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Plant stress hormones suppress the proliferation and induce apoptosis in human cancer cells

O Fingrut and E Flescher

Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel

Cellular stressors induce various outcomes including inhibition of cell proliferation and cell death. Sodium salicylate (SA), a plant stress hormone, can suppress the proliferation or cause apoptosis in certain mammalian cancer cells. Plant stress hormones are activators of cellular responses, including cell death, to diverse stress situations in plants. Thus, we hypothesized that plant stress hormones share the ability to adversely affect cancer cells. We found that the plant stress hormone SA suppressed proliferation of lymphoblastic leukemia, prostate, breast and melanoma human cancer cells. Jasmonic acid (JA), a plant stress hormone belonging to the Jasmonate family, induced death in lymphoblastic leukemia cells and caused suppression of cell proliferation in the other human cancer cells mentioned above. Another member of the Jasmonate family, methyl jasmonate (MJ), induced death in each of the cell lines. Plant stress hormones did not affect normal human lymphocytes, in contrast to their strong effect on lymphoblastic leukemia cells. JA and MJ caused apoptotic death, as determined by characteristic nuclear morphology, flow cytometric DNA profile and elevation of caspase-3 activity. Finally, mice bearing EL-4 lymphoma and treated with MJ, survived for significantly ($P = 0.00953$) longer periods of time than untreated mice. These findings suggest that plant stress hormones may potentially be a novel class of anti-cancer drugs.
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Introduction

Stress caused by UV radiation, osmotic shock, cytotoxic drugs, heat, etc. can influence cells in different ways. In response to those stresses cells could lose a variety of functions, or even die. On the other hand, some of the cells can overcome the stress and adapt to the new condition.[1–5]

Cells can die in two different ways: necrosis and apoptosis. Necrosis is caused by outside damage, recognized by destruction of plasma membrane and intracellular organelles and molecules. Necrotic cells, when microscopically observed, appear swollen with broken plasma membrane, and release cytoplasmic contents to the neighboring tissue. Necrosis can happen in a few seconds.[6]

In apoptosis, biochemical and morphological events are usually organized in a cascade of very specific and controlled steps. The beginning of apoptosis is marked by chromatin condensation that occurs in parallel to the shrinking of the cell. Fragmentation of the nucleus, transformation of the cell surface, and complete splitting of the cell contents to apoptotic bodies attached to the membrane, accompany those changes.[7] One of the important features of apoptosis, in most cell lines, is DNA fragmentation, catalyzed by endogenic endonucleases.[8]

Correspondence: E Flescher, Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel; Fax: +972 (3) 640–9160
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Final events of apoptosis include activation of specific cytoplasmic proteases named caspases.[6] In mammalian cells, caspases are activated in a cascade, resulting in inappropriate activation or fast damage to structural proteins, as well as to important signaling processes, homeostasis and repair enzymes.[9] Caspase-3 is essential for a number of morphological and biochemical events associated with apoptosis.[10] The process of apoptosis is slower then necrosis and happens in a few hours or days, depending on the inducer. This kind of death may be regarded as 'cell suicide'.[6] Chemotherapeutic drugs work usually by induction of apoptosis in cancer cells.[11]

Sodium salicylate (SA) induces apoptosis in cell lines of human myeloid leukemia, through activation of caspase-3,[12] and in FS-4 fibroblasts.[13] SA can also inhibit growth of breast cancer cells.[14] SA is a plant stress hormone and a central mediator of plant defense responses to pathogens. Two other plant stress hormones: jasmonic acid (JA, Figure 1) and methyl jasmonate (MJ, Figure 1), belong to the group of natural bioregulators named *jasmonates*.[15] JA is crucial to intracellular signals in response to injury and MJ causes the induction of a proteinase inhibitor, which is accumulated in response to wounding or pathogenic attacks, at very low concentrations.[16]

A coordinated activation of programmed cell death (PCD) and defense mechanisms often accompany the antimicrobial response of plants and animals.[17] In plants, this response is termed the hypersensitive response (HR) and results in the formation of a zone of dead cells around the infection site, the synthesis of SA, and accumulation of antimicrobial agents, such as pathogenesis-related proteins and phytoalexins.[18] The layers of dead cells that surround the site of pathogen entry are thought to function as a physical barrier that inhibits further proliferation and spread of the pathogen.[19]

Because the plant stress hormone SA induces in mammalian cells intracellular biochemical events typical of stress response,[13,20] and apoptosis,[12,13] we hypothesized that plant stress hormones in general induce pathways responsible for stress-induced cellular damage in mammalian cells. This study is focused on evaluating the possibility of using plant stress

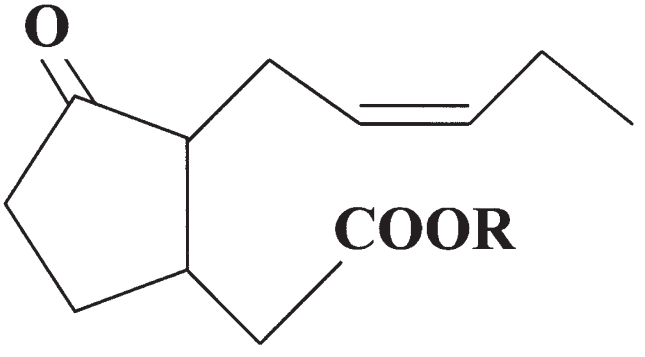

Figure 1 Structure of jasmonates. Jasmonic acid (R = H), methyl jasmonate ($R = CH_3$).

hormones for suppressing and killing cancer cells representing major types of human malignancies. To the best of our knowledge, jasmonates have never been studied as anti-cancer agents.

Materials and methods

Reagents

All the reagents used were purchased from Sigma Chemicals (St Louis, MO, USA) unless otherwise stated. JA (3-oxo-2-(2-pentenyl) cyclopentaneacetic acid) and MJ (methyl 3-oxo-2-(2-pentenyl) cyclopentaneacetic acid) were dissolved in ethanol.

Cells and animals

Molt-4 is a human T lymphoblastic leukemia cell line. SK-28 are human melanoma cells. The LNCaP cell line is an androgen-responsive human prostate adenocarcinoma. The MCF7 cell line is a human breast carcinoma. The EL-4 is a mouse T lymphoma cell line. All the cell lines were purchased from ATCC (Rockville, MD, USA).

Preparation of lymphocytes from the peripheral blood: Mononuclear cells (MNC) from venous blood of healthy donors were prepared by Ficoll–Hypaque (Phamacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The resultant mononuclear cell preparation was allowed to adhere to plastic dishes to remove macrophages. The non-adherent peripheral blood lymphocytes were used further in the experiments.[21]

All cell cultures were performed in RPMI-1640 with 10% fetal calf serum (Biological Industries, Beit-Haemek, Israel), and cells (except for Molt-4 and lymphocytes from the peripheral blood) were allowed to adhere prior to every treatment mentioned below.

In vivo study: Six-week-old male C57BL/6J mice were kept in cages under standard food and housing conditions during the experiments. 5×10^5 EL-4 cells were injected intraperitoneally (i.p.) into C57BL/6J mice in order to produce tumor growth. Methyl jasmonate was administered orally to animals. The compound was administered daily for 3 months by gavage. The methyl jasmonate was dissolved in a lipid formulation, Lipofundin (B Braun Melsungen, Melsungen, Germany). The control mice were treated with the vehicle alone. The survival of mice was monitored daily.

Cytotoxicity and cellular proliferation assays

Inhibition of cell proliferation was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). Upon completion of a given experiment, 20 μl of a mixture (20:1) of MTS (a tetrazolium compound, at a final concentration of 333 μg/ml) + phenazine methosulfate (at a final concentration of 25 μM) were added to each well of the 96-well plate for 1 h at 37°C. This allowed for the development of the reaction in which dehydrogenases reduce the MTS in metabolically active cells. Since the cells were not washed before the addition of MTS, we did not have any problem with potentially loosely adherent or non-adherent cells. Soluble MTS formazan product at wavelength 490 nm was measured with the CERES 900 HDi ELISA reader (Bio-Tek Instruments, Highland Park, VT, USA). Optical density is directly proportional to the number of living cells in culture. Cytotoxicity (%) was calculated in the following way: [(OD of control cells – OD of drug-treated cells)/OD of control cells] $\times$ 100. Since the same number of cells was aliquoted into each well initially, decreased optical density in wells containing treated cells reflects cellular death and/or decrease in the rate of proliferation. To distinguish between these two possibilities we employed an additional cytotoxicity assay which detects cell death by lack of trypan blue exclusion (see below).

Cell death was determined by trypan blue exclusion. Cells were incubated with 0.1% trypan blue for 2–5 min and the percentage of dead cells (those, which did not exclude the dye) was determined microscopically.

Cell cycle analysis

Molt-4 cells were harvested from 24-well plates. Half a million cells per sample were fixed in 0.1% paraformaldehyde + 0.1% Triton X-100 and stored at 4°C. On the day of analysis, the cells were incubated with 0.1 mg/ml propidium iodide (a DNA probe) for 20 min at room temperature and analyzed by flow cytometry in FACSORT (Becton Dickinson, Mountain View, CA, USA).

Analysis of caspase-3 activity

Molt-4 cells were treated with increasing concentrations of JA and MJ for different periods of time, and the enzymatic activity of caspase-3 was determined using the caspase-3 (CPP32) protease assay kit (PharMingen, San Diego, CA, USA) as suggested by the manufacturer. Briefly, 2×10^6 cells were lysed and resuspended in 100 μl of reaction buffer containing a fluorogenic caspase-3 (CPP32) substrate Ac-DEVD-AMC. Reactions were incubated at 37°C for 2 h and samples were assayed at excitation wavelength of 360 nm and emission wavelength of 460 nm, in the FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT, USA).

Microscopic determination of apoptosis

Following exposure, the Molt-4 cells were harvested and prepared for microscopic analysis. Cells were fixed in 3% paraformaldehyde and 0.1% Triton X-100, in phosphate-buffered saline (PBS) for 1 h. Fixed cells were stained for 10 min with DAPI (1 μg/ml) in PBS. Nuclei were analyzed by fluorescence microscopy (model A $\times$ 70 TRF; Olympus Optical, Japan), to detect qualitatively the essential morphological characteristics of apoptosis, such as condensation and fragmentation of chromatin.

Statistical analysis

The statistical significance of the results was determined (where appropriate) by two-tailed Student's *t*-test, $n = 3$. Results are presented as means ± standard deviation.

Survival data were analyzed using a Mantel–Cox test.

Results

Cytotoxicity of plant stress hormones to human transformed cell lines

Four transformed cell lines of different histologic lineages were exposed to the plant stress hormone SA. The cells were incubated with SA at concentrations ranging from 0.5 to 3 mM for 24 h. As is shown in Figure 2a, all the cell lines responded in a dose-dependent fashion to SA. SA is a nonsteroidal anti-inflammatory drug and its highest non-toxic pharmacological concentration used in humans is approximately 3 mM.[22] In order to compare with SA, we worked in the same range of concentrations (0.5–3 mM) with two additional plant stress hormones (JA and MJ).

We examined the ability of JA to affect cancer cells. Molt-4, LNCaP, MCF7 and SK28 were exposed to JA (0.5–3 mM) for 24 h and the cytotoxicity of the compound was determined. It was found that the order of sensitivity to JA was Molt-4>SK-28>LNCaP>MCF7 (Figure 2b). The responsiveness to JA was dose-dependent.

In addition to JA we examined MJ, the methylated form of JA. The results presented here show that MJ caused the highest level of cytotoxicity. For instance, 0.5 mM of MJ induced 87.5% cytotoxicity in Molt-4 cells (Figure 2c). The other cell lines responded to MJ in a dose-dependent way. The order of sensitivity was Molt-4>LNCaP>SK28>MCF7 (Figure 2c). Appropriate controls established that ethanol (in which JA and MJ were dissolved) by itself did not induce any cytotoxicity. Since MJ at 0.5 mM was already very toxic towards Molt-4 cells within 24 h, we performed a dose-response analysis at earlier times. As can be seen in Figure 3, the response to increasing doses of MJ at 2, 4 and 6 h of incubation is much more gradual than that at 24 h of incubation.

The cytotoxicity assay we employed does not differentiate between suppression of cellular proliferation and cell death. Therefore, Molt-4, LNCaP, MCF7 and SK28 cells were treated with SA, JA and MJ for 24 h and the viability of the cells was determined by the trypan blue exclusion method. SA induced suppression of cell proliferation in all cell lines, while JA induced death in lymphoblastic leukemia cells and suppression of proliferation in the other cells. Most effectively, MJ induced death in every cell line. The results presented here show that MJ is more effective in killing human transformed cell lines than its non-methylated form JA (Figure 2b, c).

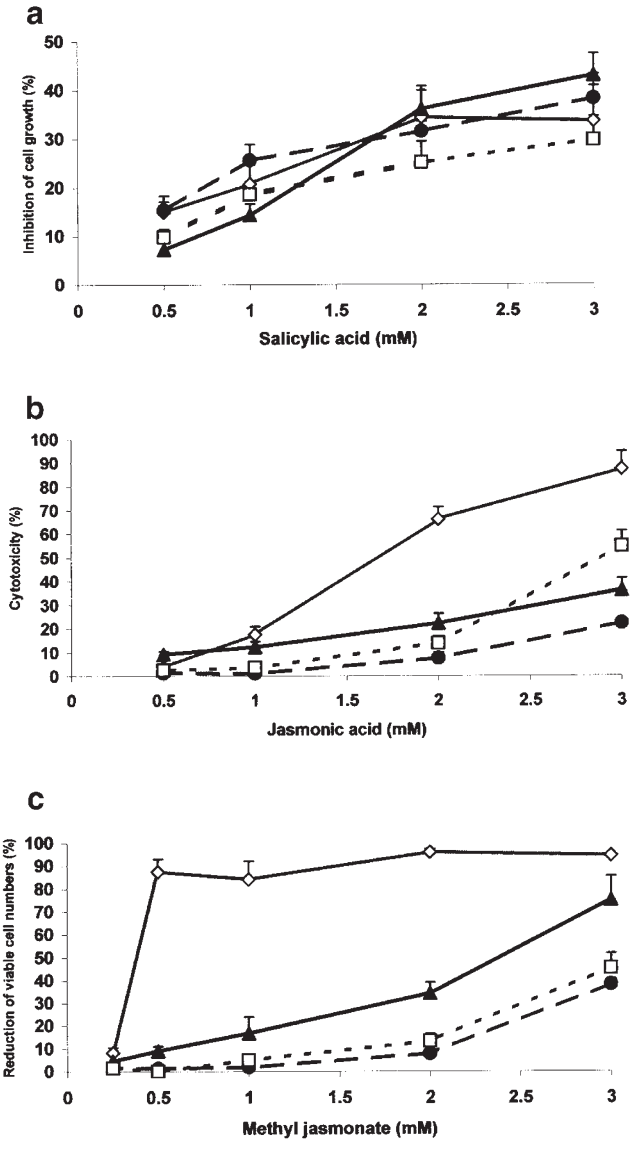

Figure 2 Cytotoxicity of SA, JA and MJ towards human transformed cell lines. Molt-4 cells (at 1.5×10^4/well) were seeded in 96-well plates. LNCaP, MCF7 and SK-28 (at 4×10^3/well) were seeded in 96-well plates and allowed to adhere overnight. (a) SA at the indicated concentrations was added for 24 h. (b) JA at the indicated concentrations was added for 24 h. In the case of Molt-4, cytotoxicity reflects a reduction of viable cell numbers, while for the other cell lines it reflects cellular growth inhibition. (c) MJ at the indicated concentrations was added for 24 h. Optical density representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. (◇) Molt-4 cell; (□) SK28; (▲) LNCaP; (●) MCF7. Cytotoxicity of SA was significant, $P < 0.05$, in Molt-4, SK28 and MCF7 cells at all concentrations and in LNCaP cells from 1 mM and higher. Cytotoxicity of JA was significant, $P < 0.05$, in Molt-4 cells from 1 mM and higher, in LNCaP and SK28 cells from 2 mM and higher, and in MCF7 cells at 3 mM. Cytotoxicity of MJ was significant, $P < 0.01$, in Molt-4 cells at all concentrations and in MCF7 cells at 3 mM; and at $P < 0.05$ in LNCaP and SK28 cells from 2 mM and higher.

Characterization of the damage induced by plant stress hormones on Molt-4 cells

Since Molt-4 leukemia cells exhibited the highest sensitivity to plant stress hormones, we further analyzed the mechanism of Molt-4 cell death.

We determined the type of death induced by JA and MJ in Molt-4 cells. Molt-4 cells were incubated with JA and MJ for 2, 4 and 14 h, and levels of the apoptosis-mediating caspase-3 were determined. Dose-dependent elevation of caspase-3 activity was observed (Figure 4a, b). Incubation with JA for 2 h did not produce any increase in the caspase-3 activity. After 14 h of exposure to MJ, the extent of death at 1 mM and above is such that caspase-3 activity is decreased (Figure 4b). Since caspase-3 activity is a specific marker of the apoptosis process,[10] our results suggest that JA and MJ induced apoptotic death in Molt-4 cells. To confirm this, Molt-4 cells were treated with JA (2 mM) and MJ (0.5 mM) for 14 h, and analyzed by fluorescence microscopy in order to detect essential morphological characteristics of apoptosis. Treatment with JA and MJ induced condensation and fragmentation of chromatin (Figure 5b,c). These results confirm that JA and MJ caused apoptotic death in Molt-4 cells.

In addition, we analyzed the influence of JA and MJ on the

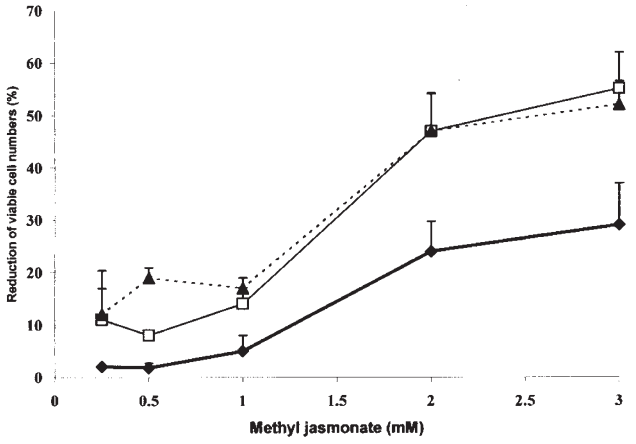

Figure 3 Cytotoxicity of MJ towards Molt-4 cells during short periods of incubation. Molt-4 cells (at 1.5×10^4/well) were seeded in 96-well plates. MJ at the indicated concentrations was added for 2 (◆), 4 (□) or 6 (▲) h. Optical density representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. Cytotoxicity of MJ was significant, $P < 0.05$, from 2, 1 and 0.5 mM and up, for the 2, 4 and 6 h incubation periods, respectively.

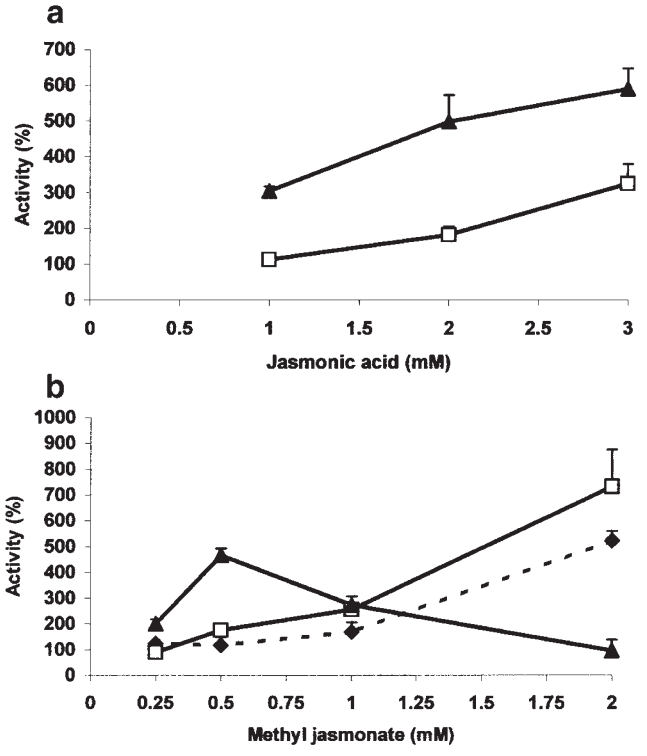

Figure 4 Activation of caspase-3 by JA and MJ. Molt-4 cells (2×10^6/sample) were incubated with JA and MJ. (a) JA at the indicated concentrations was added for 4 and 14 h. (b) MJ at the indicated concentrations was added for 2, 4 and 14 h. The enzymatic activity of caspase-3 was determined by the caspase-3 (CPP32) protease assay kit. (◆) 2 h; (□) 4 h, (▲) 14 h. JA and MJ increased caspase-3 activity significantly, $P < 0.05$, at all concentrations and times. The basal level of caspase-3 activity in untreated cells was 5 pmol/min/10^6 cells.

DNA contents of the nuclei of Molt-4 cells. As is shown in Figures 6 and 7, the plant stress hormones JA and MJ induced significant alterations in Molt-4 cell cycle: appearance of a hypodiploid apoptotic peak and accumulation of necrotic cells at the left-hand end of the X axis.

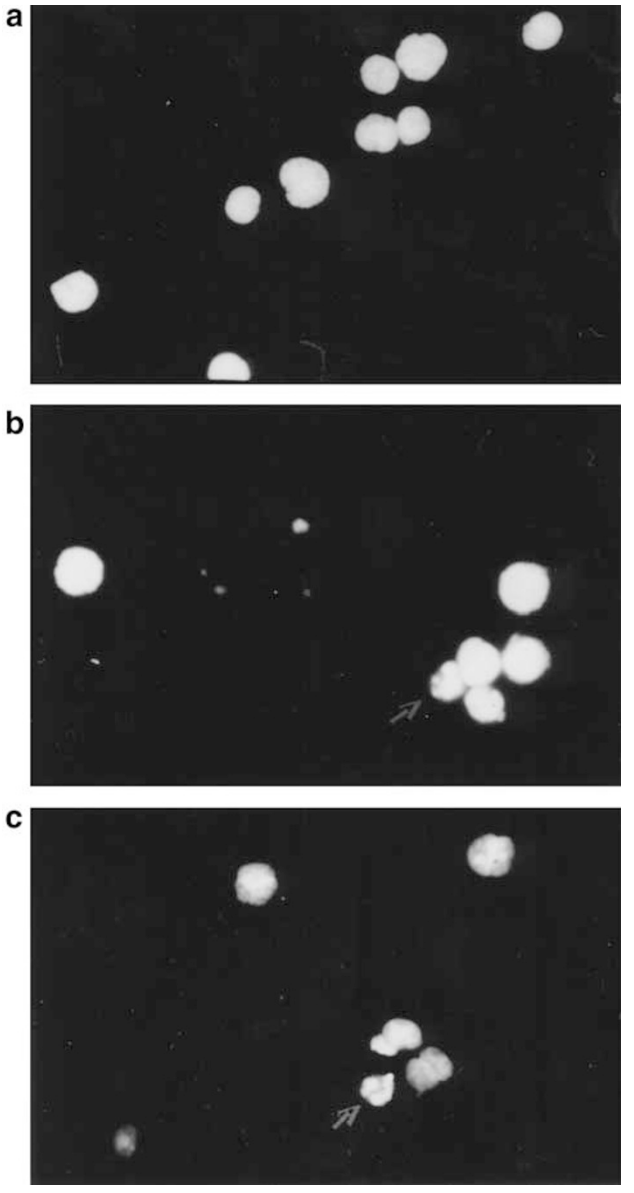

Figure 5 Morphological changes in the nuclei of Molt-4 cells treated with JA and MJ. (a) untreated Molt-4 cells. (b) Molt-4 cells treated with JA at 2 mM for 14 h. (c) Molt-4 cells treated with MJ at 0.5 mM for 14 h. Fixed cells (5×10^5/sample) were stained for 10 min with DAPI (1 μg/ml). Nuclei were analyzed by fluorescence microscopy, at a magnification of 1:400. The arrows point to characteristic apoptotic nuclei.

Specificity of plant stress hormones cytotoxicity

The results presented in this article suggest that plant stress hormones possess the ability to adversely affect cancer cells. Therefore, we studied the effect of these plant products on normal cells. Normal lymphocytes were separated from human peripheral blood. Before treatment with SA, JA and MJ, normal lymphocytes were stimulated by TPA and PHA, causing human lymphocytes to proliferate[21] (a characteristic of the transformed cells). Normal blood lymphocytes were practically uninfluenced by plant stress hormones, in contrast to the Molt-4 transformed lymphoblastic leukemia cells (Figure 8a, b and c). Similar to these results, quiescent lymphocytes were not affected by SA, JA and MJ (data not shown).

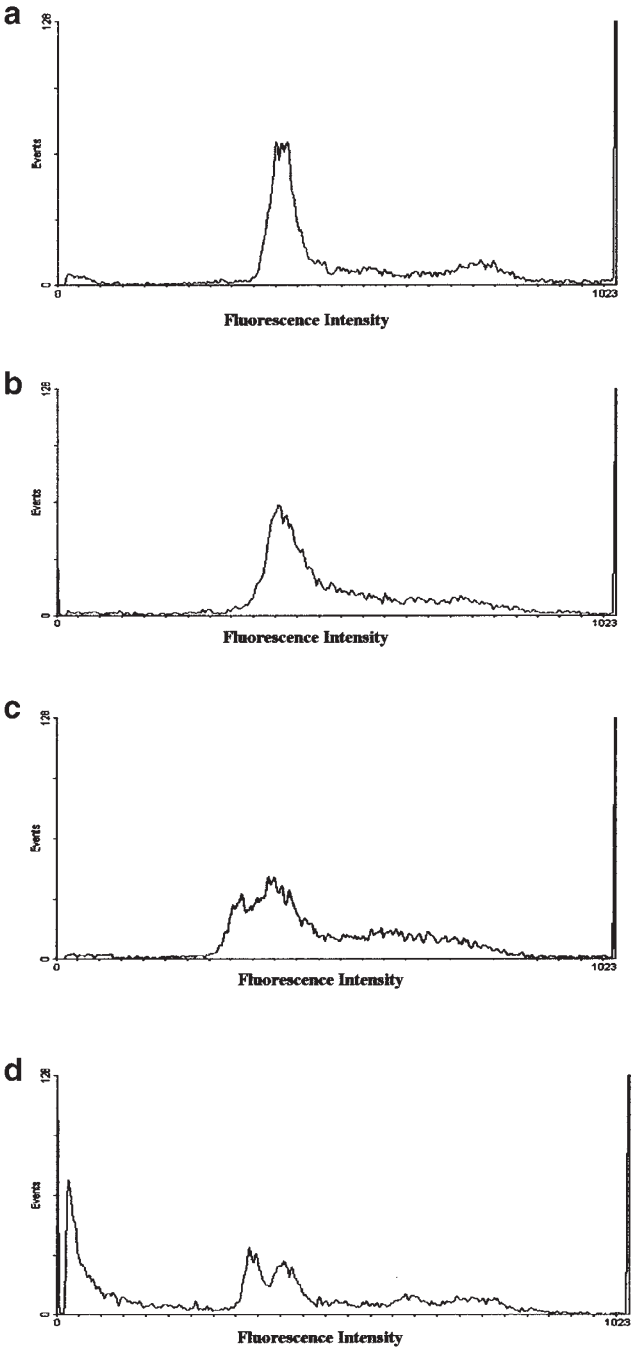

Figure 6 JA treatment and cell cycle analysis. Molt-4 cells (3×10^5/well) were seeded in 24-well plates. (a) Untreated cells. (b) Cells treated with JA at 2 mM for 2 h. (c) Cells treated with JA at 2 mM for 14 h. (d) Cells treated with JA at 2 mM for 24 h. DNA contents reflecting cell cycle stage, were determined by flow cytometry (Fluorescence Intensity). The y axis (Events) represents numbers of cells containing different quantities of DNA.

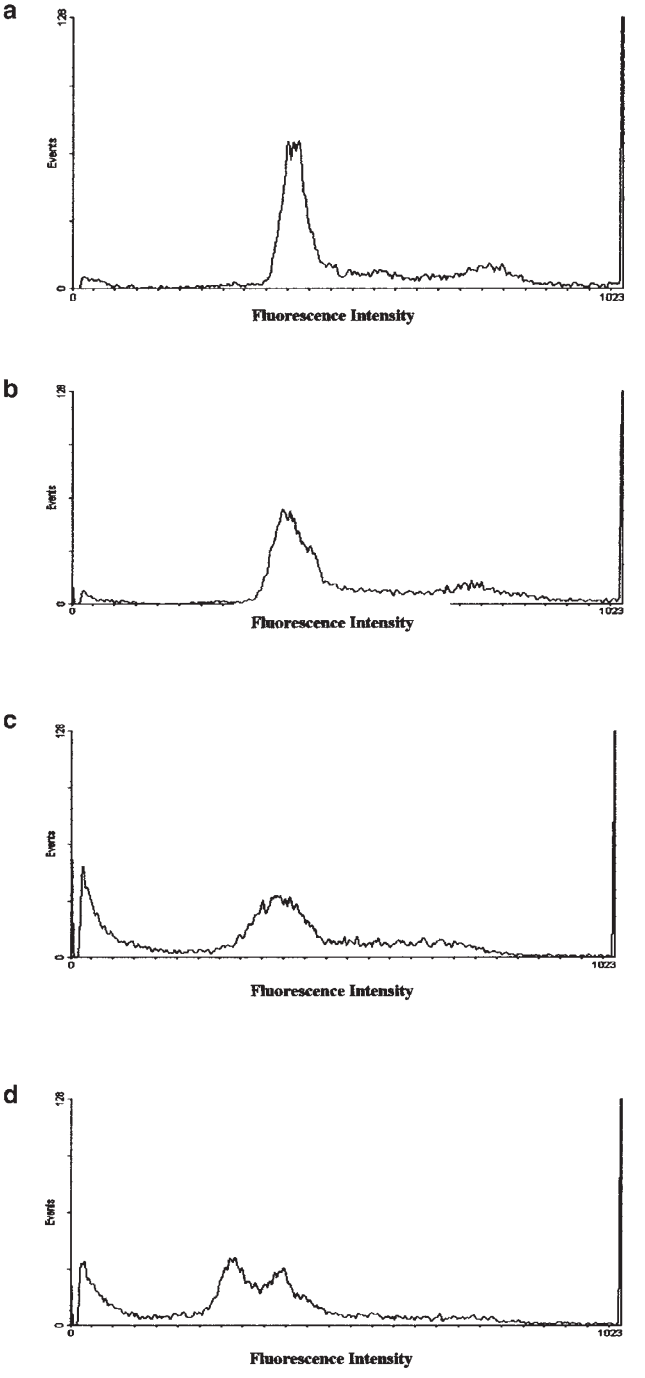

Figure 7 MJ treatment and cell cycle analysis. Molt-4 cells (3×10^5/well) were seeded in 24-well plates. (a) Untreated cells. (b) Cells treated with MJ at 0.5 mM for 2 h. (c) Cells treated with MJ at 0.5 mM for 14 h. (d) Cells treated with MJ at 0.5 mM for 24 h. DNA contents reflecting cell cycle stage, were determined by flow cytometry (Fluorescence Intensity). The y axis (Events) represents numbers of cells containing different quantities of DNA.

Cytotoxicity of plant stress hormones towards EL-4 mouse lymphoma cells in vitro, and effect of MJ on the survival of C57BL/6 mice bearing EL-4 lymphoma in vivo

EL-4 cells were incubated with SA, JA and MJ at concentrations ranging from 0.5 to 3 mM for 24 h. As is shown in Figure 9a, b and c, EL-4 responded in a dose-dependent fashion to all plant stress hormones. The results presented here show that MJ caused the highest level of cytotoxicity. We determined if plant stress hormones cause cell death or inhibition of cell proliferation. EL-4 cells were treated with SA, JA and MJ for 24 h and the viability of the cells was determined by the trypan blue exclusion method. SA induced suppression of cell proliferation in EL-4 lymphoma, while JA and MJ induced death.

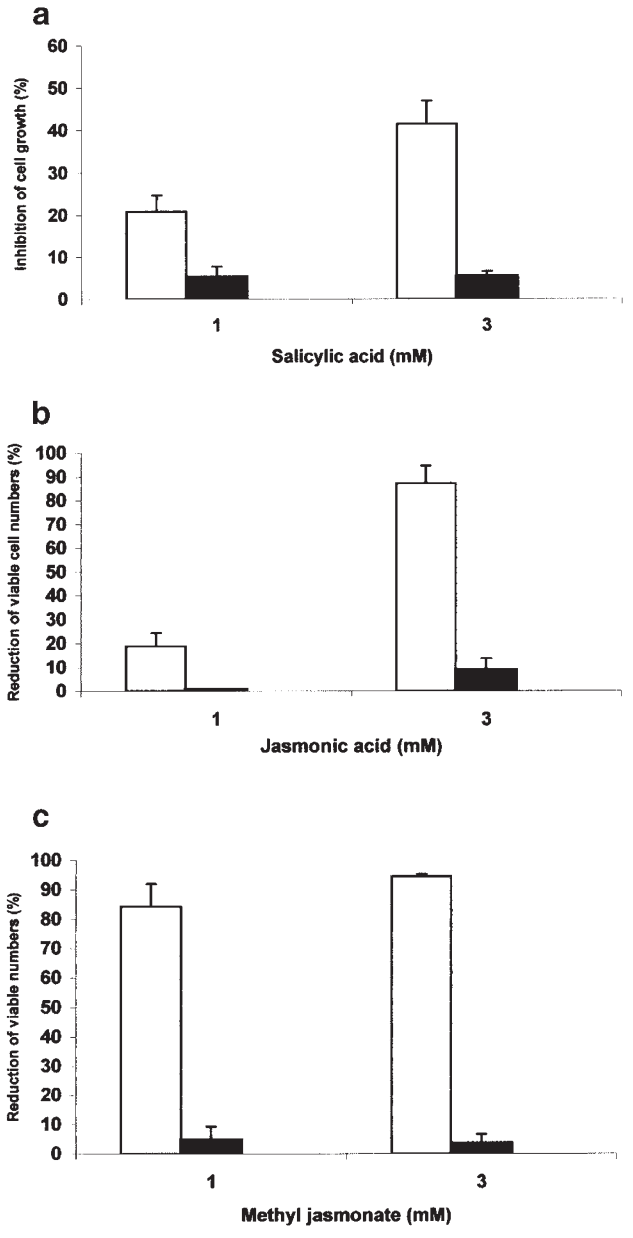

Figure 8 Effect of SA, JA and MJ on Molt-4 cells and on normal lymphocytes. Molt-4 cells (at 1.5×10^4/well) were seeded in 96-well plates (open bars). Normal lymphocytes were preincubated with PHA (0.8 μg/ml) and TPA (5 ng/ml) for 48 h, and seeded (at 1.5×10^4/well) in 96-well plates (solid bars). (a) SA at 1 mM and 3 mM was added for 24 h. (b) JA at 1 mM and 3 mM was added for 24 h. (c) MJ at 1 mM and 3 mM was added for 24 h. Optical density representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. Every plant stress hormone induced significant cytotoxicity in Molt-4 cells, $P < 0.05$, while none of the hormones induced any significant cytotoxicity in normal lymphocytes.

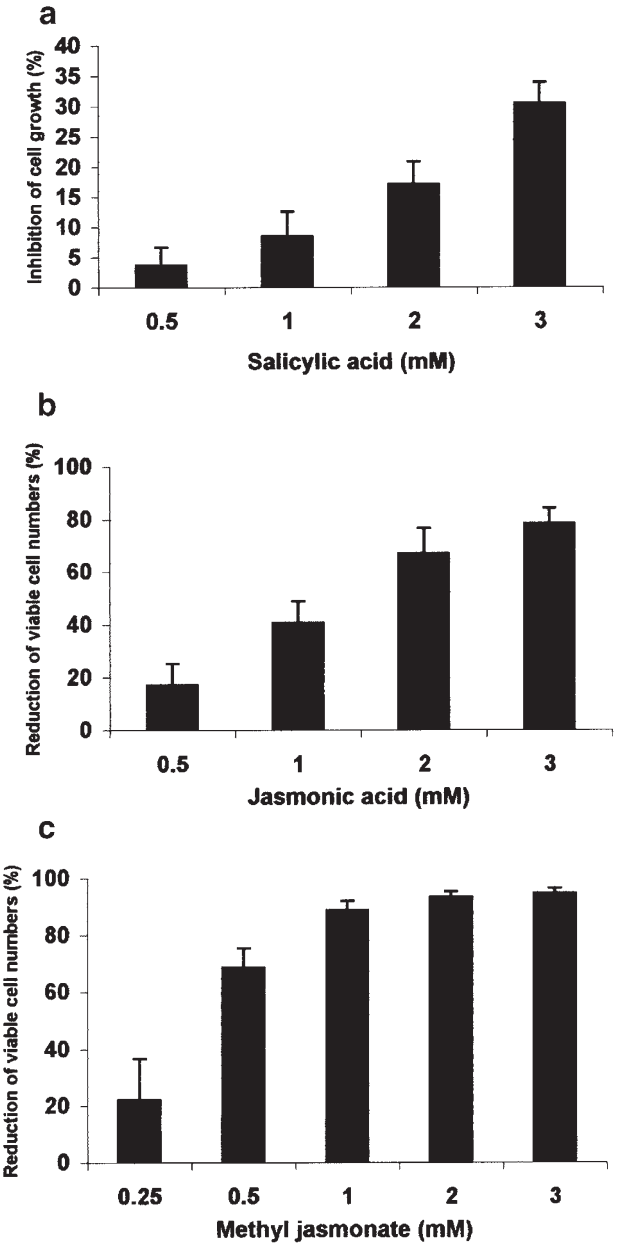

Figure 9 Cytotoxicity of SA, JA and MJ towards mouse T lymphoma cells. EL-4 cells (at 1.5×10^4/well) were seeded in 96-well plates. (a) SA at the indicated concentrations was added for 24 h. (b) JA at the indicated concentrations was added for 24 h. (c) MJ at the indicated concentrations was added for 24 h. Optical density representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. Every plant stress hormone induced significant cytotoxicity in EL-4 cells, $P < 0.05$.

Next, we examined the effect of MJ *in vivo*. MJ was chosen because of its high toxicity towards the mouse lymphoma cells. Syngeneic EL-4 lymphoma cells (5×10^5) were injected i.p. into a test group and a control group of C57BL mice. Methyl jasmonate was administered orally and the effect on the rate of survival was analyzed. Methyl jasmonate was used at 236 mg/kg body weight. We found in preliminary experiments that this dose, and even a two times higher dose, did not cause any apparent toxic effects, ie we did not detect any changes in the activity, feeding and general appearance of the treated mice. Referring to Figure 10, a Kaplan–Meier survivorship function graph is shown, illustrating the cumulative percentage of survival in each group, as the experiment progressed. As is shown in Figure 10, treatment of mice bearing EL-4 lymphoma with MJ caused a significant increase in survival ($P = 0.00953$). Forty-five percent of the treated mice are still alive more than 5 months after the inoculation of the tumor cells. When mice were treated with a methyl jasmonate dose of 47.2 mg/kg body weight, no difference in survival was recorded in comparison to the control group.

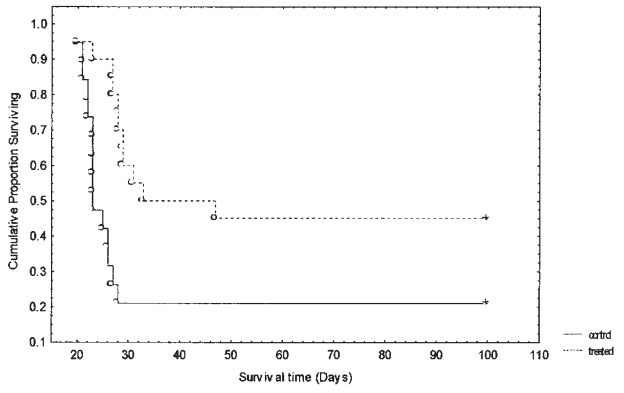

Figure 10 Survival of mice after injection of 5×10^5 EL-4 cells. Cumulative survival data were compiled from two separate experiments and presented by a Kaplan–Meier survivorship function graph. (——) Control untreated, EL-4-bearing mice; (– – –) EL-4-bearing mice treated with 236 mg/kg body weight MJ given orally. Statistical analysis was performed by the Mantel–Cox test. Comparing the control group with the MJ-treated group yielded $P = 0.00953$, $n = 20$.

Discussion

The aim of this study was to examine the effect of structurally diverse plant stress hormones, jasmonates and salicylate, on cell proliferation and viability in different cancer cell lines. There were four major findings. Firstly, all the stress hormones studied here share the ability to adversely affect cancer cells. Secondly, jasmonates caused apoptotic death in Molt-4 cells. Thirdly, SA and jasmonates do not cause damage to normal lymphocytes. Fourthly, jasmonates are effective not only *in vitro*, but also in an animal model of lymphoma, significantly increasing the survival rate.

SA induces intracellular biochemical events typical of stress response in mammalian cells, such as activation of p38 MAPK,[13] and has been found to affect two stress-associated transcription factors, NF-kB[23] and the heat shock transcription factor, HSF.[24] In plants, SA is a stress hormone that serves as an endogenous signal for activation of several plant defense responses, including transcription of the pathogenesis-related genes in response to infection and injury.[25] SA, as well as acetylsalicylic acid (aspirin), belongs to the nonsteroidal anti-inflammatory drugs (NSAID), which are known to act by inhibiting the synthesis of prostaglandins.[26] In addition, NSAID have potent chemopreventive activity.[27–29] This activity has been ascribed to their growth-inhibitory properties,[30] to their ability to induce differentiation,[31] and to their ability to induce apoptosis.[32] In the present study we found that SA inhibits cell proliferation of different cancer cells from 20 to 40%, depending on the cell line (Figure 2a). This finding is in agreement with similar reported observations, where SA inhibited growth of breast cancer cell lines,[14] rat hepatoma and human fibroblasts cultures.[33] A possible interpretation of our data is that SA causes stress in cancer cells, resulting in suppression of proliferation in those cells.

JA and MJ were studied in comparison to SA in order to determine whether the effects of SA on cancer cells are common to plant stress hormones. Jasmonates are widespread in the plant kingdom.[15] The results of the present study showed that while cancer cells from various origins responded to plant stress hormones, their response was differential. From among the tested cell lines, Molt-4 responded strongly to JA (90% cytotoxicity at 3 mM) and MJ (87.5% cytotoxicity at 0.5 mM). In terms of relative susceptibility, Molt-4 cells are followed by SK28, LNCaP and MCF7 cells, in this order. Differential susceptibility of the different cell lines to the plant stress hormones suggests a specificity of the influence of those compounds on the cells.

There is also a difference between SA and jasmonates in their influence on cancer cell lines. SA causes inhibition of cell proliferation in the tested cell lines, JA causes cell death in Molt-4 cells and inhibition of cell proliferation in SK28, LNCaP and MCF7 cells, whereas MJ causes death in all cell lines. These differences could be explained by different structures of plant stress hormones and/or by difference in biochemical events that those compounds induce in the cells. It is important to mention that there is no evidence of using JA, MJ and their derivatives as anti-cancer agents.

Our findings demonstrate that JA and MJ cause apoptotic death in Molt-4 cells, based on the rise in caspase-3 activity, which is one of the features of apoptosis,[10] and on characteristic morphology. In addition, flow cytometric analysis exhibited a characteristic hypodiploid peak to the left of the G_0/G_1 peak. An accumulation of dead cells is also evident at the left-hand end of the x axis in Figures 6 and 7. These are characteristic necrotic cells. These cells may appear either due to necrotic death caused by Jasmonates or secondary to apoptotic death[34] caused by these compounds. Thus, while we demonstrate that Jasmonates induce apopotic death, our results suggest that these plant hormones may also cause necrotic death in Molt-4 cells.

SA was reported to induce apoptosis and activation of caspases in myeloid leukemia cell lines[12] and in B cell chronic lymphocytic leukemia cells.[35] There is also evidence that SA enhances apoptosis in human pancreatic cancer[36] and causes apoptosis in FS-4 cells via p38.[13] In those studies different cell lines undergo apoptosis on incubation with concentrations of salicylates higher than those achieved in plasma of patients treated for inflammatory disorders. In our study we used concentrations of salicylates comparable to those achieved in the plasma. This can explain the difference between studies where SA induced apoptosis, and our studies showing SA to suppress cellular proliferation but not to induce cell death.

Many plant genes that respond to environmental and developmental changes are regulated by jasmonic acid, which is derived from linolenic acid by the octadecanoid pathway. It was shown that plant defense responses to certain wavelengths of ultraviolet radiation require activation of the octadecanoid defense signaling pathway.[37] The process of releasing linolenic acid from the membrane into the cell and converting it to JA, is analogous to signaling pathways in mammalian cells where releasing of arachidonic acid from the membrane results in synthesis of eicosanoids, for example prostaglandins.[38] Prostaglandins of the A and J series, which contain a cyclopentanone ring structure, are potent inhibitors of cell proliferation *in vitro* and are able to suppress tumorigenicity *in vivo*.[39,40] The ability of these compounds to cause growth arrest in a diverse range of tumor cell lines has raised the possibility that they might be useful for the treatment of human cancer.[41] Therefore, the similarity between jasmonates and prostaglandins, both are cyclopentanones, may be relevant to the potency of JA and MJ against cancer cells.

Molecular mechanisms involved in apoptosis induced by SA, are only partially understood. Salicylates induce mitogen activated protein kinase (MAPK) pathways, major pathways by which extracellular stimuli are transduced into intracellular responses.[42,43] There is substantial evidence suggesting that

MAP kinases play important signaling roles in plants.[43] In tobacco suspension cells, SA induces a 48-kDa MAP kinase that may be involved in the defense response of tobacco to TMV infection.[44] SA causes activation of JNK in COS-1 and HT-29 cell lines[20] and activation of p38.[13] Thus, activation of JNK and p38 MAPKs may be involved in the anti-cancer actions of salicylates.

Mammalian cells generate reactive oxygen species (ROS) which, physiologically, are catabolized by antioxidant enzymes.[45] However, in particular conditions they can also lead to oxidative events implicated in various cellular processes such as apoptosis.[46] Recent studies have indicated that reactive oxygen species such as superoxide ions and hydrogen peroxide, play a central role in the activation and propagation of pathogen-induced PCD in plants.[47] PCD that occurs during the HR is accompanied by an increase in the production of ROS and lipid peroxidation.[48] It was found that MJ does not cause PCD in plants, but increases the sensitivity of plants to PCD through ROS by suppression of anti-oxidative enzymes.[49] Possibly, suppression of antioxidative enzymes contributes to the anti-cancer effects of JA and MJ.

In the present study, we compared the influence of plant stress hormones on transformed lymphocytes (Molt-4 cells) *vs* normal lymphocytes from the peripheral blood. Normal lymphocytes stimulated by TPA/PHA to induce proliferation, were not influenced by SA and jasmonates, in contrast to transformed lymphocytes. These data support the potential use of plant stress hormones as selective anti-cancer agents.

The effect of MJ was tested *in vivo*. We have demonstrated that survival rates were significantly higher for the group of mice injected with EL-4 lymphoma and then treated with MJ, *vs* untreated mice. This finding strongly supports our suggestion that plant stress hormones can potentially be used as new anti-cancer agents.

In conclusion, the present study demonstrates that the plant stress hormones SA, JA and MJ cause suppression of proliferation and death in various cancer cell lines, without affecting normal lymphocytes. In addition, MJ was shown to increase significantly the survival of lymphoma-bearing mice. These results suggest that plant stress hormones may share a cytotoxic potential towards cancer cells.

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