cells, the IC50 was reduced to 0.5 μmol/l, 1200-fold shift in IC50 (Fig. 3b). This indicated that treatment of breast cancer cells with plant compounds increased cisplatin cellular toxicity.

Earlier reports on combination studies with POH and cisplatin showed that POH sensitized prostate cancer cells to cisplatin and activated the Fas-mediated death pathway and decreased mitochondrial membrane potential [7,21]. Cisplatin was reported to release TNF-α into the cell culture medium and activated TNFR1 in proximal tubular cells [22,23]. Cisplatin in combination with compound herbal medicinal prescription showed synergestic effects in proliferation inhibition in ovarian cancer cells (SKOV3). Mechanistic studies indicated TNFR1 activation in the SKOV3 cells [24]. In our previous studies, it was determined that MJ activated TNFR1 in prostate and breast cancer cells and decreased membrane potential in breast cancer cells [25,26]. In addition to that, TNF-α release (Fig. 6b) was measured in combination treatments and did not observe any significant increase in TNF-α release compared with the control (Fig. 6c). Taken together, all these observations and the combination studies show that POH, MJ, and cisplatin treatment activates TNFR1 and decreases mitochondrial membrane potential. The proposed mechanism for growth inhibition with combination treatment includes activation of TNFR1 as well as mitochondrial membrane potential decrease by cisplatin, MJ, and POH.

Cancer patients have disadvantages with single-agent therapy such as side effects and severe toxicity. Combination studies involving two or more agents to reduce toxicity and improve efficacy of chemotherapy will provide new avenues for cancer treatment. It is possible that three compounds studied here could have independent metabolic fates once in the human body. However, it has been proposed earlier that POH and MJ induce mitochondrial-mediated apoptosis, hence by combining compounds, which induce similar signaling pathways at very-low dosages with cisplatin would be advantageous and could be less toxic to cancer cells. Future animal and in-vivo studies would evaluate drug distribution and different fates of these compounds in the body. In

addition to that, scheduling and mechanistic studies at different doses need to be carried out to improve the efficacy of cisplatin.

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References

1 American Cancer Society. *Cancer facts & figures*. Atlanta, Georgia: American Cancer Society, Inc.; 2007.
2 Elegbede JA, Flores R, Wang RC. Perillyl alcohol and perillaldehyde induced cell cycle arrest and cell death in BroTo and A549 cells cultured in vitro. *Life Sci* 2003; **73**:2831–2840.
3 Ezekwudo D, Shashidharamurthy R, Devineni D, Bozeman E, Palaniappan R, Selvaraj P. Inhibition of expression of anti-apoptotic protein Bcl-2 and induction of cell death in radioresistant human prostate adenocarcinoma cell line (PC-3) by methyl jasmonate. *Cancer Lett* 2008; **270**:277–285.
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 Bardon S, Picard K, Martel P. Monoterpenes inhibit cell growth, cell cycle progression, and cyclin D1 gene expression in human breast cancer cell lines. *Nutr Cancer* 1998; **32**:1–7.
6 Gómez-Contreras PC, Hernández-Flores G, Ortiz-Lazareno PC. In vitro induction of apoptosis in U937 cells by perillyl alcohol with sensitization by pentoxifylline: increased Bcl-2 and Bax protein expression. *Chemotherapy* 2006; **52**:308–315.
7 Rajesh D, Howard SP. Perillyl alcohol mediated radiosensitization via augmentation of the Fas pathway in prostate cancer cells. *Prostate* 2003; **57**:14–23.
8 Rajesh D, Stenzel RA, Howard SP. Perillyl alcohol as a radio-/chemosensitizer in malignant glioma. *J Biol Chem* 2003; **278**:35968–35978.
9 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.
10 Yeruva L, Pierre KJ, Carper SW, Elegbede JA, Toy BJ, Wang RC. Jasmonates induce apoptosis and cell cycle arrest in non-small cell lung cancer lines. *Exp Lung Res* 2006; **32**:499–516.
11 Heyfets A, Flescher E. Cooperative cytotoxicity of methyl jasmonate with anti-cancer drugs and 2-deoxy-D-glucose. *Cancer Lett* 2007; **250**:300–310.
12 Galluzzi L, Kepp O, Tajeddine N, Kroemer G. Disruption of the hexokinase-VDAC complex for tumor therapy. *Oncogene* 2008; **27**:4633–4635.
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 Tsuruya K, Ninomiya T, Tokumoto M, Hirakawa M, Masutani K, Taniguchi M, *et al*. Direct involvement of the receptor-mediated apoptotic pathways in cisplatin-induced renal tubular cell death. *Kidney Int* 2003; **63**:72–82.
15 Ramesh G, Reeves WB. TNFR2-mediated apoptosis and necrosis in cisplatin induced acute renal failure. *Am J Physiol Renal Physiol* 2003; **285**:F610–F618.
16 Ramesh G, Kimball SR, Jefferson LS, Reeves WB. Endotoxin and cisplatin synergistically stimulate TNF-{alpha} production by renal epithelial cells. *Am J Physiol Renal Physiol* 2007; **292**:F812–F819.
17 Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998; **281**:1305–1308.
18 Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 2001; **11**:372–377.
19 Mathiasen IS, Hansen CM, Foghsgaard L, Jaattela 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.
20 Jin Z, El-diery WS. Overview of cell death signaling pathways. *Cancer Biol Ther* 2005; **4**:139–163.
21 Smolnikar K, Loffek S, Schulz T, Michna H, Diel P. Treatment with the pure antiestrogen faslodex (ICI182780) induces tumor necrosis factor receptor 1 (TNFR1) expression in MCF-7 breast cancer cells. *Breast Cancer Res Treat* 2000; **63**:249–259.
22 Dhanalakshmi S, Agarwal P, Glode LM, Agarwal R. Silibinin sensitizes human prostate carcinoma DU145 cells to cisplatin- and carboplatin-induced growth inhibition and apoptotic death. *Int J Cancer* 2003; **106**:699–705.
23 Jing XB, Cai XB, Hu H, Chen SZ, Chen BM, Cai JY. Reactive oxygen species and mitochondrial membrane potential are modulated during CDDP-induced apoptosis in EC-109 cells. *Biochem Cell Biol* 2007; **85**:265–271.
24 Suo YP, Wang BY, Hong Z, Yin RT, Wang DQ, Peng ZL. Comparative study of that synergistic effect of cisplatin combined with compound herbal medicinal prescription for tonic quality and activating blood circulation is on SKOV3 cell proliferation and apoptosis. *Sichuan Da Xue Xue Bao Yi Xue Ban* 2006; **37**:542–546.
25 Yeruva L, Elegbede JA, Carper SW. Methyl jasmonate decreases membrane fluidity and induces apoptosis through tumor necrosis factor receptor 1 in breast cancer cells. *Anticancer Drugs.* 2008; **19**:766–776.
26 Yeruva L, Pierre KJ, Bathina M, Elegbede A, Carper SW. Delayed cytotoxic effects of methyl jasmonate and cis-jasmone induced apoptosis in prostate cancer cells. *Cancer Invest* 2008; **26**:890–899.