Apoptosis (2006) 11:2225–2235
DOI 10.1007/s10495-006-0287-2

Parthenolide-induced apoptosis in multiple myeloma cells involves reactive oxygen species generation and cell sensitivity depends on catalase activity

Wei Wang · Masaaki Adachi · Rina Kawamura · Hiroki Sakamoto · Toshiaki Hayashi · Tadao Ishida · Kohzoh Imai · Yasuhisa Shinomura

Published online: 17 October 2006

Abstract The sesquiterpene lactone, parthenolide (PTL), possesses strong anticancer activity against various cancer cells. We report that PTL strongly induced apoptosis in 4 multiple myeloma (MM) cell lines and primary MM cells ($CD38^+$ high), but barely induced death in normal lymphocytes ($CD38^{-/+}$low). PTL-mediated apoptosis correlated well with ROS generation and was almost completely inhibited by L-N-acetylcysteine (L-NAC), indicating the crucial role of oxidative stress in the mechanism. Among 4 MM cell lines, there is considerable difference in susceptibility to PTL. KMM-1 and MM1S cells sensitive to PTL possess less catalase activity than the less sensitive KMS-5 and NCI-H929 cells as well as normal lymphocytes. A catalase inhibitor 3-amino-1,2,4-triazole enhanced their PTL-mediated ROS generation and cell death. The siRNA-mediated knockdown of catalase in KMS-5 cells decreased its activity and sensitized them to PTL. Our findings indicate that PTL induced apoptosis in MM cells depends on increased ROS and intracellular catalase activity is a crucial determinant of their sensitivity to PTL.

Keywords ROS · Parthenolide · Multiple myeloma · Catalase · NADPH oxidase

W. Wang · M. Adachi (✉) · R. Kawamura · H. Sakamoto
Division of Molecular Oncology and Molecular Diagnosis, Graduate School of Medicine, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-Ku, Sapporo 060-8543 Japan
e-mail: adachi@sapmed.ac.jp

M. Adachi · T. Hayashi · T. Ishida · K. Imai · Y. Shinomura
First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan

Introduction

The sesquiterpene lactone, parthenolide (PTL), is the principal active component in feverfew (*Tanacetum parthenium*), an herbal medicine used for fever, migraine and arthritis [1]. PTL has anti-microbial, anti-inflammatory and anti-cancer activities [2, 3], which may depend on a wide range of PTL-mediated intracellular signals, dividable into two categories. One is an inhibitory effect on inflammatory responses, such as inhibition of NF-κB and STAT-3-mediated signals [4–8]. The other is the induction of intracellular oxidative stress, which is manifested by elevation of reactive oxygen species (ROS) levels and activation of c-Jun N-terminal kinase (JNK) [4, 9, 10]. In addition, PTL increases phosphorylation of p53 and activates its proapoptotic functions [11]. Although these various functions have been identified, the full mechanism of the anticancer activity of PTL remains obscure.

Recent reports indicate that PTL, either alone or combined with other agents, has a strong anti-cancer activity against a wide variety of cancer cells, such as pancreatic and breast carcinoma cells [12, 13]. Importantly, PTL is more potent against human acute myelogeneous leukemia cells, since it much more specifically induces apoptosis in leukemia cells than in normal hematopoietic progenitor $CD34^+$ $CD38^-$ cells [14]. This suggests that PTL might be a highly potent anticancer agent in a broad range of hematopoietic malignant cells.

Multiple myeloma (MM) cells are mostly quiescent and nucleotide analogs, interfering with DNA replication, are thus not effective. Recently, two new classes of drugs have been identified, i.e., one is the potential NF-κB inhibitor Bortezomib (PS-341), which specifically inhibits proteasome [15, 16], and the other drug is thalidomide, which has an antiangiogenic action and potentially inhibits NF-κB [17, 18]. These promising data encourage further studies

to discover novel anti-MM agents based on the molecular inhibition of NF-κB.

In addition to inhibiting NF-κB, Bortezomib extensively increases ROS generation [19, 20], making its activities similar to those of PTL as described above, which encourages us to consider that PTL may be a good candidate as a novel anti-MM agent. We here found that PTL induces apoptosis in all 4 MM cell lines examined, and the concentrations required to cause extensive cell death were much less than doses toxic to normal lymphocytes. It is noted that there was considerable difference in sensitivity to PTL and two MM cells highly susceptible to PTL, showed more extensive increase of ROS generation and lower catalase activity than the other less-sensitive MM cells and normal lymphocytes. In addition, siRNA-mediated knockdown of catalase expression or catalase inhibitor pretreatment sensitized MM cells to PTL. We conclude that MM cells are sensitive to PTL, but their sensitivity is substantially different, and catalase is a crucial determinant of sensitivity to PTL.

Materials and methods

Cell isolation and culture

Human myeloma MM1S, KMM-1, KMS-5 and NCI-H929 cells, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), were grown in RPMI1640 supplemented with 10% fetal calf serum (FCS). Normal lymphocytes and MM bone marrow cells were obtained from volunteer donors and MM patients, respectively, with informed consent and subjected to Ficoll-Paque (Pharmacia Biotech, Piscataway, NY) density gradient separation to isolate mononuclear cells. The characteristics of the two MM patients are as follows; age: 82 and 83 years, CD38: 62.5% and 93.3%, CD56: 5.8% and 76.6% in the specimens, respectively. To maintain cell viability, peripheral lymphocytes were cultured with IL-2 (200 U/ml), which is kindly provided by Shionogi Chemical Pharmacy (Osaka, Japan). To evaluate cell viability, cells were mixed with the same volume of 0.4% trypan blue solution, and immediately examined to determine whether they could exclude the dye under light microscopical observation. Apoptotic death was evaluated by nuclear fragmentation in 46'-diamidino-2-phenylindole-2 HCl (DAPI)-staining cells. After fixation in 70% ethanol for 10 min, cells were stained with DAPI (0.5 μg/ml) for 10 min, and observed under UV-light using a confocal microscope (R2100AG2, Bio-Rad, Melville, NY).

Flow cytometry

Cell death in primary MM BM cells was evaluated by 7-aminoactinomycin (7-AAD; BD Bioscience Pharmingen, Mountain View, CA). After 24 h of PTL treatment, cells were labeled with anti-CD38 PE (phycoerythrin; Becton Dickinson) for 15 min, washed and further incubated with 7-AAD for 15 min followed by analysis using a FACScan flow cytometry (Becton Dickinson). The percent cell death was defined as 7-AAD$^+$ cells on gates set for CD38$^+$ (High) or CD38$^{-/+}$ (Low) population.

Western blots

Cells were lyzed by RIPA buffer (100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1% NP-40 and 50 mM Tris-HCl [pH 7.2]), separated by 10 to 15% SDS-PAGE gels (50 μg/lane). Western blots were performed by a standard enhanced chemiluminescence (ECL) method (Amersham) using the following antibodies: anti-14–3-3 and anti-HSC70 (Santa Cruz Biotechnology; Santa Cruz, CA); anti-Bcl-xL and anti-heme oxygenase-1 (BD Bioscience Pharmingen); anti-catalase (Sigma, St Louis, MO). 14–3-3 or HSC70 expression shows the same amount of protein loaded in each lane.

DNA fragmentation assay

Low-molecular weight genomic DNA extracted with the lysis buffer (0.5% Triton X-100, 10 nM EDTA and 10 mM Tris-HCl, pH 7.4) was treated with 400 μg/ml of RNase A and Proteinase K for 1 h at 37°C, isopropanol precipitated and subjected onto 1% agarose gels, which were stained with 1 μg/ml of ethidium bromide.

ROS detection

Following treatment, cells were incubated with 10 μM 5-(and-6)-carboxy-2′,7′- dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) C-400 (Molecular Probes; Eugene, OR) for 30 min, after which they were washed, and further incubated with comPTLete medium for 2 to 3 h. Alternatively, cells were incubated for 30 min with 10 μM 2-[6-(4-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF, Daiichi Pure Chemicals Co., Ibaraki, Japan), which is believed to detect superoxide selectively [21] and thereafter washed with PBS. ROS generation was determined using a FACScan flow cytometry using CellQuest Software™, and fluorescent signals were disptlayed as histograms. L-N-acetylcystein (L-NAC), a NADPH oxidase inhibitor diphenylene iodonium (DPI) [22] and a catalase inhibitor 3-amino-1,2,4-triazole (AT), were purchased from Sigma.

Detection of mitochondrial membrane potential ($\Delta\Psi$m)

Cells were incubated with 0.5 μM MitoTracker Orange CM-H_2TMRos (Molecular Probes) for 30 min, after which they were washed with PBS, $\Delta\Psi$m was determined using a FACScan flow cytometer (Becton Dickinson). MitoTracker Or-

ange CM-H_2TMRos does not fluorescence until it enters an actively respiring cell, where they are oxidized to fluorescent mitochondron-selective probes, and the fluorescent signals (FL2) were disptlayed as histograms or graphs by calculating the mean fluorescence intensity. $\Delta\Psi$m was also determined with prewarmed DePsipher^{TM} Kit (Trevigen, Gaithersburg, MD).

Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted with TRIzol (BRL Life and Technologies, MD). The cDNAs were amptlified from 2 μg of total RNA using ThermoScript RT-PCR System with oligo (dT) 12–18 (Invitrogen, Carlsbad, CA), analyzed on 2% agarose gel and confirmed by nucleotide sequencing. The following primer pairs were used for RT-PCR: Bcl-xL: 5′-atg tctcagagcaaccggga-3′and 5′-ggaattcccatagagttccacaaaagtatc -3′; GAPDH: 5′-cgaccactttgtcaagctca-3′ and 5′-aggggtctacat ggcaactg-3′.

ROS scavenger enzyme assays

Catalase and glutathione peroxidase (GPX) enzymetic activity was determined using BIOXYTECH Catalase-520 and GPX-340, respectively (OXIS International Inc, Oregon) according to the manufacture's protocol.

Small RNA interference

The 21-nt duplex small interfering (si) RNA pools for catalase (siGENOME SMARTpool M-010021), and control siRNAs (random; 5′-NNACTCTATCTGCACGC TGAC-3′) were purchased from Dharmacon (Lafayette, CO). Cells (5×10^5 cells/well in a 12-well plate) were incubated for 24 h, and transfected either with catalase siRNA or control random siRNA (siRandom) duplexes (80 nmoles each) using LipofectAMINE 2000. After 48–60 h, cells were used for analysis for western blots and catalase activity. Transfection efficiency (usually $> 50\%$) was assessed in parallel wells by transfection with pEGFP expression vector (BD Biosciences Clontech, Mountain View, CA).

Statistical analysis

Statistical analysis was evaluated using Student's t test (SPSS® program version 10.1;San Rafael, CA). $P<0.05$ was considered statistically significant.

Results

PTL induces apoptosis in MM cells

We first investigated the effects of PTL on primary MM bone marrow (BM) cells and four different MM cells. At 24 h incubation, more than 10 μM PTL treatment strongly induced cell death in CD38$^{+(High)}$ BM MM cells, but far less death in CD38$^{-/+(Low)}$ normal BM lymphocytes obtained from two independent MM patient (Fig. 1a). After 48 h, 1.25 μM PTL treatment began to reduce cell viability in two out of four MM cell lines, whereas normal peripheral lymphocytes showed no appreciable toxicity at 5 μM and only began to demonstrate weak toxicity at 10 μM PTL, which strongly induced death in all MM cell lines. Thus, overall MM cells were more sensitive to PTL than normal BM cells and peripheral lymphocytes (Fig. 1b). However, sensitivity of MM cells to PTL was somehow different, i.e., MM1S and KMM-1 cells were more sensitive than the other two KMS-5 and NCI-H929 cells.

Indeed, after treatment with 2.5 μM PTL, cells with nuclear fragmentation strongly increased in MM1S and KMM-1 cells (Fig. 1c) and the mitochondrial membrane potential (MMP) was profoundly decreased in these cells (Fig. 1d). In KMS-5 and NCI-H929 cells, 2.5 μM PTL however did not show distinct effect on nuclear fragmentation and loss of MMP (data not shown).

PTL-induced ROS generation is crucial for its proapoptotic effect

As pointed out in several reports [9, 11, 14], ROS generation may be important for PTL-induced apoptosis in MM cells. We thus monitored intracellular ROS levels after PTL treatment. KMM-1 and MM1S cells showed large increases of ROS levels 10 h after 2.5 μM PTL treatment and thereafter the increased ROS declined, while much smaller increases of ROS were observed in KMS-5 and NCI-H929 cells under the same treatment (Fig. 2a). We next monitored the expression of oxidative stress-inducible heme oxygenase-1 (HO-1). HO-1 was strongly induced by PTL treatment (2.5 μM) in KMM-1 and MM1S, but was not apparent in KMS-5 and NCI-H929 cells (Fig. 2b), which was consistent with the levels of increased ROS generation. In KMM-1 and MM1S cells, distinct HO-1 induction was observed in a dose-response manner when more than or equal to 1.25 μM PTL was added (Fig. 2c). The concentrations correspond well to those required for affecting cell survival rates as shown in Fig. 1b. These results strongly suggest that the increased ROS may be crucial for the PTL-mediated proapoptotic effect in MM cells. In contrast, we found Bcl-xL expression was barely affected by PTL (Fig. 2d) and steady-state Bcl-xL mRNA and protein expression levels were not linked to the susceptibility of the four MM cells to PTL (Fig. 2e), although a previous study had suggested a link between Bcl-xL expression and susceptibility to PTL in cholangiocarcinoma cells [11].

We next investigated whether oxidative stress is crucial for PTL-mediated apoptosis. To explore this, the free

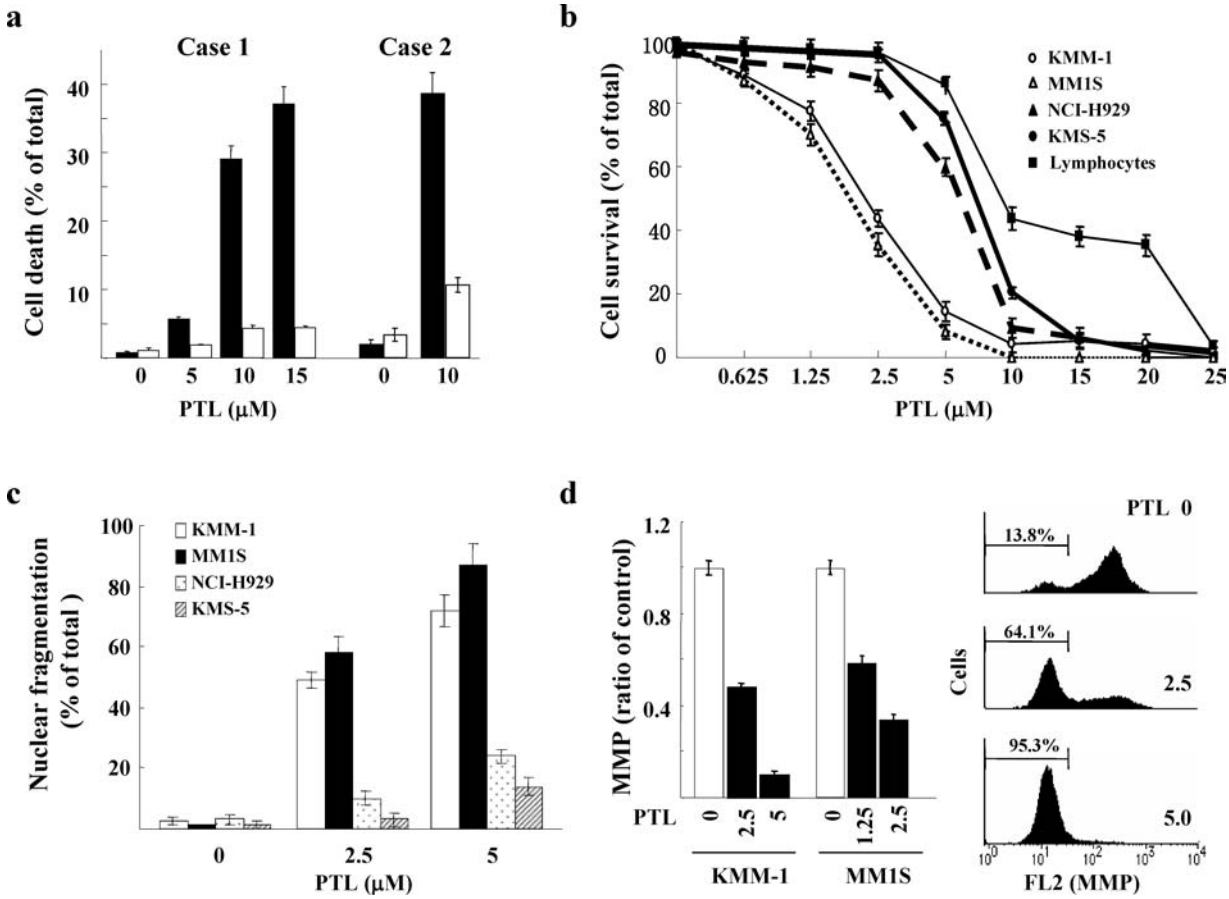

Fig. 1 PTL-induced apoptosis in MM cells. (**a**) Cell death. Viability of $CD38^{+}$(high) MM (closed) and $CD38^{-/+}$(low) normal lymphocytes (open bars) of BM cells in MM patients was analyzed by 7-AAD staining assay 24 h after PTL treatment (μM). (**b**) Trypan blue exclusion assay. Cell viability of KMS-5 (thick), NCI-H929 (thick broken), KMM-1 (thin), MM1S cells (thin broken line) and normal peripheral lymphocytes (closed square) was evaluated after 48 h incubation with PTL at the indicated concentrations. (**c**) DAPI staining. Cells with fragmented nuclei were detected by confocal microscopy after 48 h at indicated doses of PTL. (**d**) Disruption of $\Delta\Psi$m. Cells were treated with the indicated doses (μM; closed) of PTL for 48 (KMM-1) or 36 (MM1S) h, then loaded with MitoTracker Orange CM-H_2TMRos, which becomes actively fluorescent after intact mitochondrial oxidation, and its intracellular mean fluorescence intensities (MFIs) were measured. Representative histograms of KMM-1 and numbers of cell (% of total) with disruption of $\Delta\Psi$m are shown. All data display the mean $\pm$ S.D. of data from three separate experiments

radical scavenger L-N-acetylcystein (L-NAC) was incubated with the cells prior to PTL treatment. In susceptible KMM-1 and MM1S cells, L-NAC pretreatment almost completely inhibited ROS generation and HO-1 induction by PTL (Fig. 3a and b). Importantly, L-NAC profoundly inhibited PTL-induced cell death and nuclear fragmentation (Fig. 3c and d). In addition, L-NAC strongly inhibited PTL-induced DNA fragmentation and loss of MMP (Fig. 4a and b), confirming that elevated ROS is necessary for PTL-induced apoptosis in these two cells. We also investigated the effects of L-NAC in KMS-5 and NCI-H929 cells. As described above, these cells were less sensitive to PTL, but higher PTL concentrations induced ROS generation and L-NAC substantially inhibited PTL-induced ROS generation and subsequent nuclear fragmentation (Fig. 4c and d).

PTL-mediated ROS generation is sensitive to DPI

PTL induces ROS generation in a wide variety of cells [9, 11, 14], but the mechanism(s) for the ROS generation remains unclear. To determine the possible involvement of cellular NADPH oxidases in PTL-mediated ROS generation, we tested the effect of diphenylene iodonium chloride (DPI), a specific inhibitor of flavonoid-containing enzymes such as NADPH oxidase and nitric oxide synthase [23]. DPI pretreatment in a dose of 2.5 μM strongly inhibited PTL-mediated ROS generation in MM1S and KMM-1 cells (Fig. 5a). DPI in this concentration showed no significant effect on cell viability, and almost completely inhibited PTL-induced nuclear fragmentation and HO-1 induction (Fig. 5b and c). These findings suggest that that NADPH oxidase activation may be involved in PTL-mediated ROS generation.

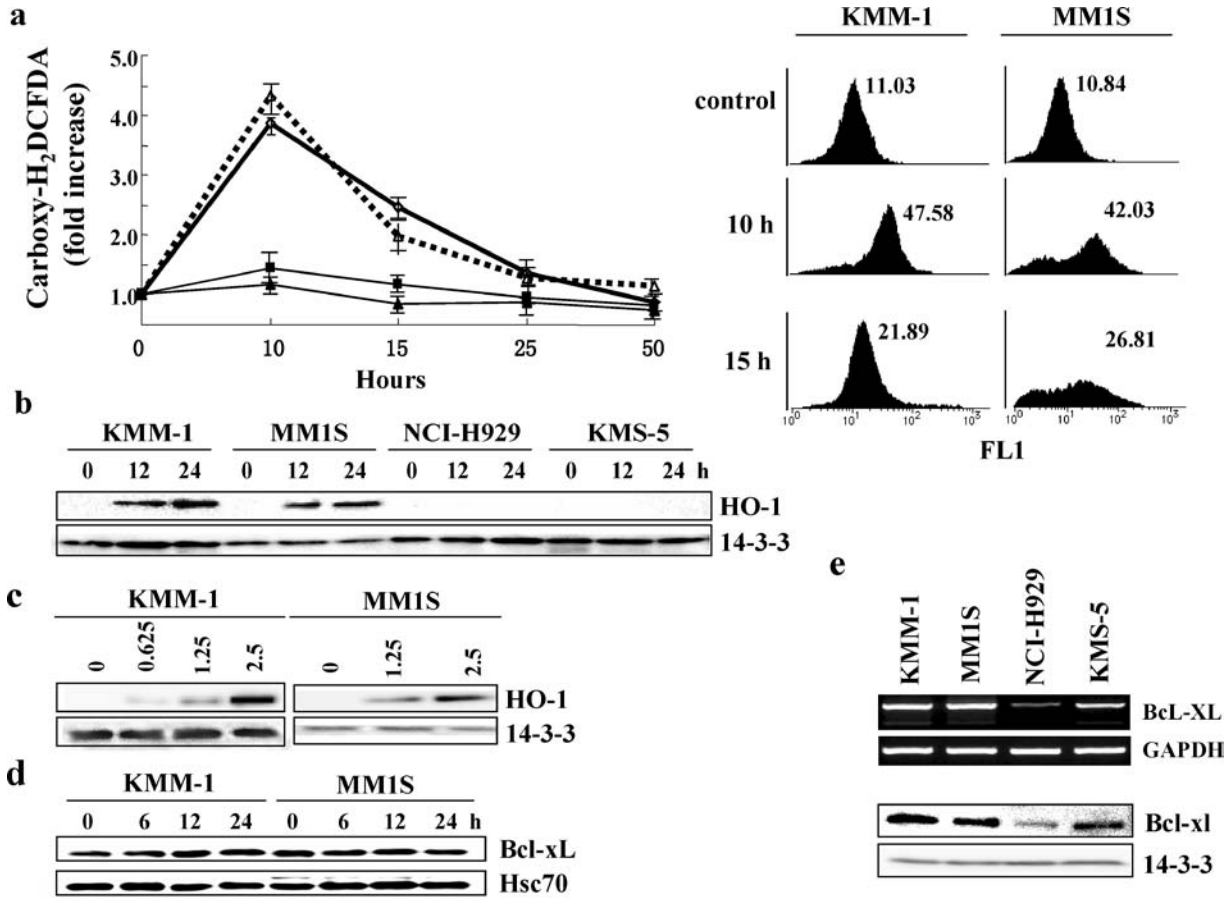

Fig. 2 PTL-induced ROS generation in MM cells. (**a**) ROS generation. Carboxy-H_2DCFDA fluorescent signals at the indicated hours after 2.5 μM PTL treatment. MM1S (thick), KMM-1 (broken), NCI-H929 (thin; closed square) and KMS-5 (thin line; closed triangle). Representative histograms of KMM-1 and MM1S are shown and numbers indicate MFIs. (**b**) Oxidative stress. Cells were harvested after treatment with 2.5 μM PTL for 12 to 24 h. HO-1 expression was evaluated by western blot analysis. (**c**) Dose-response. After incubation with 0.625 to 2.5 μM PTL for 24 h, HO-1 expression was evaluated by western blots. (**d**) Effect of PTL on Bcl-xL. Bcl-xL expression in KMM-1 and MM1S cells treated with 2.5 μM PTL for 24 h was evaluated by western blots. (e) Steady-state Bcl-xL expression. Bcl-xL expression levels of 4 MM cells were evaluated by RT-PCR (upper) and western blots (lower panels). GAPDH expression shows the same amount of RNA loaded in each lane

We further monitored ROS generation using a novel fluorescence probe 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF), which selectively detects highly reactive oxygen species (hROS) such as the hydroxyl radical ·OH and reactive intermediates of peroxidase [21]. PTL treatment clearly increased HPF-detectable hROS and the increase was strongly inhibited by both L-NAC and DPI in MM1S cells (Fig. 5d), confirming the inhibitory effects of both L-NAC and DPI on PTL-induced ROS generation. Similar results were also observed in KMM-1 cells (data not shown).

Different sensitivity to PTL in the four MM cells

We next explored the mechanisms underlying the different susceptibility to PTL among the four MM cells. As described above, 2.5 μM PTL induced distinct increases of ROS generation in the sensitive MM1S and KMM-1 cells, but much smaller increases in the less-sensitive KMS-5 and NCI-H929 cells. This suggests that different antioxidant activities may be involved in the susceptibility to PTL. Therefore, we monitored susceptibility of these cells to different chemical agents. We previously demonstrated that a non-steroidal anti-inflammatory drug (NSAID) sulindac and its metabolites extensively induce ROS generation [20]. Indeed, sulindac clearly increased ROS generation in all four MM cells, but the increased ROS levels were higher in MM1S and KMM-1 cells compared with KMS-5 and NCI-H929 cells (Fig. 6), resembling the effects of PTL. In contrast, two other anti-MM agents, a specific topoisomerase II inhibitor VP-16 and thalidomide did not apparently increase ROS generation and the sensitivity of all four MM cell types to these agents was comparable. These results support our idea that oxidative stress may be crucial for the proapoptotic effect of PTL and the levels of oxidative stress may reflect different sensitivities to PTL, and further

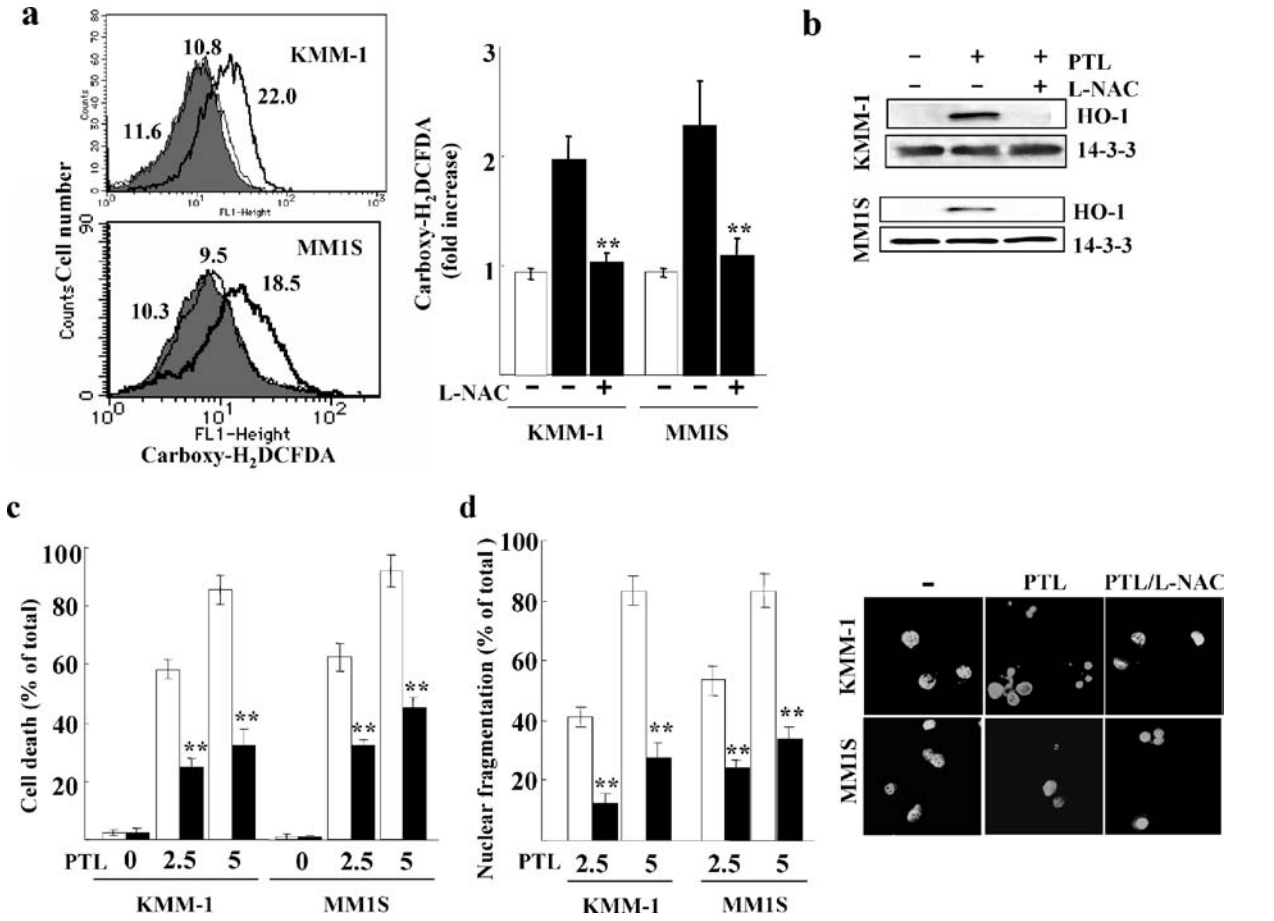

Fig. 3 Effect of L-NAC on PTL-induced oxidative stress. (**a**) Effect of L-NAC on ROS generation. KMM-1 and MM1S cells were pretreated with 5 mM L-NAC for 3 h, incubated with 2.5 μM PTL (closed bars) for 12 h, and ROS generation was detected. Representative histograms of MFIs in KMM-1 and MM1S are shown; 10.8 and 9.5 (control; gray), 22.0 and 18.5 (PTL treatment; thick), 11.6 and 10.3 (L-NAC/PTL treatment; thin line), respectively. (**b**) Effect of L-NAC on PTL-induced HO-1. Cells were harvested 24 h after treatment with 2.5 μM PTL with or without 5 mM L-NAC pretreatment. HO-1 expression was evaluated by western blots. (**c**) Effect of L-NAC on PTL-induced cell death. Cell death was evaluated by trypan blue exclusion assay after 48 h incubation with the indicated doses of PTL with (closed) or without (open columns) 5 mM L-NAC pretreatment. (**d**) DAPI staining. After treatment as (C), cells with fragmented nuclei were counted at 48 h. Representative confocal pictures are shown. Error bars represent mean $\pm$ S.D. of three different experiments and **, $P < 0.01$ compared with PTL-treated cells

suggest it unlikely that drug-resistant activity is crucial for the difference.

Different antioxidant activities in MM cells

Next, we compared the antioxidant scavenger activity in MM cells. Generated ROS are detoxified by antioxidant scavenger enzymes, such as glutathione peroxidase (GPX) and catalase. Interestingly, catalase expression levels were much lower in the MM1S and KMM-1 cells (Fig. 7A) and its steady-state activities were quite low in MM1S and KMM-1 cells compared with NCI-H929, KMS-5 and normal lymphocytes (Fig. 7B). GPX activity was relatively higher in normal lymphocytes compared with MM cells, though NCI-H929 GPX activity was equivalent (Fig. 7C). Overall, the antioxidant activities of most MM cells appear to be lower than normal lymphocytes, and catalase activity is notably low in KMM-1 and MM1S cells.

Knockdown of catalase sensitized KMS-5 to PTL

From these results, we hypothesized that catalase activity may be crucial for susceptibility to PTL in these MM cells. Therefore, we first investigated effect of catalase inhibitor, 3-amino-1,2,4-triazole (AT), on PTL-mediated ROS generation. AT treatment augmented PTL-mediated ROS generation (Fig. 8a), nuclear fragmentation (Fig. 8b) and loss of MMP (Fig. 8c) in both KMS-5 and NCI-H929 cells. We further transfected KMS-5 cells, which express catalase abundantly but GPX much less, with siRNA duplex mixtures which specifically bind to catalase mRNAs. The siRNA-catalase SMARTpool decreased catalase expression

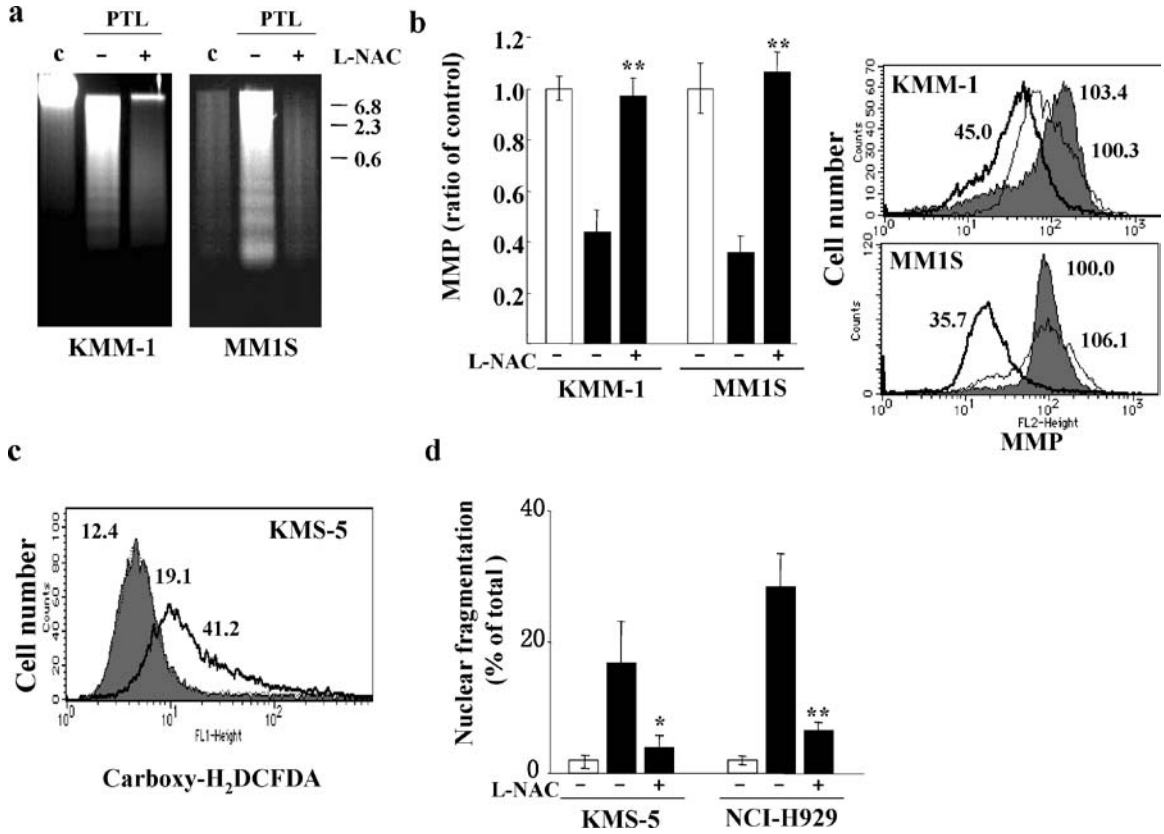

Fig. 4 Effect of L-NAC on PTL-induced apoptosis. (**a**) DNA fragmentation. Cells were treated with 2.5 μM PTL for 48 (KMM-1) or 36 h (MM1S) with or without 5 mM L-NAC pretreatment and low-molecular weight DNAs were harvested. Molecular weight markers are shown in Kbs. (**b**) Disruption of $\Delta\Psi$m. Cells were treated with 2.5 μM PTL (closed) for 36 h with or without 5 mM L-NAC pretreatment. $\Delta\Psi$m was evaluated with MitoTracker Orange CM-H_2TMRos. Representative histograms of control (gary), PTL (thick) or PTL/L-NAC (thin line) treatment and MFIs are shown. (**c**) Effect of L-NAC on ROS generation in KMS-5 cells. At 12 h PTL treatment (10 μM) with or without 5 mM L-NAC pretreatment, MFIs of carboxy-H_2DCFDA fluorescence in KMS5 cells is 12.4 (control; gray), 41.2 (PTL treatment; thick), 19.1 (L-NAC/PTL treatment; thin line). (**d**) DAPI staining. After treatment with 5 μM PTL (closed bars) with or without 5 mM L-NAC pretreatment, cells with fragmented nuclei were counted at 48 h. Error bars represent mean $\pm$ S.D. $*$, $P<0.05$, $**$, $P<0.01$ compared with PTL-treated cells

and its activity (Fig. 8d). The decreased catalase expression by siRNA-catalase transfection enhanced ROS generation after PTL (10 μM) treatment (Fig. 8e) and increased nuclear fragmentation at 5 μM PTL (Fig. 8f). Thus, catalase inhibition enhanced PTL-mediated ROS generation and subsequent apoptosis, strongly suggesting that catalase activity affects susceptibility to PTL in MM cells.

Discussion

We here demonstrate that an active component of medicinal plants, PTL, induces apoptosis in four different MM cell lines, and the concentrations required for its proapoptotic effect are less than those that induce toxic effects in normal lymphocytes and hematopoietic BM cells. Our results encourage the belief that PTL can be applied clinically in the chemotherapeutic strategy for these MM cells.

Though MM cells are sensitive to PTL, their sensitivity considerably differs. We also found different sensitivity to PTL in colon cancer cells (data not shown), suggesting that these observations are commonly observed. It is noted that PTL-mediated elevation of ROS levels correlated well with their sensitivity. PTL-sensitive MM cells were also sensitive to another ROS inducer, sulindac, but sensitivity to the non-ROS inducing anticancer agents, VP16 and thalidomide, was comparable in all four MM cells. These data may help exclude the possible involvement of the multidrug resistant transporter p-glycoprotein and other drug resistant ABC transporters functions [23, 24] in the mechanism determining different sensitivity to PTL.

We found that catalase activity was significantly lower in the sensitive MM cells compared with less-sensitive MM cells and normal lymphocytes, and that inhibition of catalase activity or knockdown of catalase expression increased sensitivity to PTL. Similar scavenger enzyme GPX activity also corresponded with the susceptibility to PTL, but

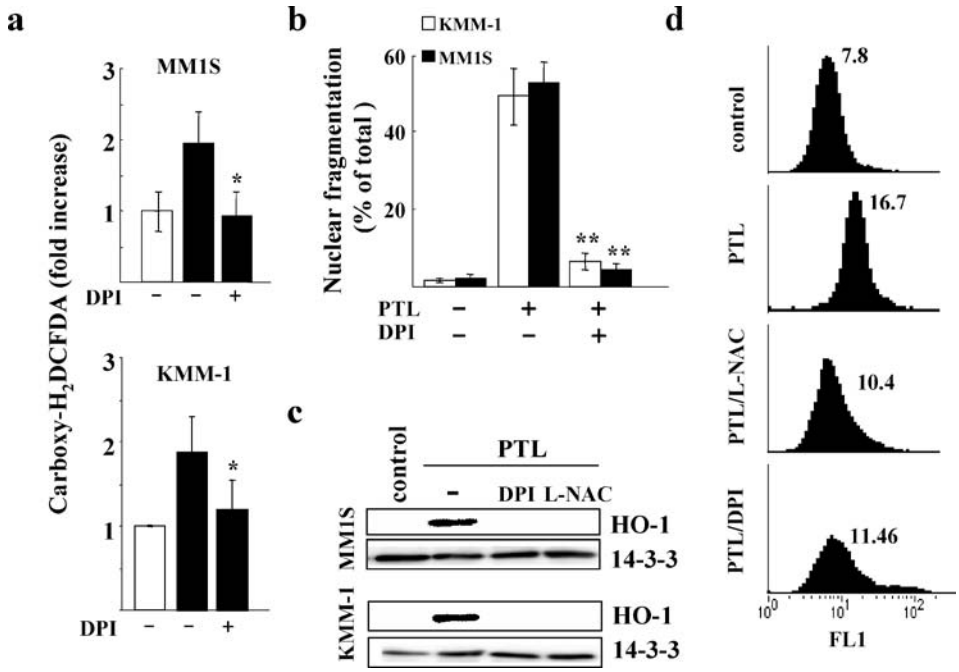

Fig. 5 Effect of DPI on PTL-induced ROS generation. (**a**) Relative ROS generation in MM1S and KMM1 cells treated with 2.5 μM PTL (closed bars) for 12 h with (+) or without 2.5 μM DPI pretreatment. (**b**) Effect of DPI on apoptosis. Nuclear fragmented cells (% of total) after 48 h treatment (2.5 μM PTL / 2.5 μM DPI pretreatment) were evaluated by DAPI staining. $*$, $P<0.05$, **, $P<0.01$ compared with PTL-treated cells. (**c**) Oxidative stress. Cells were harvested after treatment with 2.5 μM PTL for 24 h with 2.5 μM DPI or 5 mM L-NAC pretreatment. HO-1 expression was evaluated by western blots. (**d**) HPF-mediated ROS detection. After treatment with 2.5 μM PTL for 12 h with 2.5 μM DPI or 5 mM L-NAC pretreatment, MM1S cells were loaded with HPF and ROS generation was detected. Numbers indicate MFIs of HPF

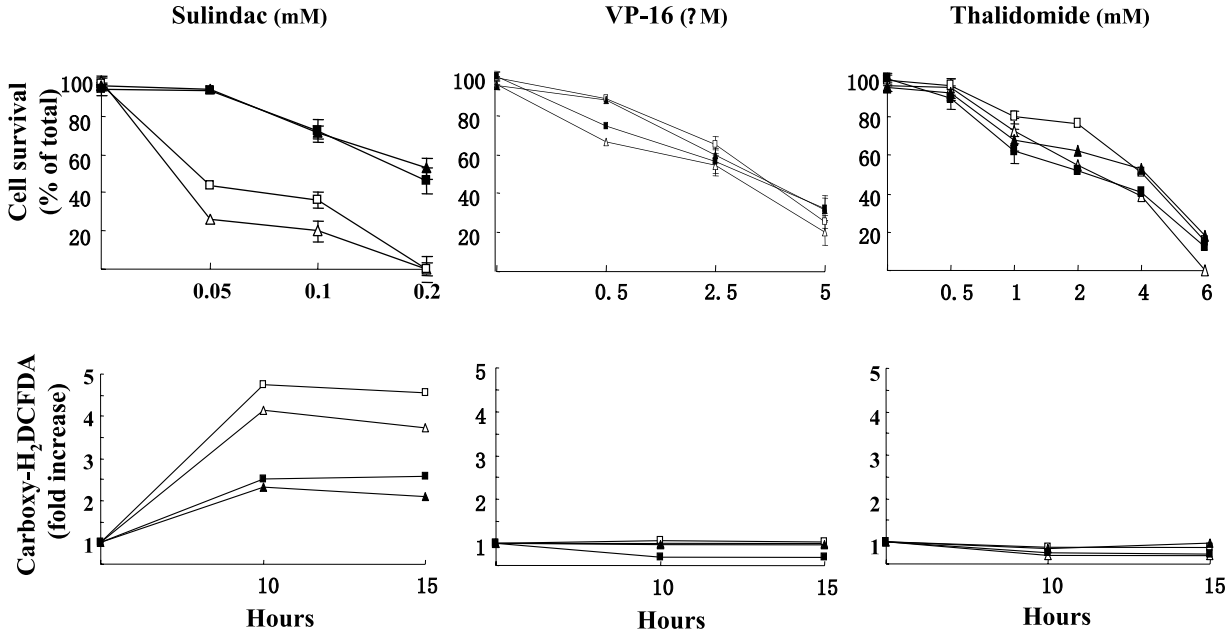

Fig. 6 Cell viability and ROS generation after various treatments. Cells were treated with the indicated agents at the indicated doses for 48 h and their viability was evaluated by trypan blue exclusion assay (upper). Cells were treated with 0.05 mM sulindac, 2.5 μM VP-16, or 1 mM thalidomide for 10 or 15 h and ROS generation was detected with Carboxy-H_2DCFDA (lower panels). MM1S (open triangle), KMM-1 (open square), KMS-5, (closed triangle) and NCI-H929 (closed square). Error bars represent mean $\pm$ S.D

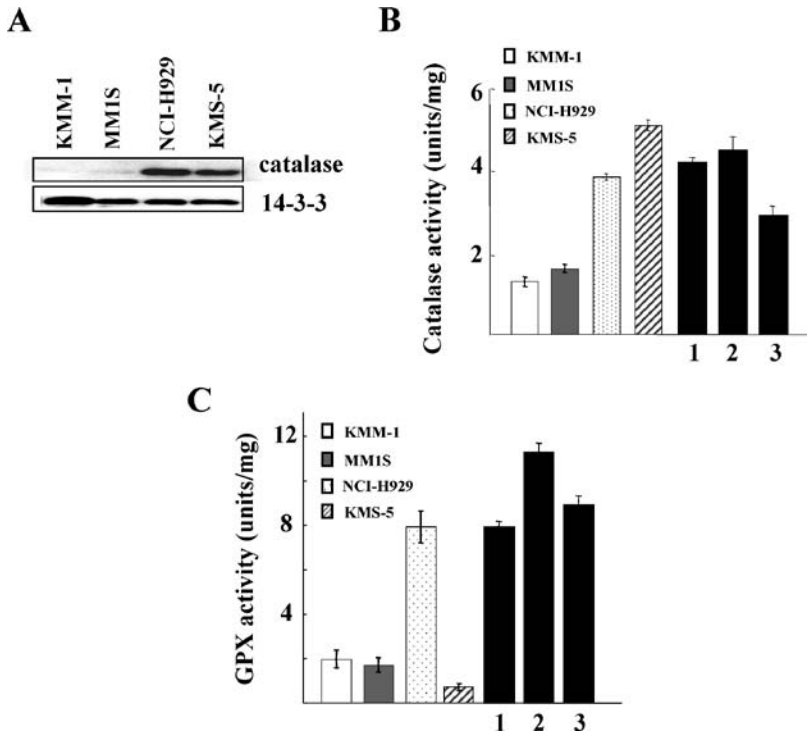

Fig. 7 Catalase expression and activity. Catalase expression (**A**) in 4 MM cells. Catalase activity (**B**) and GPX activity (**C**) in 4 MM cells and 3 independent normal peripheral lymphocytes (1–3; closed columns). Columns display the mean ± S.D. of data from three separate experiments

did not correspond well for KMS-5 cells. Although there are many scavenger enzymes, we thus think that catalase activity appears to be a central molecule determining the susceptibility. Moreover, our idea is supported by several data, i.e., cells with high concentrations of catalase have low levels of endogenous H_2O_2, while the other cells with low catalase concentrations have high endogenous H_2O_2 levels [25] and catalase transgenic mice can be resistant to oxidative stress inducers, but not other proapoptotic inducers [26]. In addition, a recent study indicates that catalase, but not superoxide dismutase (SOD), can increase the number of granulocytes in murine BM in vitro cultures [27], suggesting a crucial role of catalase in proliferation and maintenance of hematopoietic cells.

Although a previous suggestion was that expression levels of antiapoptotic Bcl-xL may affect the susceptibility to PTL [11], Bcl-xL expression was not altered by PTL treatment and its expression levels did not correlate well with sensitivity to PTL at least in the four MM cells we examined. Thus, we concluded that Bcl-xL expression has little influence on susceptibility to PTL in MM cells. In addition, our data are inconsistent with several reports suggesting that its inhibitory effect on NF-κB is crucial for PTL-mediated apoptosis [4–7, 12, 14]. It is noted that an increase of ROS generation was very rapid and apoptosis was already detectable 24 h after PTL treatment in MM cells, but PTL did not decrease Bcl-xL expression, one of the important target gene of NF-κB-mediated signals [28]. In addition, PTL-mediated apoptosis was totally blocked by antioxidant L-NAC. Based on these results, it is unlikely that inhibition of NF-kB plays central roles in its proapoptotic effects on MM cells. In this context, it is important to clarify how PTL rapidly and strongly induces ROS generation in MM cells.To address this question, we investigated the effect of DPI on PTL-mediated ROS generation and showed its inhibitory effect (Fig. 5d). This suggests that the NADPH oxidase activation [29, 30] is associated with the PTL-mediated ROS generation in MM cells. Since the precise expression pattern and functions of the NADPH oxidases in MM cells remain uncertain, we are currently investigating these issues.

It also remains uncertain how oxidative stress induces apoptosis in MM cells. Oxidative stress can activate the stress-activated MAP kinase, c-Jun NH_2-terminal kinase (JNK). However, data concerning JNK activation is conflicting, i.e., PTL activates JNK in breast cancer cells [10], but inhibits UVB-induced JNK activation in epidermal cells [31]. Since JNK is a strong apoptotic mediator, it is a logical candidate for PTL-mediated apoptosis. However, we could detect neither a distinct increase of JNK phosphorylation in any of the four PTL-treated MM cells nor any inhibition by the JNK specific inhibitor SP600125 on PTL-mediated death (data not shown), suggesting its marginal contribution to PTL-mediated apoptosis and different susceptibility.

In sum, our data strongly suggest that PTL strongly induces apoptosis in MM cells, but exhibits much less adverse effect on normal lymphocytes and BM cells. Since its proapoptotic activity appears to be mediated through

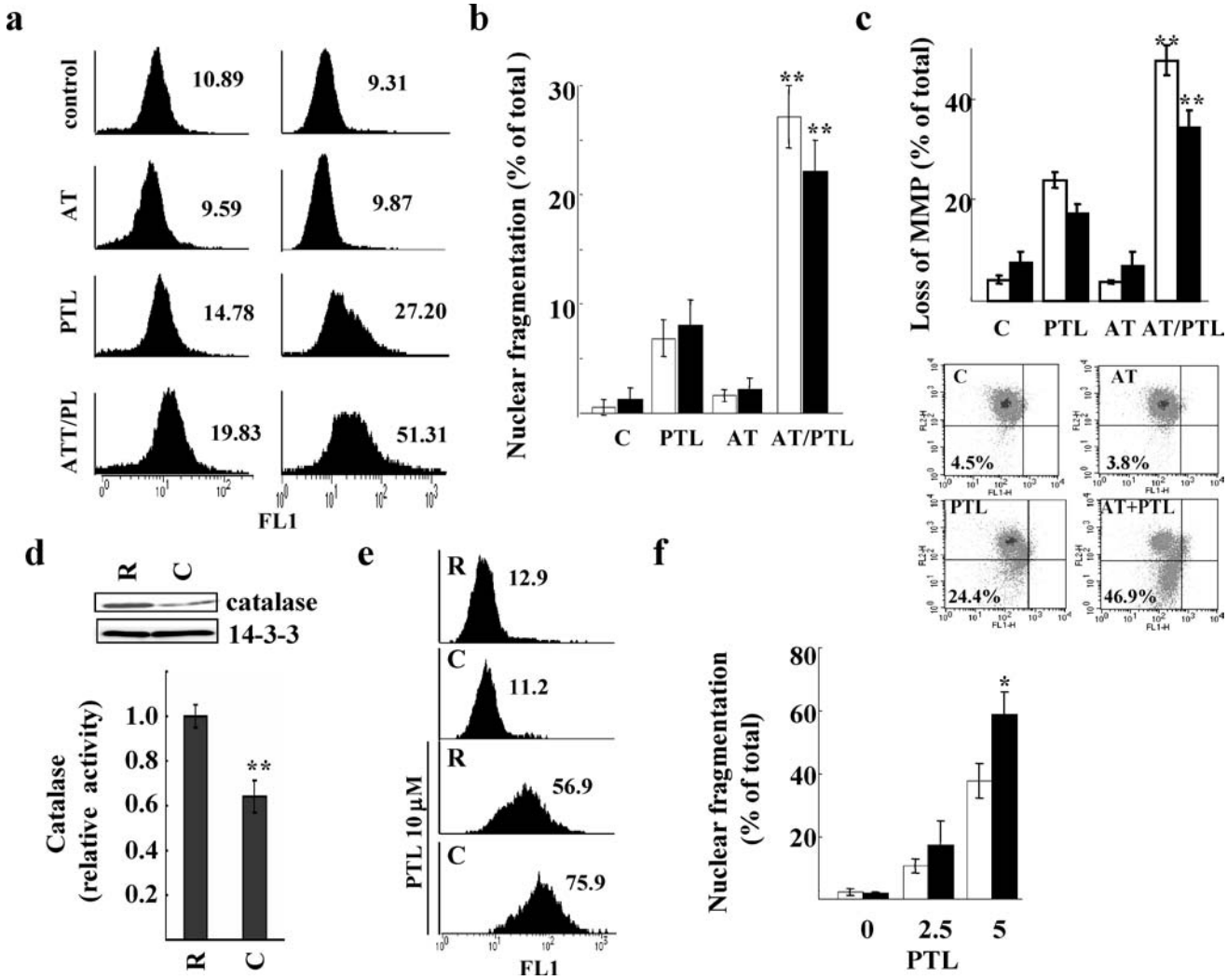

Fig. 8 Effect of catalase inhibition on PTL-mediated death. (**a**) Cells were treated with 5 (NCI-H929; left) or 10 (KMS-5; right) μM PTL for 12 h with or without 20 mM AT pretreatment (1 h). Representative histograms of carboxy-H_2DCFDA fluorescent signals are shown and numbers indicate their MFIs. (**b**) DAPI staining. At 24 h after incubation with 5 (NCI-H929; open) or 10 (KMS-5; closed) μM PTL with or without 20 mM AT, cells with nuclear fragmentation were counted. (**c**) Loss of MMP ($\Delta\Psi$m). KMS-5 and NCI-H929 were treated as (B), $\Delta\Psi$m was measured at 24 h after PTL treatment. Representative data of KMS-5 are shown and numbers indicate rate of apoptotic cells (lower panel). *, $P<0.05$ **, $P<0.01$ compared with cells treated with PTL alone. (**d**) Catalase expression (upper panel) and its activity (lower panel) were measured at 48 h and 52 h, respectively, after transfection with catalase SMARTpool (C)- or random-siRNA (R). (**e**) ROS generation in KMS-5 transfectant (R, si-random; C, si-catalase) cells treated with 10 μM PTL for 12 h. Representative histograms of carboxy-H_2DCFDA fluorescent signals are shown and numbers indicate their MFIs. (**f**) DAPI staining. After transfection with si-Catalase (closed) or si-Random (open column), KMS-5 cells were incubated for 18 h and nuclear fragmentation was counted 48 h after treatment with PTL (μM). *, $P<0.05$ **, $P<0.01$ compared with siRandom-transfected cells. Columns display the mean $\pm$ S.D. of data from three separate experiments

induction of oxidative stress, this action is similar to Bortezomib [32–34]. It is thus important to investigate cross-resistance of MM cells to both agents. We also enlighten catalase activity as a factor affecting susceptibility to PTL and inhibition of its activity may enhance the sensitivity. These results imply that its combination with catalase inhibitory agents may enable PTL as a more potent anticancer agent against MM cells and catalase activity may be useful for prediction of PTL-mediated anticancer effects. Our data thus provide useful clues to new powerful anti-MM agents.

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We are grateful to Dr. P. Olley (Sapporo Medical University School of Medicine) for critical reading of this manuscript.

References

1. Knight DW (1995) Feverfew: chemistry and biological activity. Nat Prod Rep 12:271–276

2. Zhang S, Ong CN, Shen HM (2004) Critical roles of intracellular thiols and calcium in parthenolide-induced apoptosis in human colorectal cancer cells. Cancer Lett 208:143–153
3. Woynarowski JM, Konopa J (1981) Inhibition of DNA biosynthesis in HeLa cells by cytotoxic and antitumor sesquiterpene lactones. Mol Pharmacol 19: 97–102
4. Zhang S, Lin ZN, Yang CF, Shi X, Ong CN, Shen HM (2004) Suppressed NF-{kappa}B and sustained JNK activation contribute to the sensitization effect of parthenolide to TNF-{alpha}-induced apoptosis in human cancer cells. Carcinogenesis 25:2191–2199
5. Kwok BH, Koh B, Ndubuisi MI, Elofsson M, Crews CM (2001) The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IkappaB kinase. Chem Biol 8:759–766
6. Hehner SP, Hofmann TG, Droge W, Schmitz ML (1999) The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-kappa B by targeting the I kappa B kinase complex. J Immunol 163:5617–5623
7. Garcia-Pineres AJ, Castro V, Mora G, Schmidt TJ, Strunck E, Pahl HL, Merfort I (2001) Cysteine 38 in p65/NF-kappaB plays a crucial role in DNA binding inhibition by sesquiterpene lactones. J Biol Chem 276:39713–39720
8. Sobota R, Szwed M, Kasza A, Bugno M, Kordula T (2000) Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. Biochem Biophys Res Commun 267:329–333
9. Wen J, You KR, Lee SY, Song CH, Kim DG (2002) Oxidative stress-mediated apoptosis: the anticancer effect of the sesquiterpene lactone parthenolide. J Biol Chem 277:38954–38964
10. Nakshatri H, Rice SE, Bhat-Nakshatri P (2004) Antitumor agent parthenolide reverses resistance of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand through sustained activation of c-Jun N-terminal kinase. Oncogene 23:7330–7344
11. Kim JH, Liu L, Lee SO, Kim YT, You KR, Kim DG (2005) Susceptibility of cholangiocarcinoma cells to parthenolide-induced apoptosis. Cancer Res 65:6312–6320
12. Yip-Schneider MT, Nakshatri H, Sweeney CJ, Marshall MS, Wiebke EA, Schmidt CM (2005) Parthenolide and sulindac cooperate to mediate growth suppression and inhibit the nuclear factor-kappa B pathway in pancreatic carcinoma cells. Mol Cancer Ther 4:587–594
13. Sweeney CJ, Mehrotra S, Sadaria MR, Kumar S, Shortle NH, Roman Y, Sheridan C, Campbell RA, Murry DJ, Badve S, Nakshatri H (2005) The sesquiterpene lactone parthenolide in combination with docetaxel reduces metastasis and improves survival in a xenograft model of breast cancer. Mol Cancer Ther 4:1004–1012
14. Guzman ML, Rossi RM, Karnischky L, Karnischky L, Li X, Peterson DR, Howard DS, Jordan CT (2005) The sesquiterpene lactone parthenolide induces apoptosis of human acute myelogenous leukemia stem and progenitor cells. Blood 105:4163–4139
15. Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, Rajkumar SV, Srkalovic G, Alsina M, Alexanian R, Siegel D, Orlowski RZ, Kuter D, Limentani SA, Lee S, Hideshima T, Esseltine DL, Kauffman M, Adams J, Schenkein DP, Anderson KC (2003) A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 348:2609–2617
16. Ma MH, Yang HH, Parker K, Manyak S, Friedman JM, Altamirano C, Wu ZQ, Borad MJ, Frantzen M, Roussos E, Neeser J, Mikail A, Adams J, Sjak-Shie N, Vescio RA, Berenson JR. (2003) The proteasome inhibitor PS-341 markedly enhances sensitivity of multiple myeloma tumor cells to chemotherapeutic agents. Clin Cancer Res 9:1136–1144
17. Singhal S, Mehta J, Desikan R, Ayers D, Roberson P, Eddlemon P, Munshi N, Anaissie E, Wilson C, Dhodapkar M, Zeddis J, Barlogie B (1999) Antitumor activity of thalidomide in refractory multiple myeloma. New Engl J Med 341:1565–1571
18. Keifer JA, Guttridge DC, Ashburner BP, Baldwin Jr AS (2001) Inhibition of NF-kappa B activity by thalidomide through suppression of IkappaB kinase activity. J Biol Chem 276:22382–22387
19. Ling YH, Liebes L, Zou Y, Perez-Soler R (2003) Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to Bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells. J Biol Chem 278:33714–33723
20. Minami T, Adachi M, Kawamura R, Zhang Y, Shinomura Y, Imai K (2005) Sulindac enhances the proteasome inhibitor bortezomib-mediated oxidative stress and anticancer activity. Clin Cancer Res 11:5248–5256
21. Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T (2003) Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J Biol Chem 278:3170–3175
22. Begonja AJ, Gambaryan S, Geiger J, Aktas B, Pozgajova M, Nieswandt B, Walter U (2005) Platelet NAD(P)H-oxidase-generated ROS production regulates alphaIIbbeta3 -integrin activation independent of the NO/cGMP pathway. Blood 106:2757–2760
23. Ambudkar SV, Sauna ZE, Gottesman MM, Szakacs G (2005) A novel way to spread drug resistance in tumor cells: functional intercellular transfer of P-glycoprotein (ABCB1). Trends Pharmacol Sci 26:385–387
24. Polgar O, Bates SE (2005) ABC transporters in the balance: is there a role in multidrug resistance? Biochem Soc Trans 33:241–245
25. Scandalios JG (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res 38:995–1014
26. Chen X, Liang H, Van Remmen H, Vijg J, Richardson A (2004) Catalase transgenic mice: characterization and sensitivity to oxidative stress. Arch Biochem Biophys 422:197–210
27. Gupta R, Karpatkin S, Basch RS (2006) Hematopoiesis and stem cell renewal in long-term bone marrow cultures containing catalase. Blood. 107:1837–1846
28. Bernal-Mizrachi L, Lovly CM, Ratner L (2006) The role of NF-{kappa}B-1 and NF-{kappa}B-2-mediated resistance to apoptosis in lymphomas. Proc Natl Acad Sci U S A 103:9220–9225
29. Geiszt M, Leto TL (2004) NOX The Nox family of NAD(P)H oxidases: host defense and beyond. J Biol Chem 279:51715–51718
30. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD (1999) Cell transformation by the superoxide- generating oxidase Mox1. Nature 401:79–82
31. Won YK, Ong CN, Shi X, Shen HM (2004) Chemopreventive activity of parthenolide against UVB-induced skin cancer and its mechanisms. Carcinogenesis 25:1449–1458
32. Ling YH, Liebes L, Zou Y, Perez-Soler R (2003) Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to Bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells. J Biol Chem 278:33714–33723
33. Fribley A, Zeng Q, Wang CY (2004) Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. Mol Cell Biol 24:9695–9704.
34. Perez-Galan P, Roue G, Villamor N, Montserrat E, Campo E, Colomer D (2006) The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. Blood 107:257–264