Jasmonates: Novel Anticancer Agents Acting Directly and Selectively on Human Cancer Cell Mitochondria

Ronit Rotem,[1] Alina Heyfets,[1] Orit Fingrut,[1] Dorit Blickstein,[2] Mati Shaklai,[2] and Eliezer Flescher[1]

[1]Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel and [2]Rabin Medical Center Beilinson Campus, Felsenstein Medical Research Center, Petach Tikva, Israel

Abstract

We reported previously that jasmonates can kill human cancer cells. Many chemotherapeutic drugs induce mitochondrial membrane permeability transition, membrane depolarization, osmotic swelling, and release of cytochrome *c*, involving the opening of the permeability transition pore complex (PTPC). Because jasmonates exert their cytotoxic effects independent of transcription, translation, and p53 expression, we hypothesized that these compounds may act directly on mitochondria. Mitochondrial membrane depolarization was determined by flow cytometry, and cytochrome *c* release by Western blotting. Mitochondria were isolated by mechanical lysis and differential centrifugation. Cytotoxicity was measured by a tetrazolium-based assay, and mitochondrial swelling by spectrophotometry. Jasmonates induced membrane depolarization and cytochrome *c* release in intact human cancer cell lines. Jasmonates induced swelling in mitochondria isolated from Hep 3B hepatoma cells, but not in mitochondria isolated from 3T3 nontransformed cells or from normal lymphocytes, in a PTPC-mediated manner. Methyl jasmonate induced the release of cytochrome *c* from mitochondria isolated from cancer cell lines in a PTPC-mediated manner, but not from mitochondria isolated from normal lymphocytes. A correlation was found between cytotoxicity of methyl jasmonate and the percentage of leukemic cells in the blood of patients with chronic lymphocytic leukemia (CLL). Jasmonates induced membrane depolarization in CLL cells, and swelling and release of cytochrome *c* in mitochondria isolated from these cells. In conclusion, jasmonates act directly on mitochondria derived from cancer cells in a PTPC- mediated manner, and could therefore bypass premitochondrial apoptotic blocks. Jasmonates are promising candidates for the treatment of CLL and other types of cancer. (Cancer Res 2005; 65(5): 1984-93)

Introduction

Jasmonates are a group of plant stress hormones (1). Upon exposure to stress (e.g., wounding and pathogens), jasmonates are produced in plants and cause the induction of a proteinase inhibitor (2). A coordinated activation of programmed cell death and defense mechanisms often accompany the antimicrobial response of plants (3). We have recently discovered that jasmonates can suppress the proliferation of human cancer cells and induce their death. Methyl jasmonate induced death in breast and prostate carcinoma cells, as well as in melanoma, lymphoma, and leukemia cells (4). Furthermore, we determined that jasmonates are capable of killing cancer cells in a manner independent of cellular mRNA transcription, protein translation (5), and p53 expression.[3] Finally, methyl jasmonate significantly increased the life span of lymphoma-bearing mice (4).

Apoptotic cell death can be mediated via several pathways. One pathway involves the engagement of so-called death receptors belonging to the tumor necrosis factor receptor superfamily. A cascade of proteolytic digestion occurs, involving caspases, resulting in cell death. However, caspase-8 may digest the Bid protein to yield a truncated form that can induce mitochondrial damage, eventually leading to cell death. In addition, many agents (including chemotherapeutic drugs) induce cellular stress, which may also lead to mitochondrial perturbation and, finally, cell death. A mechanism causing the mitochondrial dysfunction mentioned above has been proposed, consisting of mitochondrial membrane permeability transition, dissipation of the inner membrane potential, osmotic swelling of the matrix, rupture of the outer mitochondrial membrane, release of cytochrome *c* and other apoptogenic proteins from the mitochondria, and formation of the caspase-3 activation complex, the apoptosome (6, 7). Permeability transition involves the opening of a channel named permeability transition pore complex (PTPC). The main components of this pore are adenine nucleotide translocator and cyclophilin D in the inner membrane of the mitochondria, and voltage-dependent anion channel and peripheral benzodiazepine receptor in the outer mitochondrial membrane. The PTPC is formed in regions of contact between the inner and outer mitochondrial membranes. Prolonged opening of the PTPC leads to the above-mentioned effects, exposes the cytosol to the contents of the mitochondria, and culminates in cell death (6, 7). Interestingly, mitochondrial permeability transition can lead to both apoptosis as well as necrosis (8).

A novel chemotherapeutic approach has been explored in recent years. This approach is based on cytotoxic molecules that induce direct perturbation of mitochondria, thereby circumventing upstream proapoptotic pathways that may be mutated or lacking in the cancer cell. Many of these cytotoxic molecules act directly on the PTPC, and in some cases, their actual target molecule has been identified (6, 9–11). In fact, several agents targeting the PTPC exhibited beneficial effects in animals and humans bearing tumors, including mice with xenografts of lung carcinoma and cholangiocarcinoma, as well as patients with ovarian cancer (11). Arsenic trioxide is an ancient drug that has been recognized to act directly on cancer cell mitochondria (presumably by acting as a thiol-oxidizing agent) and induce apoptosis in various types of cancer

Requests for reprints: Eliezer Flescher, Department of Human Microbiology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel. Phone: 972-3-640-6063; Fax: 972-3-640-9160; E-mail: flascher@post.tau.ac.il.
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[3] E. Flescher, unpublished data.

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cells including esophageal carcinoma and acute promyelocytic leukemia (12–15). Several proteins that are either components of PTPC or at least recognized as associated with PTPC have been identified as targets of mitochondriotoxic agents. Because members of the Bcl-2 family are known to regulate PTPC opening, proteins belonging to the family and antisense oligonucleotides targeting Bcl-2 are potential proapoptotic agents (6). Notably, Bcl-2 family proteins such as Bax and Bak may also form pores in mitochondrial membranes (and induce cytochrome *c* release) without association with PTPC. Thus, modulation of Bcl-2 family proteins can affect cell death in two modes: dependent or independent of PTPC (6, 7, 16–22). Several adenine nucleotide translocator ligands have been found to induce direct mitochondrial membrane permeabilization. These include lonidamine, arsenite, and the retinoid derivative CD437 (23, 24). In addition, the HIV-1 viral protein T can cross the mitochondrial outer membrane through the voltage-dependent anion channel and interact with adenine nucleotide translocator to form an ion channel and bring about permeability transition and apoptosis (25, 26). Ligands of the peripheral benzodiazepine receptor (e.g., PK11195 and benzodiazepine 4′-chlorodiazepam) have also been shown to cause apoptosis, supporting the suggestion that this PTPC protein may serve as a molecular target for anticancer agents (6, 27).

Since we determined that jasmonates exert their cytotoxic effects independent of cellular transcription, translation, and p53 expression we suspected that these compounds may act directly on mitochondria. The findings presented in this article suggest that jasmonates are mitochondriotoxic toward human cancer cell lines, in a PTPC-mediated mechanism. In addition, they perturb mitochondria isolated from leukemic cells derived from patients with chronic lymphocytic leukemia (CLL) but not those isolated from normal blood lymphocytes.

Materials and Methods

Materials. Reagents were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise stated. Jasmonic acid [3-oxo-2-(2-pentenyl)cyclopentaneacetic acid], methyl jasmonate [methyl 3-oxo-2-(2-pentenyl)cyclopentaneacetic acid], and *cis*-jasmone [3-methyl-2-(2-pentenyl)-2-cyclopenten-1-one], as well as 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$, Molecular Probes, Eugene, OR], ceramide C8, and cyclosporine A, were dissolved in ethanol.

Two PTPC inhibitors were used in this study. The first is cyclosporine A, which binds to cyclophilin D (a member of a family of peptidylpropyl cis-trans isomerases) that is an integral part of the PTPC located in the mitochondrial inner membrane. The cytoprotective effect of cyclosporine A may result (*a*) from its interaction with cyclophilin D leading to inhibition of mitochondrial permeability transition or (*b*) from the ability of cyclosporine A to inhibit calcineurin-mediated dephosphorylation of the Bcl-2 family protein BAD. The latter action of cyclosporine A results in the inhibition of the proapoptotic effect of BAD (28). Nevertheless, because the proapoptotic action of BAD requires translocation from cytosol to mitochondria (29), this mechanism is not relevant to our experimental setting in which isolated mitochondria were studied. Therefore, inhibition of mitochondrial permeability transition by cyclosporine A in our experiments can be attributed to the ability of cyclosporine A to bind cyclophilin D and interfere with the PTPC opening. A second PTPC inhibitor we used was bongkrekic acid. This compound is a ligand of adenine nucleotide translocator, also located in the mitochondrial inner membrane. Bongkrekic acid prevents apoptosis and is a specific inhibitor of mitochondrial permeability transition (30).

Cells. Molt-4 is a human T lymphoblastic leukemia cell line maintained in RPMI 1640 supplemented with 10% FCS. Hep 3B is a human liver carcinoma cell line maintained in DMEM supplemented with 2 mmol/L glutamine, 2 mmol/L sodium pyruvate, nonessential amino acids, and 10% FCS. 3T3 is a human fibroblast immortal, nontransformed cell line maintained in the same medium as Hep 3B cells.

Lymphocytes from venous blood of healthy donors or patients with CLL were prepared by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation (4). The peripheral blood lymphocytes were maintained in RPMI 1640 supplemented with 10% FCS and used further in the experiments. The percentage of CD5+/CD19+ (leukemic) cells in the samples from patients with CLL was determined by flow cytometry, using monoclonal mouse anti-human antibodies, CD5 FITC/CD19 PE by DAKO (Glostrup, Denmark). Written informed consent was obtained from the blood donors and the investigation was approved by the local institutional review board.

Measurement of Mitochondrial Membrane Potential in Intact Cells. Cells were incubated at 37°C and 5% CO_2 with the appropriate jasmonate. The PTPC inhibitors were added 30 minutes before the addition of methyl jasmonate. Carbonyl cyanide 3-chlorophenylhydrazone, a carbonyl cyanide that served as a positive control, was added at 10 μmol/L immediately before the loading with $DiOC_6(3)$. The cell suspension was incubated with 0.4 nmol/L $DiOC_6(3)$, a fluorescent probe used to measure mitochondrial membrane potential, until the time of analysis (usually 30 minutes).

Cytotoxicity Assay. Cytotoxicity was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Upon completion of a given experiment, MTS (a tetrazolium compound) at 333 μg/mL + phenazine methosulfate (at 25 μmol/L) were added to each well of the 96-well plate for 1 hour at 37°C. This allowed for the development of the reaction in which dehydrogenases reduce the MTS in metabolically active cells. The soluble MTS formazan product was measured at 490 nm by the CERES 900 HDi ELISA reader (Bio-Tek Instruments, Inc, Highland Park, VT). Absorbance is directly proportional to the number of living cells in culture. Cytotoxicity (%) was calculated in the following way: [(absorbance of control cells − absorbance of drug-treated cells)/absorbance of control cells] × 100.

Cell Lysis and Isolation of Mitochondria. Mitochondria were isolated from the cells by mechanical lysis and differential centrifugation (31, 32). Briefly, cells were washed with cold PBS at 4°C and centrifuged at 450 × *g*. The pellet was resuspended in cold isolation buffer (75 mmol/L sucrose, 20 mmol/L HEPES, 225 mmol/L mannitol, 0.5 mmol/L EDTA, pH 7.2), and the cells were disrupted by homogenization. Nonlysed cells and nuclei were spun down by centrifugation at 750 × *g* for 20 minutes. The supernatant was further spun at 10,000 × *g* for 10 minutes twice. The pellet, designated as the mitochondrial fraction, was suspended in assay buffer (140 mmol/L KCL, 10 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.5 mmol/L KH_2PO_4, 20 mmol/L HEPES, 0.5 mmol/L EGTA; adjusted to pH 7.2 with KOH). The isolation of mitochondria was determined by measurement of succinate dehydrogenase. The assay buffer was supplemented with 1 mg/mL rotenone and 10 mmol/L succinate immediately before use.

Determination of Cytochrome *c* Release from Isolated Mitochondria. Mitochondria (isolated from 15 × 10^6 cells per sample) were incubated in 1.5-mL Eppendorf tubes at 37°C for various periods. Inhibitors of PTPC were added 15 minutes before the addition of methyl jasmonate. Following the incubation, tubes were centrifuged at 10,000 × *g*. The supernatant contained the cytochrome *c* released from the mitochondria (cytosolic fraction), and the pellet consisted of the mitochondrial fraction. The proteins in these fractions were separated by SDS-PAGE on a 15% Tris-glycine gel, followed by immunoblotting using specific antibodies against cytochrome *c* (1:1000, BD PharMingen, San Diego, CA) at 4°C, overnight. Antigen-antibody complexes were stained with horseradish peroxidase–conjugated antibody (1:1000, Santa Cruz Biotechnology, CA) and enhanced chemiluminescence (ECL) reagent, and exposed to ECL film (Eastman Kodak, Rochester, NY). Immunoblot images were digitized and the optical densities of specific antigen-antibody complexes were quantified using the Gene Gnome Imaging System supported by the Gene Tools software package (both from Syngene, Frederick, MD).

Determination of Cytochrome *c* Release from Intact Cells. Cells were treated with methyl jasmonate, and whole-cell lysates were prepared as

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described before (5). Lysates were spun at 10,000 × g for 10 minutes. The resultant pellet was designated as the mitochondrial fraction and the supernatant as the cytosolic fraction. Protein samples were separated by SDS-PAGE and cytochrome c was determined as mentioned above.

Determination of Mitochondrial Swelling. Purified mitochondria were isolated as described above. Mitochondria suspensions (at 100 μg protein per well) were incubated in 96-well plates at 25°C in assay buffer (140 mmol/L KCl, 10 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.5 mmol/L KH_2PO_4, 20 mmol/L HEPES, 0.5 mmol/L EGTA; adjusted to pH 7.2 with KOH) supplemented with 1 mg/mL rotenone and 10 mmol/L succinate. Various compounds were added after 10 minutes of preincubation. The PTPC inhibitors cyclosporine A or bongkrekic acid were added upon initiation of the preincubation period. Mitochondrial swelling was measured spectrophotometrically. This method equates mitochondrial membrane permeability transition with high-amplitude swelling of the mitochondria. Mitochondrial swelling results in a decrease in absorbance monitored at 540 nm.

Statistical Analysis. Statistical significance was determined using two-tailed Student's t test. Correlation was calculated using Excel statistical package.

Results

Jasmonates Perturb Mitochondria in Intact Cells. Our initial approach to the issue of mitochondrial involvement in jasmonate-induced cancer cell death was to evaluate perturbation of mitochondrial membrane potential. To that end, we measured this parameter in cells treated with three jasmonates: methyl jasmonate, *cis*-jasmone, and jasmonic acid. As can be seen in Fig. 1*A*, each of the jasmonates induced membrane depolarization in Molt-4 cells and their relative potency correlated with their respective cytotoxic effects on these cells (4). Jasmonates are plant stress hormones and we worked with a range of concentrations based on the plasma concentrations achieved upon administration of a well-studied plant stress hormone, salicylic acid. Salicylic acid is a nonsteroidal anti-inflammatory drug. Whereas most nonsteroidal anti-inflammatory drugs (such as indomethacin) act in the micromole per liter range, salicylates act in the low millimole per liter range (33). The highest nontoxic pharmacologic concentration of salicylate used in humans is approximately 3 mmol/L (33). The range of concentrations used here was shown previously to induce cancer cell death while sparing normal lymphocytes (4). Indeed, these concentrations can be achieved in the plasma of mice given methyl jasmonate i.v. without causing any overt toxicity.[4] We chose to further analyze the potent jasmonate derivative methyl jasmonate. The findings regarding membrane depolarization in intact cells do not necessarily imply that the jasmonates act directly upon mitochondria. Therefore, we isolated mitochondria from Molt-4 cells to measure direct effects on mitochondria. Because the yield of mitochondria isolation from Molt-4 cells was not sufficient for measurements of mitochondrial swelling (but was sufficient for the purpose of measuring cytochrome c release), we sought an additional cancer cell model. We identified Hep 3B human hepatoma cells as an appropriate cell type yielding enough mitochondria for the purpose of swelling assays. First, we determined that methyl jasmonate is cytotoxic toward these cells (Fig. 1*B*) and that it can induce a considerable cytochrome c release (a measure of mitochondrial membrane permeability transition) in intact Hep 3B cells (Fig. 1*C*). In addition, we determined that methyl jasmonate induced mitochondrial membrane depolarization in Hep 3B cells (Fig. 1*D*). Once the validity of Hep 3B as a target for jasmonate-induced cytotoxicity was confirmed, we evaluated the effects of methyl jasmonate on mitochondria isolated from Molt-4 and Hep 3B cells.

Methyl Jasmonate Is Directly Toxic to Mitochondria Isolated from Human Cancer Cell Lines in a PTPC-Mediated Fashion. To evaluate permeability transition in isolated mitochondria, we subjected these organelles to methyl jasmonate treatment and measured two characteristic phenomena: release of cytochrome c and mitochondrial swelling. Furthermore, to assess the involvement of PTPC in methyl jasmonate–induced permeability transition, we used inhibitors of the PTPC opening that act via interaction with proteins in the mitochondrial inner membrane: cyclosporine A and bongkrekic acid.

Methyl jasmonate induced the release of cytochrome c from mitochondria isolated from Molt-4 (Fig. 2*A*) and from Hep 3B (Fig. 2*B*) cells in a time-dependent manner. For instance, the level of cytochrome c in mitochondria treated with methyl jasmonate for 60 minutes decreased by about 80% (Fig. 2*A* and *B*). Thus, our findings indicate that methyl jasmonate directly affects mitochondria isolated from cancer cells and induces permeability transition. On the other hand, methyl jasmonate did not induce release of cytochrome c from mitochondria isolated from normal lymphocytes (Fig. 2*C*), in agreement with the selective cytotoxic effect of methyl jasmonate against transformed cells (4). Notably, the methyl jasmonate–induced release of cytochrome c from mitochondria isolated from cancer cells was inhibited by cyclosporine A and bongkrekic acid, suggesting that the mitochondrial permeability transition induced by methyl jasmonate is PTPC mediated (Fig. 2*A* and *B*).

In addition, we determined mitochondrial swelling in mitochondria isolated from Hep 3B cells. As can be seen in Fig. 3*A*, methyl jasmonate and jasmonic acid induced swelling in correlation with their relative cytotoxic effects (4), and so did ceramide C8 serving as a positive control (34). Cyclosporine A and bongkrekic acid prevented the effect of methyl jasmonate (Fig. 3*B*). In contrast, methyl jasmonate and jasmonic acid did not induce swelling in mitochondria isolated from 3T3 human fibroblasts (Fig. 3*C*). Because these cells are immortal but nontransformed, our results suggest again that jasmonates act selectively on mitochondria isolated from transformed cells. In summary, determination of mitochondrial permeability transition by both cytochrome c release and mitochondrial swelling assays indicates that methyl jasmonate affects mitochondria isolated from transformed cells directly, in a PTPC-mediated manner.

To extend these findings to intact cells, we determined the effects of PTPC inhibitors on methyl jasmonate–induced mitochondrial perturbation and cytotoxicity in human cancer cells. Both cyclosporine A and bongkrekic acid inhibited methyl jasmonate–induced mitochondrial membrane depolarization in Molt-4 cells (Fig. 4*A*). In addition, cyclosporine A and bongkrekic acid significantly inhibited the cytotoxic effects of methyl jasmonate toward Molt-4 (Fig. 4*B*) and Hep 3B (Fig. 4*C*) cells. In the 24-hour-long cytotoxicity assay, cyclosporine A was given at 0.25 mol/L, the highest nontoxic concentration.

Jasmonates Induce Mitochondrial Perturbation in Leukemic Cells from Patients with CLL. Having shown the toxic effects of methyl jasmonate against mitochondria isolated from human cancer cell lines, we proceeded to evaluate the potential clinical relevance of our findings. To that end, we determined the ability of

[4] E. Flescher and A. Heyfets, unpublished data.

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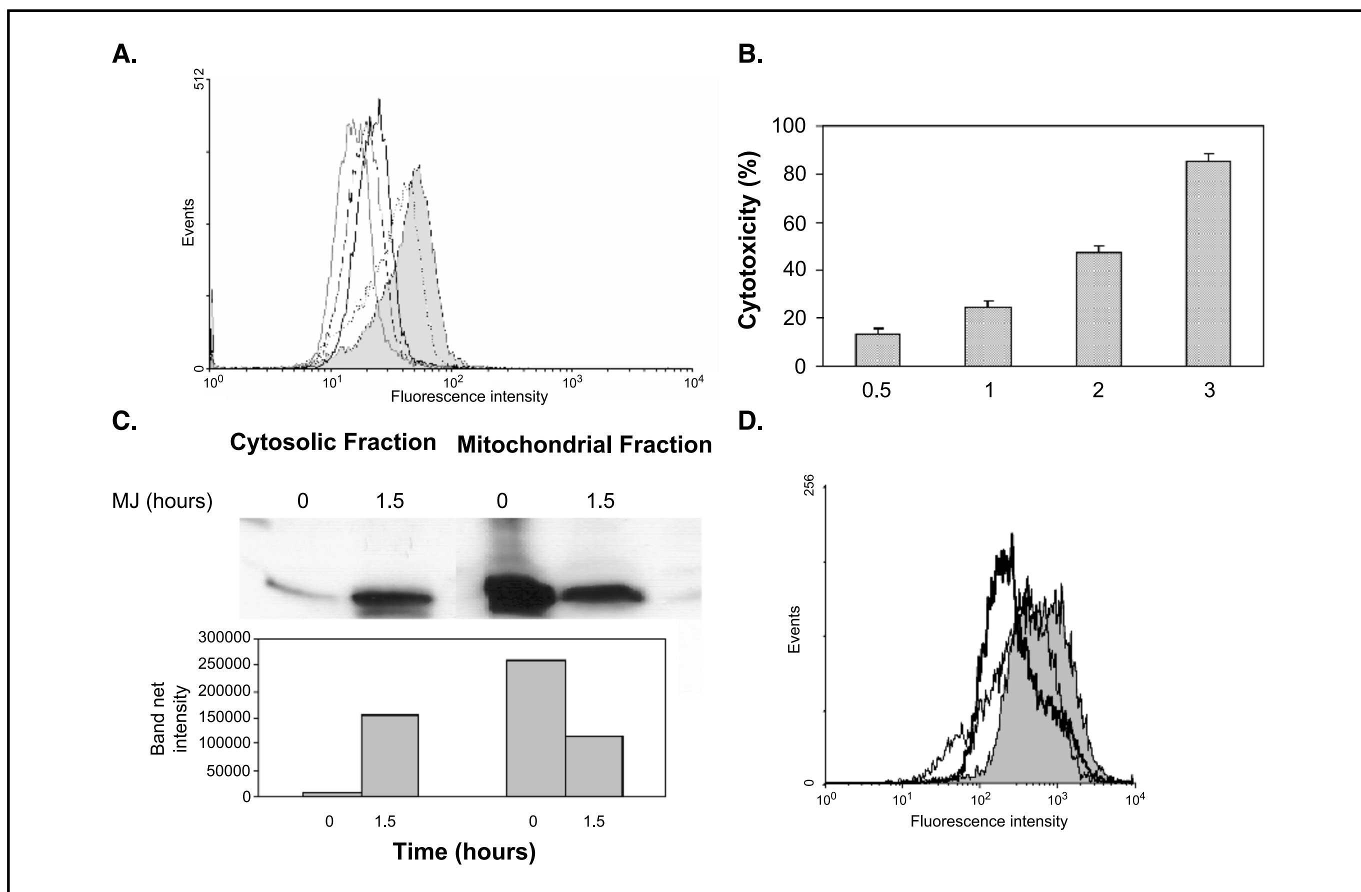

Figure 1. Effect of jasmonates on mitochondrial membrane potential and cytochrome *c* release in intact cells. *A,* 1 million Molt-4 cells were either not treated (*gray area*) or treated with a jasmonate derivative at 2 mmol/L: jasmonic acid (*dashed line*), methyl jasmonate (*bold black line*), or *cis*-jasmone (*fine black line*), for 7 hours. *Gray line,* cells treated with carbonyl cyanide 3-chlorophenylhydrazone (10 μmol/L) as a positive control. The cells were loaded with the fluorescent probe $DiOC_6(3)$ at 0.4 nmol/L. Upon mitochondrial membrane depolarization the fluorescence becomes weaker and the peak moves to the left. *B,* Hep 3B cells, at 5×10^3 cells per well, were seeded in 96-well plates. Methyl jasmonate at the indicated concentrations was added for 24 hours. Absorbance representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. $n = 3$. *C,* Hep 3B cells (at 1×10^6 per well) were seeded in 6-well plates and treated with or without (0) methyl jasmonate (*MJ*) at 1 mmol/L for 90 minutes. Whole-cell lysates were prepared and centrifuged, and proteins were separated by SDS-PAGE followed by immunoblotting using specific antibodies against cytochrome *c*. Antigen-antibody complexes were stained with horseradish peroxidase–conjugated antibody and ECL reagent and exposed to ECL film. Immunoblot images were digitized and the optical densities of specific antigen-antibody complexes were quantified. *D,* 1 million Hep 3B cells were either not treated (*gray area*) or treated with methyl jasmonate at 2 mmol/L (*dashed line*) or 3 mmol/L (*bold black line*) for 3.5 hours. The cells were loaded with the fluorescent probe $DiOC_6(3)$ at 0.4 nmol/L. Upon mitochondrial membrane depolarization the fluorescence becomes weaker and the peak moves to the left.

jasmonates to perturb mitochondria in leukemic cells freshly separated from the peripheral blood of patients with CLL. First, we evaluated the cytotoxic activity of three jasmonates against blood cells from a series of five patients with CLL. As can be seen in Fig. 5, samples from different patients responded differently to jasmonates. A dose-response relationship was evident in most cases, and jasmonic acid was not as effective as *cis*-jasmone and methyl jasmonate (in agreement with the effects of jasmonates against cancer cell lines; ref. 4). We could not find a relationship between the extent of response of a given sample, and most of the patients' characteristics (sex, age, stage of disease, and treatment). However, one parameter, that is, the percentage of leukemic cells in the sample, was an exception. Drawing the cytotoxicity of methyl jasmonate at 0.5 mmol/L against the percentage of CD5+/CD19+ leukemic cells in the lymphocyte population of the respective blood sample yielded a high correlation coefficient ($R^2 = 0.8519$; Fig. 5*D*). These results support our former (4) and current (Figs. 2 and 3) findings suggesting that jasmonates are selectively cytotoxic toward cancer cells.

Next, we analyzed the mitochondriotoxic effects of jasmonates on cells from patients with CLL, studying blood samples containing at least 90% CD5+/CD19+ lymphocytes. First, we compared the effects of different jasmonates on the mitochondrial membrane potential in intact cells from patients with CLL. As can be seen in Fig. 6*A*, jasmonates induced membrane depolarization, the relative effects of the different jasmonates being in correlation with their cytotoxic effects (Fig. 5). We then isolated mitochondria from lymphocytes obtained from the blood of patients with CLL. As can be seen in Fig. 6*B* and *C*, methyl jasmonate induced mitochondrial swelling and release of cytochrome *c* from the mitochondria to the cytosol, similar to its effects on mitochondria from cancer cell lines (Figs. 2 and 3). Thus, the direct effects of methyl jasmonate on mitochondria isolated from cell lines can be reproduced with mitochondria isolated from leukemic cells taken freshly from patients with CLL. On the other hand, mitochondria isolated from normal lymphocytes did not swell in response to methyl jasmonate, reflecting again the selective effect of methyl jasmonate on mitochondria

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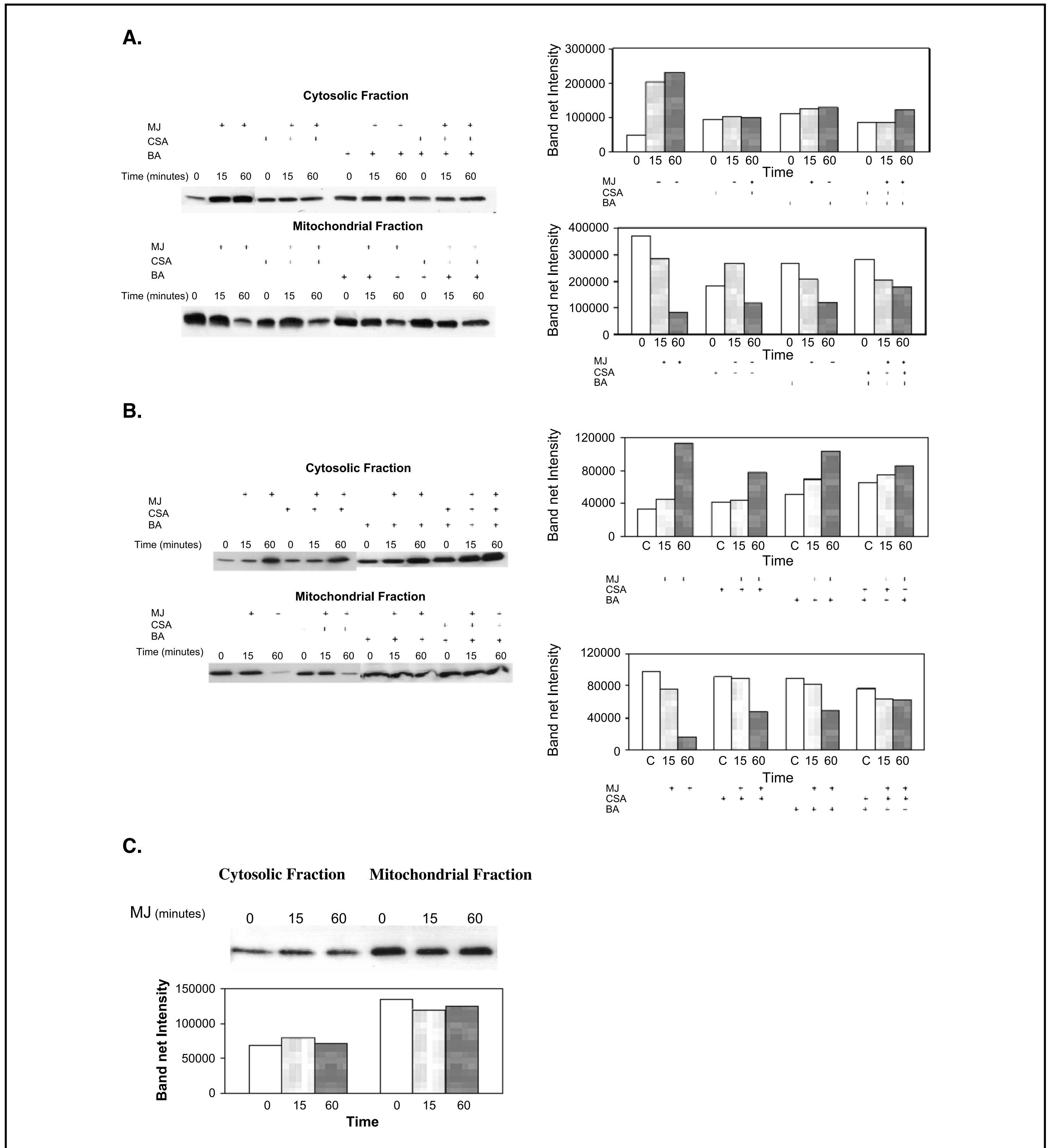

Figure 2. Methyl jasmonate induces cytochrome *c* release in mitochondria isolated from human cancer cell lines. *A,* mitochondria (isolated from 15×10^6 Molt-4 cells per sample) were incubated at 37°C for various periods. Cyclosporine A (*CSA*) at 10 μmol/L and/or bongkrekic acid (*BA*) at 50 μmol/L were added 15 minutes before the addition of methyl jasmonate (*MJ*) at 1 mmol/L. Following the incubation, mitochondrial suspensions were centrifuged at 10,000 × *g*. The supernatant contained the cytochrome *c* released from the mitochondria (cytosolic fraction), whereas the pellet consisted of the mitochondrial fraction. Proteins were separated by SDS-PAGE, followed by immunoblotting using specific antibodies against cytochrome *c*. Antigen-antibody complexes were stained with horseradish peroxidase–conjugated antibody and ECL reagent and exposed to ECL film. Immunoblot images were digitized and the optical densities of specific antigen-antibody complexes were quantified. *B,* essentially the same experiment as that depicted in *A* but with mitochondria from Hep 3B cells. *C,* similar experiment to that depicted in *A* but with mitochondria from normal peripheral blood lymphocytes.

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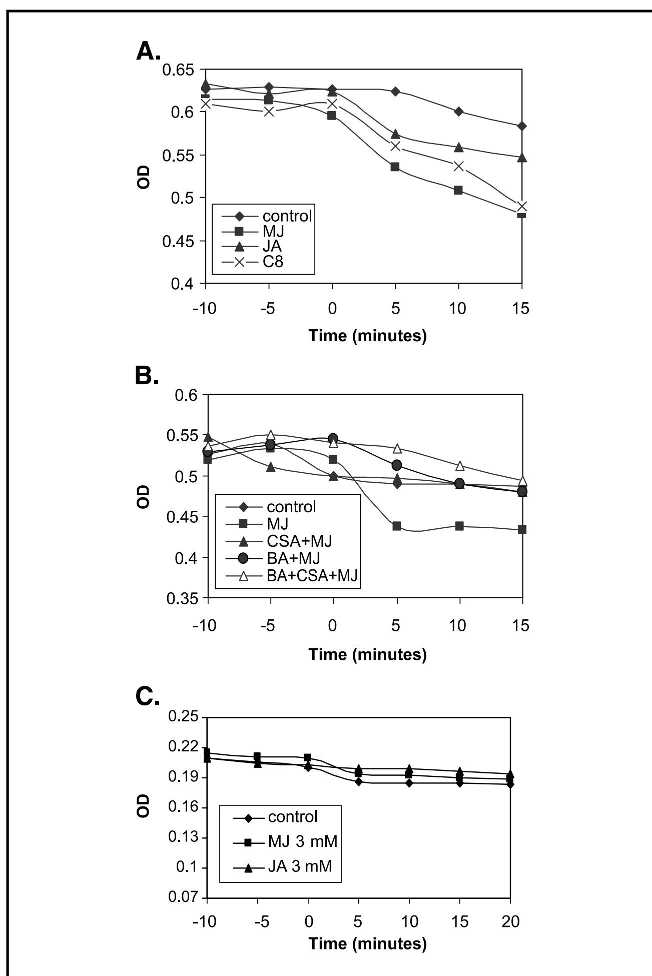

Figure 3. Methyl jasmonate induces swelling in mitochondria isolated from Hep 3B cells. Mitochondria isolated from Hep 3B cells (*A* and *B*) or 3T3 cells (*C*), 100 μg protein per well, were incubated at 25°C in 96-well plates, in assay buffer supplemented with 1 mg/mL rotenone and 10 mmol/L succinate. *A,* methyl jasmonate or jasmonic acid (at 3 mmol/L) or ceramide C8 (at 20 μmol/L) were added after 10 minutes of preincubation. The experimental groups include untreated (♦), methyl jasmonate (*MJ*)–treated (■), jasmonic acid (*JA*)–treated (▲), and ceramide C8 (*C8*)–treated (×) mitochondria. *B,* methyl jasmonate was added after 10 minutes of the preincubation period. Cyclosporine A at 5 μmol/L and/or bongkrekic acid (at 50 μmol/L) were added at the initiation of the preincubation period. The experimental groups included untreated (♦), methyl jasmonate (*MJ*)–treated (■), cyclosporine A + methyl jasmonate (*CSA + MJ*)–treated (▲), bongkrekic acid + methyl jasmonate (*BA + MJ*)–treated (•) and bongkrekic acid + cyclosporine A + methyl jasmonate (*BA + CSA + MJ*)–treated (△) mitochondria. Absorbance decreases upon mitochondrial swelling due to membrane perturbation. *C,* methyl jasmonate or jasmonic acid (at 3 mmol/L) were added after 10 minutes of preincubation. The experimental groups include untreated (♦), methyl jasmonate–treated (■), and jasmonic acid–treated (▲) mitochondria.

isolated from cancer cells (Fig. 6*B* and *D*). Furthermore, stimulating the normal lymphocytes to proliferate did not render their mitochondria sensitive to the methyl jasmonate effect (Fig. 6*D*, *bottom*).

Discussion

In this report jasmonate compounds were determined to comprise a new a family of anticancer agents acting directly on cancer cell mitochondria. The mitochondriotoxic effects (mitochondrial membrane depolarization, swelling, and cytochrome *c* release) are exhibited both against intact cells as well as against isolated mitochondria. Furthermore, mitochondria isolated from normal lymphocytes and from nontransformed fibroblasts were not perturbed by methyl jasmonate. The jasmonate effect is mediated via the mitochondrial PTPC, as judged by its inhibition with cyclosporine A and bongkrekic acid. In addition to the above-mentioned effects on the human cancer cell lines, Molt-4 leukemia and Hep 3B hepatocarcinoma, we showed the cytotoxic effects of jasmonates against *ex vivo* leukemic cells from patients with CLL. Moreover, jasmonates are mitochondriotoxic toward CLL leukemic cells and act directly on mitochondria isolated from these cells.

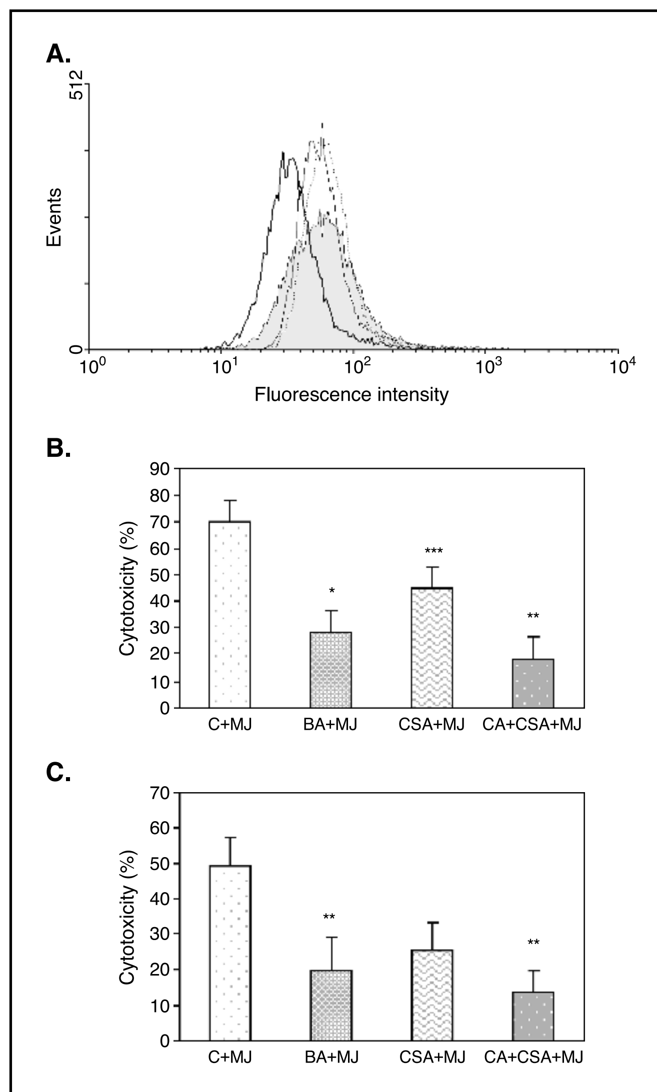

Figure 4. PTPC inhibitors prevent the cytotoxic effects of methyl jasmonate in intact Molt-4 and Hep 3B cells. *A,* 1 million Molt-4 cells were either not treated (*gray area*) or treated with methyl jasmonate at 2 mmol/L by itself (*bold black line*), methyl jasmonate + cyclosporine A at 5 μmol/L (*dashed line*), or methyl jasmonate + bongkrekic acid at 50 μmol/L (*fine black line*) for 7 hours. The cells were loaded with the fluorescent probe $DiOC_6(3)$ at 0.4 nmol/L. Upon mitochondrial membrane depolarization the fluorescence becomes weaker and the peak moves to the left. *B,* Molt-4 cells, at 1.5×10^4 per well, were seeded in 96-well plates. Methyl jasmonate at 1 mmol/L, with or without cyclosporine A (0.25 μmol/L) and/or bongkrekic acid (50 μmol/L) was added for 24 hours. Absorbance representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. $n = 3$. *C,* same as *B* but with Hep 3B cells at 5×10^3 per well. The effect of the PTPC inhibitors was statistically significant: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

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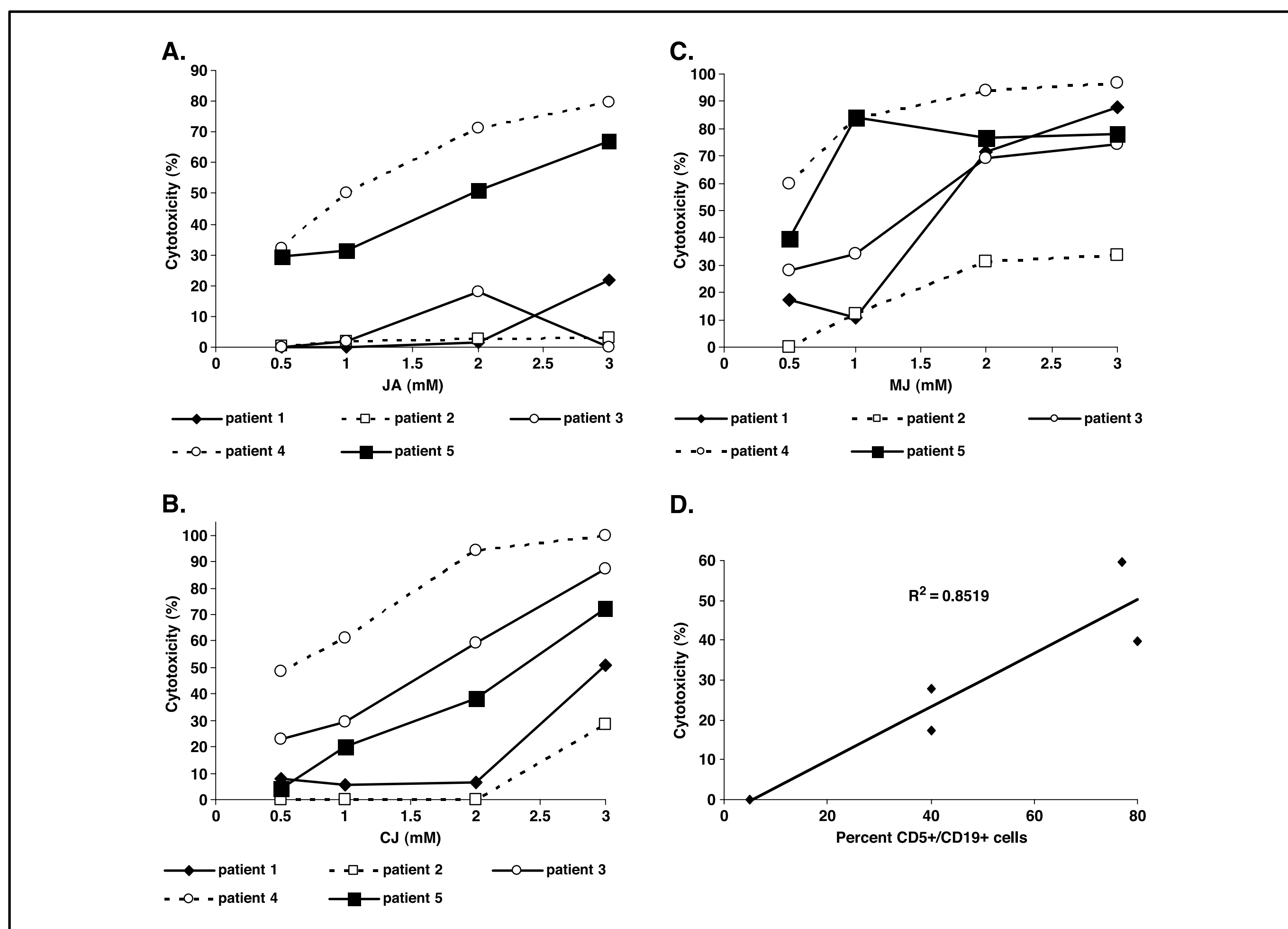

Figure 5. Jasmonates are cytotoxic toward peripheral blood lymphocytes from patients with CLL. *A,* blood lymphocytes, at 1×10^5 per well, were seeded in 96-well plates. Jasmonic acid at the indicated concentrations was added for 24 hours. Absorbance representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. $n = 3$. *B,* same as *A* but with *cis*-jasmone. *C,* same as *A* but with methyl jasmonate. *D,* the cytotoxicity at 0.5 mmol/L methyl jasmonate (*C*) was correlated with the percentage of CD5+/CD19+ cells in the respective blood sample.

The central finding presented here is that jasmonates act directly on mitochondria derived from cancer cells. Furthermore, our findings suggest that whereas PTPC opening may not be a general mechanism of apoptotic cell death (7), it is essential for jasmonate cytotoxicity. It is important to note that the involvement of PTPC in jasmonate-induced mitochondriotoxicity was not only shown in isolated mitochondria but also in intact cells, proving that it is relevant to the actual conditions under which jasmonates induce cell death.

Jasmonates are plant stress hormones, a group that includes salicylate as well. Similar to our findings regarding jasmonates, salicylate induces permeability transition in isolated mitochondria, causing swelling and membrane depolarization. Also, these effects are inhibited by cyclosporine A, suggesting the involvement of PTPC (35–38). Thus, plant stress hormones found by us to share anticancer ability (4) also share the ability to directly perturb mitochondria. In this respect, it is intriguing that a component of the PTPC, voltage-dependent anion channel, is a conserved element of the death machinery in both plant and animal cells (39).

Jasmonates are selectively cytotoxic toward transformed cells (4). Because methyl jasmonate induced cytochrome *c* release from mitochondria isolated from cancer cells but not from normal lymphocytes (Figs. 2 and 6) and induced swelling only in mitochondria isolated from cancer cells but not from non-transformed fibroblasts and normal lymphocytes (Figs. 3 and 6), it most probably exploits mitochondrial characteristics that are differentially expressed in cancer cells. Moreover, the mere fact that cells are proliferating is not sufficient to render them susceptible to methyl jasmonate because mitochondria isolated from an immortal nontransformed cell line (Fig. 3*C*), or from proliferating normal lymphoblasts (Fig. 6*D*, *bottom*), do not swell upon exposure to methyl jasmonate. Thus, our findings suggest that the cancerous transformation of cells results in their mitochondria becoming sensitive to methyl jasmonate. Several findings suggest that the composition and function of mitochondria in cancer cells and normal cells differ. These include a higher mitochondrial membrane potential, possible modulation of the expression of PTPC components, and enhanced rates of ATP generation through glycolysis rather than through oxidative phosphorylation (the Warburg effect) in cancer cells (6, 40, 41). In fact, recent studies of samples freshly taken from human tumors suggest that these cancers bear a distinct signature of mitochondrial energy generation. Liver carcinomas exhibit depletion of cellular mitochondrial contents. Tumors originating from the colon, kidney,

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breast, stomach, esophagus, and lung have significantly reduced expression of the β-catalytic subunit of the mitochondrial H+-ATP synthase (β-F1-ATPase; refs. 42, 43). Thus, even if jasmonates reach normal cells as well, their effects on mitochondria from cancer cells may be different. The difference may be related to PTPC components that jasmonates may directly interact with. Another possibility is that the defective ability of mitochondria in cancer cells to generate ATP may turn these organelles into the weak point of the cells.

CLL is the most prevalent type of adult leukemia in Western countries. Resistance to chemotherapy is a major problem in this disease, and CLL is rarely curable (44). Purine analogues induce significant clinical improvement but are associated inevitably with immune suppression, resulting in opportunistic infections (45). In addition, the combination of 9-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine) and cyclophosphamide induces myelosuppression (46). Thus, a need exists for novel compounds for the treatment of patients with CLL. We found that jasmonates are cytotoxic toward leukemic cells from patients with CLL. Furthermore, we found that treatment of these cells with jasmonates results in mitochondrial membrane depolarization.

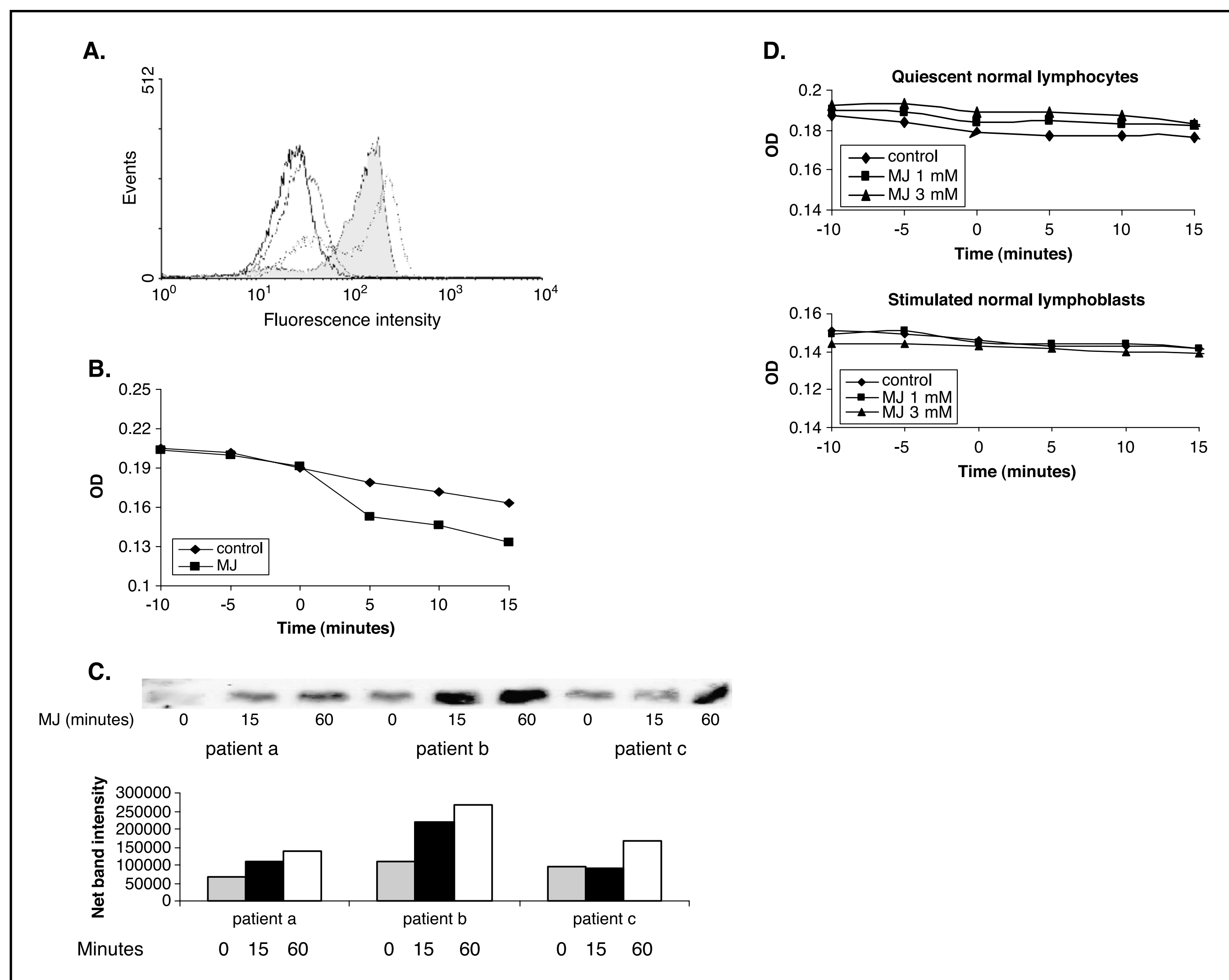

Figure 6. Jasmonates induce mitochondrial perturbation in intact leukemic lymphocytes from patients with CLL and in mitochondria isolated from these cells. *A,* 1 million leukemic cells were either not treated (*gray area*) or treated with a jasmonate derivative at 2 mmol/L: jasmonic acid (*dashed line*), methyl jasmonate (*bold black line*), or *cis*-jasmone (*fine black line*) for 7 hours. The cells were loaded with the fluorescent probe $DiOC_6(3)$ at 0.4 nmol/L. Upon mitochondrial membrane depolarization the fluorescence becomes weaker and the peak moves to the left. *B,* mitochondria isolated from leukemic lymphocytes (100 μg protein per well) were incubated at 25°C in 96-well plates in assay buffer supplemented with 1 mg/mL rotenone and 10 mmol/L succinate. Methyl jasmonate at 3 mmol/L was added after 10 minutes of preincubation. The experimental groups include untreated (♦) and methyl jasmonate–treated (■) mitochondria. Absorbance decreases upon mitochondrial swelling due to membrane perturbation. *C,* mitochondria (isolated from 15×10^6 leukemic lymphocytes per sample) were incubated at 37°C with or without (0) methyl jasmonate at 1 mmol/L for various periods. Following the incubation, mitochondrial suspensions were centrifuged at 10,000 × *g*. The supernatant contained the cytochrome *c* released from the mitochondria (cytosolic fraction). Proteins were separated by SDS-PAGE followed by immunoblotting using specific antibodies against cytochrome *c*. Antigen-antibody complexes were stained with horseradish peroxidase–conjugated antibody and ECL reagent and exposed to ECL film. Immunoblot images were digitized and the optical densities of specific antigen-antibody complexes were quantified. *D,* mitochondria isolated from quiescent or stimulated (preincubated with 0.8 μg/mL phytohemagglutinin + 5 ng/mL tetradecanoyl phorbol acetate for 48 hours) normal lymphocytes (100 μg protein per well) were incubated at 25°C in 96-well plates in assay buffer supplemented with 1 mg/mL rotenone and 10 mmol/L succinate. Methyl jasmonate at the indicated concentrations was added after 10 minutes of preincubation. The experimental groups include untreated (♦), methyl jasmonate (1 mmol/L)–treated (■), and methyl jasmonate (3 mmol/L)–treated (▲) mitochondria. Absorbance decreases upon mitochondrial swelling due to membrane perturbation.

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Several currently available and experimental anticancer agents have similarly been shown to induce mitochondrial membrane depolarization in cells from patients with CLL. These include 2-chloro-2′-deoxyadenosine (cladribine) and 2-chloro-2′-fluorodeoxyadenosine, as well as polyphenols, rolipram, forskolin, and proteasome inhibitors (47–52). Nevertheless, to the best of our knowledge, our study is only the second one to exhibit direct effects of anticancer agents on mitochondria isolated from leukemic cells derived from patients with CLL. The first study found that cladribine and 2-chloro-2′-fluorodeoxyadenosine induced membrane depolarization (48), whereas our study found that methyl jasmonate induced swelling and cytochrome *c* release in mitochondria isolated from CLL leukemic cells.

The accumulation of tumor cells in patients with CLL mostly results from a defect in the apoptotic program (53). Mechanisms accounting for this deficiency include overexpression of anti-apoptotic molecules such as Bcl-2, Mcl-1, and survivin, and impaired expression of proapoptotic molecules including death receptors and p53 (53–59). Indeed, abnormal p53 expression is predictive of shorter survival in patients with CLL (54, 55). Although p53 mutations occur only in a small number of patients with CLL, they present a major clinical problem (60). Given the direct effects of jasmonates on mitochondria, these compounds comprise a new class of anticancer agents capable of circumventing antiapoptotic mutations/deletions in premitochondrial steps of the cell death pathways (61). These apoptotic blocks are instrumental in drug and radiation resistance. Thus, the use of jasmonates is a plausible approach toward treating therapy-resistant patients with CLL and other malignant diseases. Indeed, we recently found that jasmonates are equally cytotoxic toward B lymphoma cells expressing normal or mutant p53.[3] An example to this line of thought is the mitochondriotoxic compound F16, which is capable of killing cells overexpressing Bcl-2 (62).

In conclusion, this article provides evidence that jasmonates constitute a novel group of selective anticancer agents, structurally different from any known cancer chemotherapeutics whose target organelles are the mitochondria. These compounds exhibit activity against leukemic cells from patients with CLL and are thus promising candidates for the treatment of that and other types of cancer.

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