Parthenolide induces significant apoptosis and production of reactive oxygen species in high-risk pre-B leukemia cells

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Abstract

We investigated whether parthenolide, the principal bioactive component of the herb feverfew (Tanacetum parthenium) induced apoptosis in pre-B acute lymphoblastic leukemia (ALL) lines, including cells carrying the t(4;11)(q21;q23) chromosomal translocation. Parthenolide induced rapid apoptotic cell death distinguished by loss of nuclear DNA, externalization of cell membrane phosphatidylserine, and depolarization of mitochondrial membranes at concentrations ranging from 5 to 100 μM. Using reactive oxygen species (ROS)-specific dyes, an increase in nitric oxide and superoxide anion was detected in the cells by 4 h after exposure to parthenolide. Parthenolide-induced elevation of hypochlorite anion was observed only in the two t(4;11) lines. These data suggest parthenolide may have potential as a potent and novel therapeutic agent against pre-B ALLs.

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1. Introduction

Chromosomal abnormalities in the ALL-1 gene (also known as MLL, HRX, and HTRX1) on chromosome 11 are frequently involved in childhood ALL. The chromosomal translocation t(4;11)-(q21;q23) is found in greater than 60% of infants, 2% of children, and 3–6% of adults diagnosed with ALL and the presence of this chromosomal abnormality is strongly associated with an exceedingly poor prognosis [1]. More recently, the frequency of the t(4;11) translocation in infants with ALL was placed at 85% [2]. The t(4;11) ALLs are often classified as pre-B cells, but display a mixed-lineage phenotype with both B and myeloid cell surface markers. Dual-lineage markers suggest that these leukemias may represent pluripotent hematopoietic progenitors that can potentially differentiate into lymphoid, myeloid, or other hematopoietic cells. This high-risk subgroup of ALL is highly resistant to conventional chemotherapeutics and has an exceedingly poor prognosis.

Parthenolide is a sesquiterpene lactone and the principal bioactive component of the medicinal herb...
feverfew (*Tanacetum parthenium*). Feverfew has been used for centuries as a folk medicine to treat migraines and rheumatoid arthritis [3]. Parthenolide has shown anti-inflammatory and anti-cancer activities [4,5]. Potent anti-cancer activity of this substance is due in part to its ability to inhibit the transcription factor NF-κB, thereby reducing survival potential in a number of cancer cells [6,7]. Parthenolide-induced generation of reactive oxygen species (ROS) in cancer cells has also been shown to play a role in promoting apoptotic cell death [8].

We have previously shown that a number of phytochemicals can effectively induce apoptotic cell death in cell lines that were established from patients with high-risk, B-lineage acute lymphoblastic leukemia (ALL) carrying the t(4;11)(q21;q23) chromosomal translocation, as well as other ALL lines without the translocation [9–11]. In the current study, the ability of parthenolide from the herb feverfew to induce apoptosis was examined in two t(4;11) pre-B ALL-derived cell lines and a pre-B ALL line without the translocation. We hypothesized that parthenolide would be effective in killing these leukemia cells. Parthenolide produced significant apoptotic death accompanied by mitochondrial dysfunction in the ALL lines and the t(4;11) lines were more sensitive to this agent than cells without the translocation. Parthenolide increased the production of nitric oxide and superoxide anion in the ALL lines. The production of the potent oxidant hypochlorite anion was also increased in the mixed-lineage t(4;11) lines, but not the cells without the translocation, in response to parthenolide treatment. These data suggest that parthenolide may be useful as a novel therapeutic agent against high-risk t(4;11) as well as other ALLs.

2. Materials and methods

2.1. Reagents

Parthenolide was purchased from EMD Biosciences (San Diego, CA) and stock solutions were dissolved dimethylsulfoxide (DMSO, Sigma Chemical Co., St. Louis, Mo.) before use. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was purchased from Molecular Probes (Eugene, OR) and stock solutions were prepared in DMSO. Alexa Fluor 488-conjugated Annexin V and propidium iodide (PI) were obtained from Molecular Probes and Sigma, respectively. The cell permeable dye 4-amino-5-methylamino-20, 70-difluorofluorescein diacetate (DAF-FM DA), dihydrorhodamine 123 (DHR123) were purchased from Molecular Probes.

2.2. Cell culture

SEM and RS4;11 are established cell lines from patients diagnosed with pre-B cell acute lymphoblastic leukemia (ALL) containing the chromosomal translocation t(4;11)(q21;q23) [12,13]. The REH cell line (pre-B cell ALL without the translocation) was obtained from American Type Culture Collection (Manassas, VA). All cell lines were maintained at 37 °C, 5% CO<sub>2</sub> in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma), 50 IU/ml penicillin, 50 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 1 mM sodium pyruvate, and 2 mM L-glutamine (Invitrogen). For each experiment, unless otherwise noted, the cells were split to a density of 0.5 × 10<sup>6</sup>/ml before treatment. As a control for the dissolving medium used for each chemical, an equivalent amount of the specific diluent was always added to a control cell population in every experiment (designated untreated).

2.3. Flow cytometric analysis of cell death

Immunofluorescence analysis was performed on a FACSCanto fluorescence activated cell sorter (FACS) using FACSDiva software (Becton Dickinson, Mountain View, CA). Cells were incubated with vehicle (0.1% DMSO) or concentrations of parthenolide ranging from 5 to 100 µM and aliquots from each treatment were removed at 24, 48, and 72 h. Cell death was measured by lysing the cells in a hypotonic solution containing 1 mg/ml sodium citrate, 0.1% Triton X-100, and 50 µg/ml propidium iodide (PI, Sigma) and analyzing the resulting nuclei by FACS. The extent of cell death (percentage) was determined by measuring the fraction of nuclei that contained sub-diploid DNA content. Ten thousand events were collected for each sample stained with propidium iodide.

To determine whether cell death was due to apoptosis, cells were plated in 96-well microtiter plates and treated with concentrations of parthenolide ranging from 5 to 100 µM. Control cells were treated with 0.1% DMSO. After 24 h, whole cells were stained with Alexa Fluor 488-conjugated Annexin V (Invitrogen) and PI according to the manufacturer’s protocol. All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells. Ten thousand events were collected for each sample stained with Annexin V and PI.

2.4. Analysis of mitochondrial membrane potential

JC-1 dye was used to measure changes in mitochondrial membrane potential as previously described [9]. Cells were plated in 96-well microtiter plates and treated with
concentrations of parthenolide ranging from 5 to 100 \mu M. Control cells were treated with 0.1% DMSO. After 24 h, the cells were stained with 2 \mu g/ml JC-1 for 30 min at 37 °C, washed with PBS containing 1 g/l bovine serum albumin and sodium azide (Sigma). Fluorescence analyses were performed on the FACSCanto. All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells. Ten thousand events were collected for each sample stained with JC-1.

2.5. Detection of nitric oxide and reactive oxygen species (ROS)

To measure nitric oxide, superoxide anion, or peroxyl radical formation, the cells were incubated with the cell permeable dye DAF-FM DA (1 \mu M), dihydroethidium (5 \mu M), or the lipophilic BODIPY 588/591 C11 (10 \mu M), respectively, for 30 min at 37 °C. Cells were washed and then exposed to 10 \mu M parthenolide for 4 h. After washing the cells in PBS (Ca²⁺/Mg²⁺-free, Invitrogen), the live cell populations were gated and fluorescence analyzed by FACS. To measure other ROS, cells were first incubated with 10 \mu M parthenolide for 4 h, washed, and then incubated with 10 \mu M APF, 10 \mu M HPF, or 1 \mu M DHR123 for 30 min at 37 °C. After a further wash, the live cells were gated and fluorescence of the dyes was measured by FACS. All analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregated cells. Ten thousand events were collected for each sample stained with the ROS specific dyes.

2.6. Statistical analysis

All statistical analyses were performed with GraphPad software (GraphPad Software, Inc., San Diego, CA, USA) and data were displayed as arithmetic means ± standard error (SE). \( P \) values were obtained using two-tailed \( t \)-tests with a confidence interval of 95% for evaluation of the significance of differences between treatment groups.

3. Results

3.1. Parthenolide induces apoptotic cell death in B-ALL lines

Time course and dose–response analyses were performed with two pre-B t(4;11) ALL lines SEM and RS4;11 and the pre-B ALL line REH which has no t(4;11) translocation. The cells were treated once with 0, 5, 10, 25, 50, and 100 \mu M parthenolide, and percent cell death was measured at 24, 48, and 72 h by PI staining of nuclei and evaluation by FACS (Fig. 1). Both SEM and RS4;11 cells showed greater sensitivity to parthenolide at a dose of 10 \mu M compared to REH. By 72 h post-treatment, SEM and RS4;11 showed 74.1 ± 4.5% and 93.9 ± 2.6% cell death, respectively, at a parthenolide dose of 10 \mu M compared to only 36.2 ± 7.7% observed for REH cells at this concentration. Cell death in REH cells reached 91.4 ± 3.7% at a dose of 25 \mu M parthenolide after the 72 h treatment. All increases in cell death after parthenolide treatment were statistically significant compared to untreated cells (\( P < 0.05 \)).

To determine whether the parthenolide-induced cell death observed in the leukemia cells was due to apoptosis, the cells were stained with Alexa Fluor 488-conjugated...
Annexin V and PI after 24 h of treatment with parthenolide at the concentrations described above. Annexin V binds to the phosphatidylserine that is exposed early during the apoptotic process [14]. PI is used to differentiate between early apoptotic and late apoptotic or necrotic cells, since it does not enter cells with intact membranes. Fig. 2 shows distinct populations of early apoptotic cells (Annexin V-positive, bottom right quadrant) and late apoptotic/necrotic cells (Annexin V-positive/PI-positive, top right quadrant) at concentration of 5 and 10 µM parthenolide. Populations of early apoptotic cells were observed for each dose of parthenolide used, indicating that apoptotic death was also occurring at high doses. However, due to the high number of cells that stained with both Annexin V and PI at higher doses, it cannot be ruled out that high dose parthenolide may also induce necrotic cell death.

3.2. Loss of mitochondrial membrane potential

Loss of mitochondrial membrane potential ($\Delta \Psi_{mt}$) is an early event in apoptosis [15]. The lipophilic cation JC-1 was used to determine the extent of mitochondrial membrane depolarization after treatment with different concentrations of parthenolide. JC-1 is a mitochondria-selective dye that forms aggregates in normal polarized mitochondria with an emission of 590 nm after excitation at 488 nm. Monomers of the dye form in mitochondria with depolarized membranes which emit only green fluorescence at 527 nm. The leukemia cells were untreated or treated with 0, 5, 10, 25, 50, and 100 µM parthenolide for 24 h, stained with JC-1, and analyzed by FACS. By 24 h, the percentage of cells with depolarized membranes was greatest at parthenolide concentrations between 10 and 100 µM parthenolide for SEM and RS4;11 (Fig. 3). REH cells displayed a dose dependent increase in the percentage of cells with mitochondrial dysfunction. The increases in percent of cells with mitochondrial depolarization after parthenolide treatment were statistically significant compared to untreated cells ($P < 0.05$).

3.3. Generation of nitric oxide and reactive oxygen species

The generation of ROS can play an important role in depolarizing mitochondria and inducing apoptosis. A series of ROS probes were used to identify the species that were produced in response to parthenolide. We have previously observed the production of nitric oxide during the early stages of the apoptotic process induced by several phytochemicals in the pre-B ALL lines [11]. To determine if parthenolide treatment produced increased nitric oxide production, the cells were pre-incubated with the cell-per-
meable nitric oxide binding dye DAF-FM DA and then treated with 10 μM parthenolide. Parthenolide at a concentration of 10 μM was chosen because it was the lowest concentration that showed significant cell death and depolarized mitochondrial membranes. The cells were analyzed after 4 h of treatment in order to evaluate the early responses to parthenolide. Treatment of the pre-B ALL cells with 10 μM parthenolide increased the production of nitric oxide approximately fourfold, ninefold, and sixfold for SEM, RS4;11, and REH, respectively, compared to untreated cells (Fig. 4).

Generation of other reactive oxygen species was also tested to determine the potential mediators of oxidative stress in the leukemia cells after parthenolide treatment. Overproduction of NO has been linked to the increased generation of superoxide anion [16]. Dihydroethidium has been reported to detect the presence of superoxide anion [17]. Therefore, to determine if an increase in superoxide anion was detectable, we pre-incubated the cells with DHE and treated with parthenolide. We observed an increase in the fluorescence of DHE after treatment of the cells with 10 μM parthenolide for 4 h suggesting that superoxide anion was produced. Treatment of the pre-B ALL cells with 10 μM parthenolide increased the fluorescence of the DHE from approximately 1.3- to 1.5-fold in the leukemia lines compared to untreated cells (Fig. 5).

The two dyes APF and HPF have been used to differentiate between the generation of hypochlorite anion and other ROS [18]. The dye APF detects hypochlorite anion, hydroxyl radical, and peroxynitrite, whereas HPF detects hydroxyl radicals and peroxynitrite. After treating the cells for 4 h with 10 μM parthenolide, they were subsequently stained with APF or HPF. Both SEM and RS4;11 showed statistically significant increases in the fluorescence of APF, but not HPF in response to parthenolide treatment (P < 0.05; Fig. 6). However, REH cells showed no increase in fluorescence of either APF or HPF after treatment with parthenolide.

Dihydrorhodamine (DHR) is a nonfluorescent, mitochondrial selective probe that becomes fluorescent when oxidized by hydrogen peroxide, peroxynitrite, or hypochlorous acid [19]. By 4 h, an increase in fluorescence intensity of a subpopulation of cells was observed in all three lines indicating oxidation of the dye after parthenolide treatment (Fig. 7). The lipophilic dye BODIPY 581/591 C11 was also used to detect peroxyl radicals in the lipid membrane environment. No evidence of lipid peroxidation was observed by 4 h using this dye and later time points were not tested (data not shown).

4. Discussion

Pre-B ALL with chromosomal translocation t(4;11) are commonly refractory to conventional chemotherapeutics after relapse. A need exists for novel approaches to treat high-risk leukemia. Parthenolide, derived from the medicinal herb feverfew, has shown promise as a potential new chemotherapeutic agent. In our study, we show that parthenolide induced significant depolarization of mitochondrial membranes and apoptotic cell death in several pre-B ALL lines. The pre-B ALL lines SEM and RS4;11 with the chromosomal translocation t(4;11) were particularly more susceptible to parthenolide-induced cell death at lowered concen-
trations compared to the REH line which has no translocation. Rapid apoptotic cell death was achieved at a concentration of 10 μM parthenolide in the t(4;11) lines. For REH cells, the concentration had to be increased to 25 μM to observe a similar amount of death.

The anti-cancer activity of parthenolide is partly due to the generation of oxidative stress [8]. An increase in the generation of multiple ROS was observed in the t(4;11) and non-t(4;11) ALL lines after parthenolide treatment, including nitric oxide and superoxide anion. NO reversibly inhibits cytochrome c oxidase in the electron transport chain [20,21]. Overproduction of NO has been linked to
increases in superoxide anion and other ROS, which can lead to disruption of mitochondrial membrane potential. Interaction of NO with superoxide anion can produce peroxynitrite that irreversibly inhibits the electron transport complexes I, II, and III [16]. Of particular interest is the increase in hypochlorite anion in the two t(4;11) lines SEM and RS4;11 after parthenolide treatment, but not in the REH cell line without the translocation. Hypochlorite anion is a potent oxidant and dissociative product of hypochlorous acid and has been shown to be involved in oxidative stress responses that can lead to apoptotic cell death [22,23]. Hypochlorite anion and hypochlorous acid are normally produced in myeloid cells by a myeloperoxidase-catalyzed reaction between hydrogen peroxide and chloride anion. Both SEM and RS4;11 are mixed-lineage leukemias with B and myeloid markers. Although myeloperoxidase activity has not been tested in SEM, the RS4;11 line contains a subpopulation of cells that are myeloperoxidase positive [13].

In the hepatocellular carcinoma line SH-J1, depolarization of mitochondria and apoptotic cell death induced by parthenolide were inhibited by the addition of N-acetyl-L-cysteine, a precursor of glutathione [8]. When compared to immortalized Chang liver cells, cell death of the SH-J1 cells was approximately 75% and only 20% in the Chang liver

![Graphs showing results](image)

**Fig. 6.** Parthenolide treatment increases the production of hypochlorite anion in the t(4;11) leukemia lines. The leukemia cells were untreated (black bars) or incubated with 10 μM parthenolide (white bars) for 4 h, washed, and then stained with APF or HPF for 30 min at 37 °C. Cells were analyzed by FACS. The asterisks indicate a statistically significant difference compared to untreated cells, \( P < 0.05 \). Data are representative of three separate experiments. MFI, mean fluorescent intensity.

![Graphs showing results](image)

**Fig. 7.** Generation of other ROS in response to parthenolide. The leukemia cells were incubated with 10 μM parthenolide for 4 h, washed, and then stained with 1 μM dihydrorhodamine 123 (DHR) for 30 min at 37 °C. Cells were analyzed by FACS. Data are representative of three separate experiments. The top histograms represent untreated cells stained with DHR. The bottom histograms represent cells that were treated with parthenolide. The numbers in histogram show the percentages of cells that have increased fluorescence due to the oxidation of the DHR dye.
cells after a 48 h treatment with 10 μM parthenolide. Furthermore, cellular glutathione levels were increased in Chang liver cells, but decreased in the liver carcinoma line after parthenolide treatment. Herrera et al. [24] also found an increase in glutathione levels in immortalized hippocampal cells after treatment with 5 and 10 μM parthenolide. These data suggest that in noncancerous cells, parthenolide acts as an antioxidant molecule by increasing levels of intracellular glutathione resulting in a decrease in ROS. In contrast, an increase in ROS generation in response to parthenolide appears to increase apoptotic cell death in cancer cells. These data may be extrapolated to experiments performed by Guzman et al. [25]. These authors showed 5 and 7.5 μM parthenolide induced significant apoptotic cell death in patient specimens of both acute and chronic myelogenous leukemia cells. A comparison of parthenolide treatment with that of the chemotherapeutic drug AraC showed parthenolide held a greater degree of specificity for the leukemia cells compared to normal cells. Whereas AraC killed both leukemia cells and normal cells to an equivalent extent, parthenolide showed significantly less toxicity to normal hematopoietic cells from bone marrow and cord blood [25].

Anti-cancer activity of parthenolide has also been attributed to the inhibition of the transcription factor NF-κB. In a xenograft metastasis model of breast cancer, enhanced survival of mice was observed when treated with a combination of paclitaxel [26]. The increased survival was associated with decreased lung metastasis and a decrease in NF-κB levels in residual tumors and metastasized cells. Reduction of NF-κB by parthenolide was found to increase the sensitivity of breast cancer to the chemotherapeutic drug paclitaxel [27]. Weston et al. [28] showed that pre-B ALL cells frequently have increased levels of NFκB that make them resistant to apoptotic stimuli, in this case ionizing radiation. Therefore, the use of parthenolide as a novel treatment for high-risk pre-B ALLs may be advantageous in lieu of the reports of limited toxicity to normal hematopoietic progenitor cells, antioxidant protective activity in other cell types, and the ability to inhibit the pro-survival protein NF-κB. Future investigations on the role of NFκB in the apoptotic process in the high-risk leukemia cells, as well as animal experiments will be useful for evaluating the efficacy of parthenolide as a novel therapeutic agent against this leukemia.

References


