Oncogene (2004) 23, 7330–7344

www.nature.com/onc

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

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The antitumor activity of the sesquiterpene lactone parthenolide, an active ingredient of medicinal plants, is believed to be due to the inhibition of DNA binding of transcription factors NF-κB and STAT-3, reduction in MAP kinase activity and the generation of reactive oxygen. In this report, we show that parthenolide activates c-Jun N-terminal kinase (JNK), which is independent of inhibition of NF-κB DNA binding and generation of reactive oxygen species. Parthenolide reversed resistance of breast cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Cancer cells treated with a combination of TRAIL and parthenolide underwent massive typical apoptosis and atypical apoptosis involving the loss of plasma membrane integrity. JNK activity is necessary for the parthenolide-induced sensitization to TRAIL because a dominant-negative JNK or the JNK inhibitor SP600125 reduced TRAIL plus parthenolide-induced apoptosis. Parthenolide induced phosphorylation of Bid and increased TRAIL-dependent cleavage of Bid without affecting caspase 8 activities. Cytochrome *c* but not Smac/DIABLO was released from the mitochondria in cells treated with parthenolide alone. Parthenolide through JNK increased the TRAIL-mediated degradation of the antiapoptotic protein X-linked inhibitor of apoptosis (XIAP). Enhanced XIAP cleavage correlated with increased and prolonged caspase 3 activity and PARP cleavage, suggesting that the sensitization to TRAIL involves 'feed forward' activation of caspase 3. These results identify a new antitumor activity of parthenolide, which can be exploited to reverse resistance of cancer cells to TRAIL, particularly those with elevated XIAP levels.
Oncogene (2004) **23,** 7330–7344. doi:10.1038/sj.onc.1207995
Published online 2 August 2004

Keywords: parthenolide; TRAIL; apoptosis; JNK; NF-κB; breast cancer

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Received 4 May 2004; revised 25 June 2004; accepted 27 June 2004; published online 2 August 2004

Introduction

Recent advances in our understanding of signaling pathways have resulted in the development of several molecular target-based therapies (Johnstone *et al*., 2002; Ritter and Arteaga, 2003). Signaling molecules that are targeted for therapy include members of the epidermal growth factor receptor family, MAP kinase and PI3 kinase pathways, and transcription factors such as NF-κB and STAT3 (Baldwin, 2001; Nicholson *et al*., 2001; Karin *et al*., 2002; Ritter and Arteaga, 2003). While activation of most of these signaling pathways lead to chemoresistance, a few of these signaling molecules may increase sensitivity of cancer cells to chemotherapy. For example, depending on the cell type, activation of c-Jun-N-terminal kinase (JNK) leads to increased sensitivity of cancer cells to tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL) (Herr *et al*., 1999; Deng *et al*., 2003). Inhibition of NF-κB through either overexpression of inhibitor of kappaB (IκB) or prior exposure to chemical inhibitors of NF-κB sensitizes cancer cells to TNF and chemotherapeutic drugs such as paclitaxel and CPT-11 (Wang *et al*., 1999; Patel *et al*., 2000). Chemosensitization by either mechanism involves altered function of preexisting proapoptotic molecules and/or reduced expression of antiapoptotic proteins. For example, chemosensitization by JNK may involve the phosphorylation of the proapoptotic protein Bim, activation of the Bax/Bak-dependent mitochondrial apoptotic machinery or the release of Smac/DIABLO from the mitochondria and subsequent disruption of the antiapoptotic TRAF-2-cIAP-1 complex (Deng *et al*., 2003; Lei and Davis, 2003). In contrast, chemosensitization as a consequence of NF-κB inhibition is most likely due to the reduced expression of antiapoptotic genes including cIAP-1, cIAP-2, XIAP, TRAF-1, TRAF-2, Bcl-2, Bfl-1/A1, Bcl-XL, c-FLIP and manganese superoxide dismutase (Mn-SOD) (Baldwin, 2001; Karin *et al*., 2002; Nakshatri and Goulet, 2002).

Death receptors and their ligands play a major role in physiological regulation of apoptosis (Daniel *et al*., 2001; LeBlanc and Ashkenazi, 2003; Ozoren and El-Deiry, 2003). Death receptor 4 (DR4) and death receptor 5 (DR5) and their ligand TRAIL (also called

Apo2 ligand) have received considerable attention recently because of relative cancer cell specificity of this apoptotic pathway. TRAIL is an apoptosis inducing member of the tumor necrosis factor (TNF) superfamily, which triggers apoptosis through crosstalk between components of extrinsic and intrinsic cell death pathways (LeBlanc and Ashkenazi, 2003). Normal cells are usually resistant to TRAIL. Mechanisms responsible for lack of sensitivity of normal cells to TRAIL is not known, although elevated expression of decoy receptors for TRAIL has been suggested as one such mechanism (Zhang *et al*., 2000). Recent studies suggest that JNK and NF-κB modulate TRAIL-induced apoptosis in an opposing direction. For example, NF-κB confers resistance to TRAIL by increasing the expression of decoy receptors and reducing the expression of caspases and Bid (Bernard *et al*., 2001; Eid *et al*., 2002). In contrast, JNK contributes to synergy between TRAIL and DNA-damaging agents or translational inhibitors (Sah *et al*., 2003; Vivo *et al*., 2003). Therefore, an agent that can inhibit NF-κB and/or activate JNK should make cancer cells highly susceptible to TRAIL.

The antitumor agent sesquiterpene lactone parthenolide shows several biological activities, including inhibition of NF-κB and STAT3 DNA-binding activity, inhibition of MAP kinase activity and induction of oxidative stress, followed by G2/M arrest and apoptosis (Hehner *et al*., 1998, 1999; Sobota *et al*., 2000; Wen *et al*., 2002). It is the active ingredient of the herb feverfew, which is used as a migraine prophylaxis. Recently, we have shown that parthenolide inhibits NF-κB, reduces Mn-SOD expression and sensitizes breast cancer cells to paclitaxel (Patel *et al*., 2000). This study was initiated to identify the mechanisms of parthenolide-induced cell death that are independent of NF-κB inhibition. We show that parthenolide induces JNK, which is not a consequence of inhibition of classical p65 : p50 heterodimer of NF-κB. We also show that parthenolide sensitizes breast cancer cells to TRAIL, which requires JNK activity. Parthenolide-induced sensitization correlated with phosphorylation and cleavage of Bid, degradation of X-linked inhibitor of apoptosis (XIAP), elevated caspase 3 activities, cleavage of poly-ADP ribose polymerase (PARP), and increased typical and atypical apoptosis involving loss of plasma membrane integrity. Because of its unique ability to independently induce JNK and inhibit NF-κB, parthenolide may serve as a potent chemosensitizer.

Results

Parthenolide inhibits the growth of HBL-100 cells independent of NF-κB inhibition

We have previously demonstrated chemosensitizing property of parthenolide in breast cancer cells (Patel *et al*., 2000). To determine whether parthenolide alone inhibits breast cancer proliferation, the breast cancer cell lines HBL-100, MDA-MB-231 and MDA-MB-436 cells were treated with increasing concentration of parthenolide for 48 h and cell proliferation was measured by MTS assay. Proliferation of all of the three cell lines was inhibited by parthenolide with an IC_{50} ranging from 0.8 to 1.5 μM (Figure 1a). Parthenolide-mediated decrease in HBL-100 and MDA-MB-231 cell proliferation was further confirmed by the clonogenic assay (Figure 1b).

To determine whether inhibition of breast cancer cell proliferation by parthenolide correlates with inhibition of NF-κB DNA-binding activity, we performed electrophoretic mobility shift assay (EMSA). We have previously shown NF-κB DNA binding in these three cell lines is constitutive and p50 : p65 heterodimers are the major DNA : protein complexes in these cells using antibody supershift and oligonucleotide competition assays (Nakshatri *et al*., 1997; Newton *et al*., 1999; Patel *et al*., 2000). Parthenolide reduced NF-κB DNA-binding activity in MDA-MB-231 and MDA-MB-436 but not in HBL-100 cells (Figure 1c). It is interesting that sensitivity of cells to parthenolide showed an inverse correlation to constitutive NF-κB DNA-binding activity. For example, HBL-100 cells with lowest NF-κB DNA-binding activity being more sensitive than MDA-MB-436 cells, which has highest NF-κB DNA-binding activity. No inhibition of NF-κB DNA binding in HBL-100 cells was observed even after 24 h incubation with parthenolide (data not shown). Parthenolide did not inhibit the transactivation function of the p65 subunit of NF-κB in HBL-100 cells in transient transfection assays (data not shown). The cell type specific differences in the ability of parthenolide to inhibit constitutive NF-κB could be related to mechanisms of constitutive NF-κB activation in these cells. While autocrine action of interleukin 1 alpha and epidermal growth factor receptor pathway are responsible for constitutive NF-κB activation in MDA-MB-231 and MDA-MB-436 cells, SV40 t antigen may be responsible for NF-κB activation in HBL-100 cells (Vanhamme and Szpirer, 1988; Sontag *et al*., 1997; Biswas *et al*., 2000; Nozaki *et al*., 2000). Nonetheless, our results indicate that parthenolide inhibits cell proliferation independent of NF-κB inhibition and the levels of constitutive NF-κB determine the concentration of parthenolide required to inhibit cell proliferation. Because several of the other previously described activities of parthenolide including oxidative stress, G2/M arrest and STAT3 DNA binding was observed at 5–10 μM of parthenolide, a novel function of parthenolide is responsible for inhibition of HBL-100 cell proliferation.

Parthenolide induces JNK, which is required for the inhibition of HBL-100 but not MDA-MB-231 cell proliferation

JNK plays distinct roles in cell death. Transient activation of JNK is believed to be antiapoptotic whereas persistent activation is proapoptotic (Kennedy and Davis, 2003; Lin, 2003). The duration of JNK activation is controlled in part by NF-κB because NF-κB-inducible genes such as GADD45β and XIAP have been shown to inhibit JNK activity (De Smaele *et al*.,

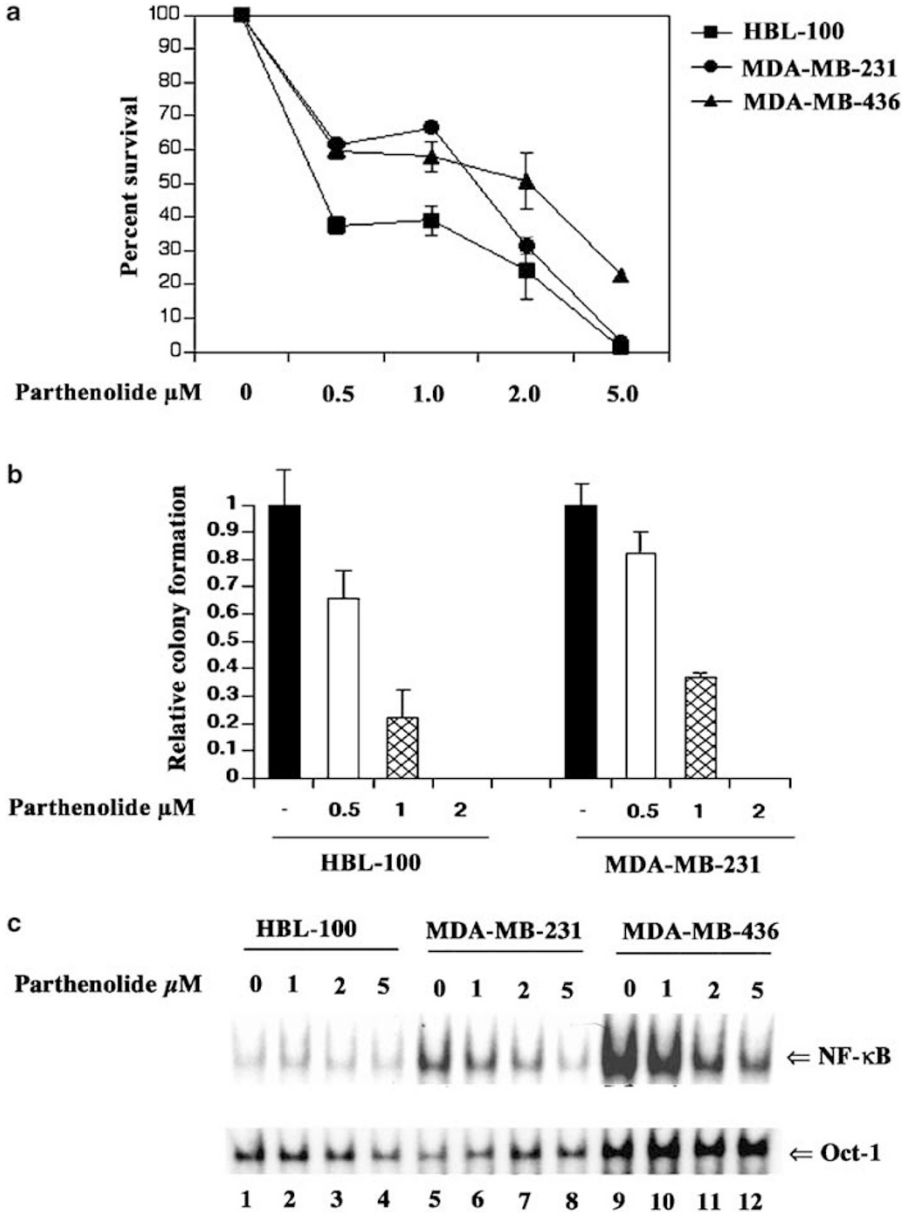


Figure 1 Parthenolide inhibits proliferation of HBL-100, MDA-MB-231 and MDA-MB-436 cells. (**a**) Cell proliferation was measured by MTS assay. Cells were treated for 48 h with indicated concentration of parthenolide. Average with standard error is shown. (**b**) Clonogenic survival of parthenolide-treated cells. The clonogenic assay was performed as described in Materials and methods. Cells were treated with indicated concentration of drugs for 24 h and number of colonies were determined after 21 days of drug treatment. (**c**) Parthenolide inhibits NF-κB DNA-binding activity in MDA-MB-231, MDA-MB-436 but not HBL-100 cells. Cells were treated with the indicated concentration of parthenolide for 3 h. Extracts were subjected to electrophoretic mobility shift assays (EMSA) with NF-κB or Oct-1 probes. NF-κB : DNA complex in these cells is a heterodimer of p50 and p65 as determined by an antibody supershift assay (data not shown)

2001; Tang *et al*., 2001). These observations prompted us to investigate whether parthenolide activates JNK. We also tested whether JNK activation by parthenolide is persistent in cells with constitutive NF-κB because of possible dual function of parthenolide (JNK activation and inhibition of NF-κB). JNK activation was measured by Western blot analysis of phosphorylated JNK and its downstream target c-Jun. Parthenolide induced JNK in all of three cell types tested (Figure 2a). Pretreatment with the JNK inhibitor SP600125 reduced parthenolide-induced phosphorylation of c-Jun in MDA-MB-231 cells (Figure 2a). Time course experiments revealed peak JNK activation at 3 h post-treatment and this activation persisted for up to 5 h (Figure 2b).

We next investigated the role of JNK in parthenolide-mediated inhibition of cell proliferation using MTS assay. Parthenolide was less efficient in inhibiting proliferation of HBL-100 cells but not MDA-MB-231 cells in the presence of SP600125 (Figure 2c, $P = 0.005$ for HBL-100 cells at 1 μM). However, SP600125 was

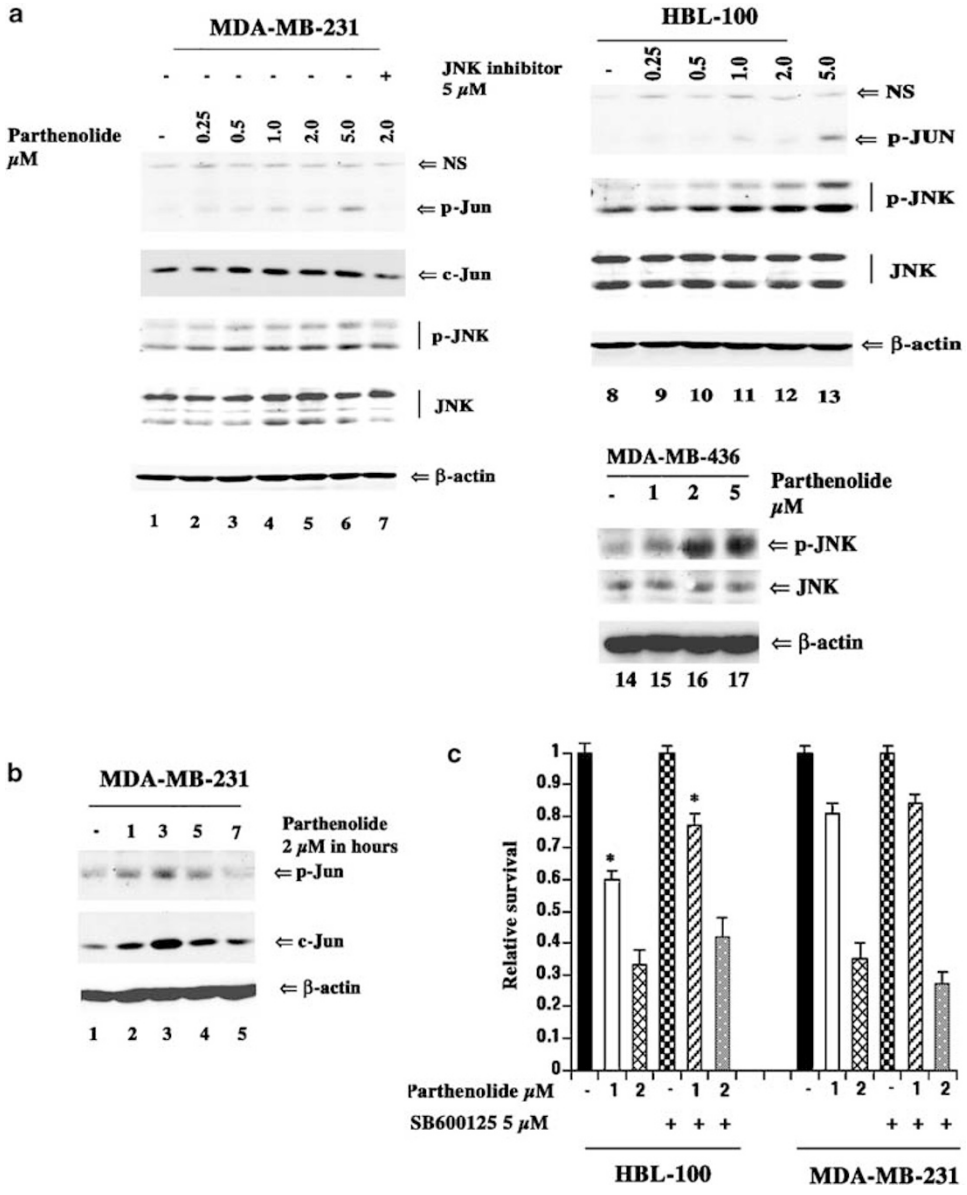


Figure 2 Parthenolide induces JNK. (**a**) Dose-dependent increases in JNK and c-Jun phosphorylation by parthenolide. MDA-MB-231, MDA-MB-436 and HBL-100 cells were treated with increasing concentration of parthenolide for 3 h and JNK activity was measured using phosphospecific JNK (p-JNK) and c-Jun (p-Jun) antibodies. Parthenolide-induced phosphorylation of both c-Jun and JNK was lower in MDA-MB-231 cells pretreated with SP600125 (5 μM) for 1 h (lane 7). NS, nonspecific. (**b**) Time course of JNK activation by parthenolide. MDA-MB-231 cells were treated with 2 μM parthenolide for the indicated time and analysed for phospho-Jun levels. (**c**) JNK activity is required for parthenolide-mediated inhibition of HBL-100 but not MDA-MB-231 cell proliferation. Cells were pretreated with SP600125 (5 μM) for 1 h followed by parthenolide treatment for 48 h. Cell death was measured by MTS assay. $^*P = 0.005$

effective only when low concentration of parthenolide was used. In contrast to the effect of SP600125, parthenolide inhibited cell proliferation in the presence of the p38 kinase inhibitor SB202190 (data not shown). Our repeated attempts to reconfirm these results in HBL-100 cells using a dominant-negative JNK1 were not successful. However, dominant-negative JNK1 failed to protect MDA-MB-231 cells from parthenolide in both MTS and clonogenic assay, which further suggests cell type specific role of JNK in parthenolide-mediated inhibition of cell proliferation (data not shown). Note that parthenolide had no effect on the phosphorylation status of the cell survival kinase AKT/PKB in these cells (data not shown). Also note that parthenolide (1–5 μM) was effective in inhibiting the proliferation of both HBL-100 and MDA-MB-231 cells in the presence of the broad-spectrum caspase inhibitor Z-VAD-FMK, suggesting the inhibition of proliferation is not due to caspase-dependent apoptosis (data not shown).

Parthenolide-induced activation of JNK is not a consequence of NF-κB inhibition

Although parthenolide induced JNK in HBL-100 cells without inhibiting NF-κB DNA-binding activity, it is still possible that inhibition of NF-κB activity, which cannot be measured with available assays, is responsible for the parthenolide-induced JNK activation. To rule out this possibility, we determined the JNK activity in embryonic fibroblasts derived from mice lacking the p65 subunit of NF-κB. Basal JNK activity was higher in p65−/− fibroblasts compared to wild-type fibroblasts (Figure 3). Parthenolide-induced JNK activity in wild-type cells was observed only at 5 μM. In contrast, JNK activation was observed at 1 μM in p65−/− cells. Overall levels of JNK was modestly higher in parthenolide-treated p65−/− cells compared to untreated cells, possibly due to stabilization of phospho-JNK. Phospho-Jun levels were also increased by parthenolide in both cell types, although lower concentration of parthenolide was required in p65−/− cells compared to wild-type cells. The p65−/− cells appears to be sensitive to parthenolide at 2–5 μM because we consistently observed lower levels of β-actin in treated cells compared to untreated cells. The basal c-Jun level was higher in p65−/− cells compared to wild type cells possibly due to increased transcription of c-Jun by phospho-Jun (Shaulian and Karin, 2002). Taken together, these results suggest that JNK activation is one of the primary functions of parthenolide and the concentration required for this activation is dependent on cellular levels of NF-κB. This observation is consistent with results in MDA-MB-436 cells, which contains highest level of constitutive NF-κB DNA-binding activity among three cell types tested (Figure 1c) and required higher concentration of parthenolide to activate JNK (Figure 2a). It is interesting that Pozarowski *et al*. (2003a) recently demonstrated NF-κB-independent cell death of Jurkat cells by parthenolide, which may be dependent on JNK activation.

Parthenolide reverses resistance of MDA-MB-231 cells to TRAIL-induced apoptosis

JNK activation and NF-κB inhibition have been shown to increase the sensitivity of cancer cells to TRAIL. Because parthenolide displayed this dual property in a cell type specific manner, we examined the effect of parthenolide on TRAIL-induced cell death. (Herr *et al*., 1999). We used the carboxyfluorescein FLICA assay to measure parthenolide ± TRAIL-induced cell death. This assay simultaneously measures the level of active caspases and the loss of plasma membrane integrity thus allowing quantitation of live, apoptotic, atypical apoptosis (apoptosis with loss of plasma membrane integrity) and necrotic cells (Pozarowski *et al*., 2003b). At 5 ng/ml, HBL-100 and MDA-MB-436 cells but not MDA-MB-231 cells were sensitive to TRAIL (Figure 4a). Although parthenolide inhibited proliferation of all cell types at a lower concentration (Figure 1a), parthenolide-induced apoptosis and necrosis was observed only with MDA-MB-231 cells at 5 μM (Figure 4a). Thus, it appears that inhibition of cell proliferation by parthenolide is not a consequence of apoptosis, which explains the failure of Z-VAD-FMK to prevent inhibition of cell proliferation in MTS assay. All three cell types showed increased typical and atypical apoptosis when treated with a combination of parthenolide and TRAIL. This is particularly evident with MDA-MB-231 cells. For example, treatment with either 2 μM parthenolide or 5 ng/ml TRAIL did not lead to significant apoptosis. In contrast, ~60% of cells were apoptotic when two drugs were combined (Figure 4a). Synergistic cell death was observed when MDA-MB-231 cells were incubated with 1 μM parthenolide and 5 ng/ml TRAIL (Figure 4b) or with 2 ng/ml TRAIL and 2 μM parthenolide (data not shown). Similarly, parthenolide increased apoptosis of MDA-MB-436 cells treated with 1 ng/ml of TRAIL (Figure 4c).

JNK activity is required for parthenolide-induced sensitization of MDA-MB-231 cells to TRAIL

We used the JNK inhibitor SP600125 to investigate the role of JNK in parthenolide plus TRAIL-induced cell death. SP600125 substantially reduced parthenolide plus TRAIL-induced cell death of MDA-MB-231 cells (Figure 5a). We generated MDA-MB-231 cells over-expressing the dominant-negative JNK1 mutant to further confirm the role of JNK in parthenolide-induced sensitization to TRAIL (Figure 5b). Although over-expression of dominant-negative JNK (JNK-dn) did not

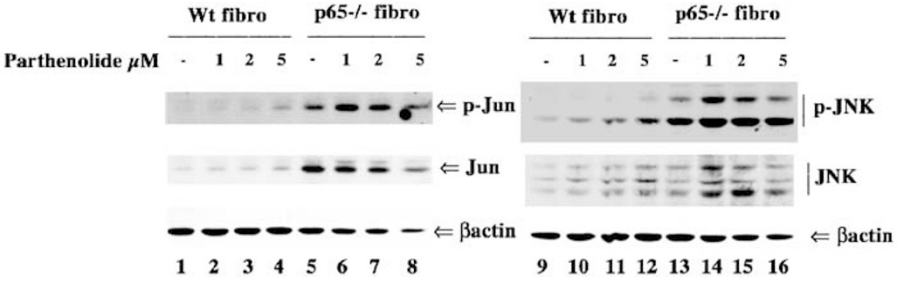
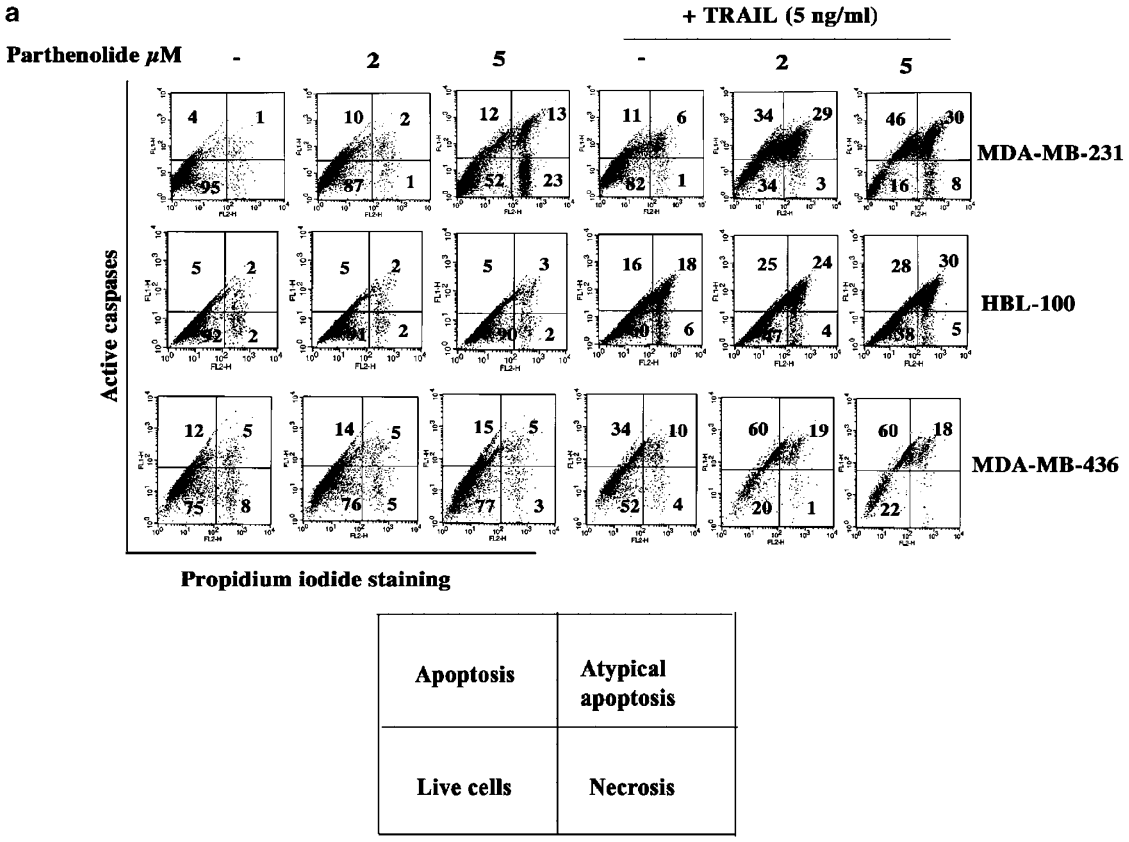
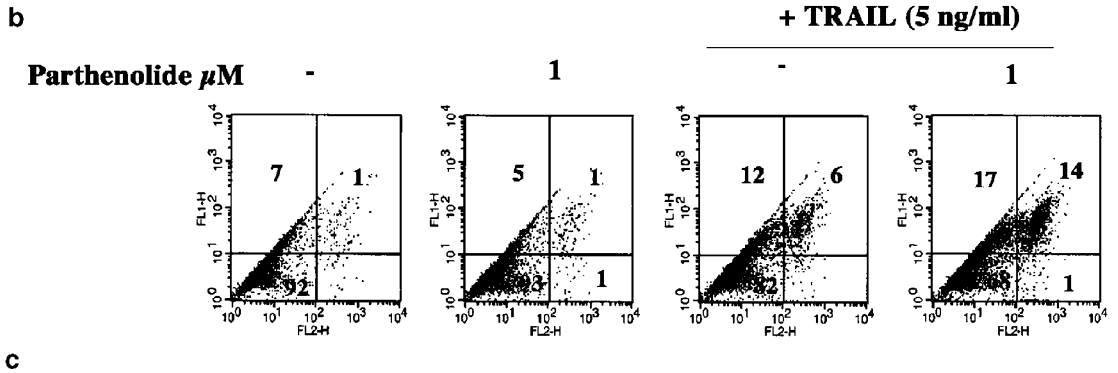


Figure 3 Parthenolide induces JNK in p65−/− fibroblasts. Fibroblasts derived from embryos lacking the p65 subunit of NF-κB and the wild-type counterpart were treated with increasing concentration of parthenolide for 3 h. JNK activity was measured using phosphospecific JNK and c-Jun antibodies





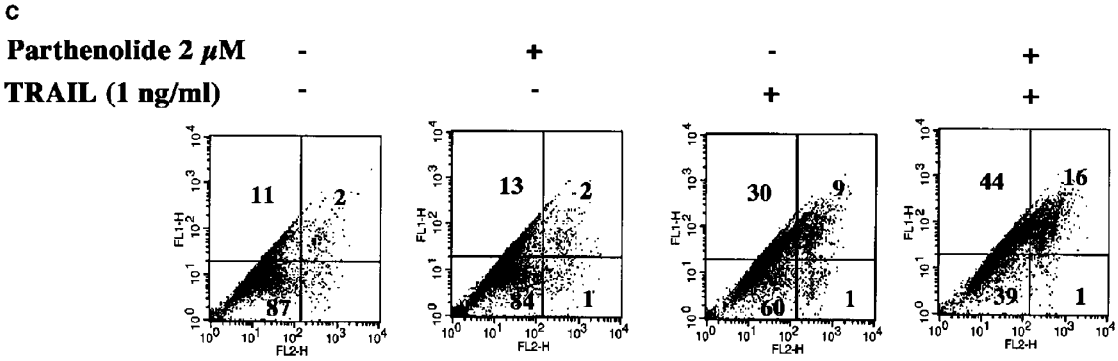


Figure 4 Parthenolide sensitizes MDA-MB-231, MDA-MB-436 and HBL-100 cells to TRAIL. (**a**) Parthenolide increases TRAIL-induced typical and atypical apoptosis. Apoptosis assay using carboxyfluorescein FLICA was performed as described in Materials and methods. Apoptosis was measured 48 h after treatment. A representative data from two to five experiments is shown. A similar result was observed when MDA-MB-231 cells were treated for only 24 h. (**b**) Parthenolide at 1 μM increases the sensitivity of MDA-MB-231 cells to TRAIL. (**c**) Parthenolide increases the sensitivity of MDA-MB-436 cells treated with 1 ng/ml of TRAIL

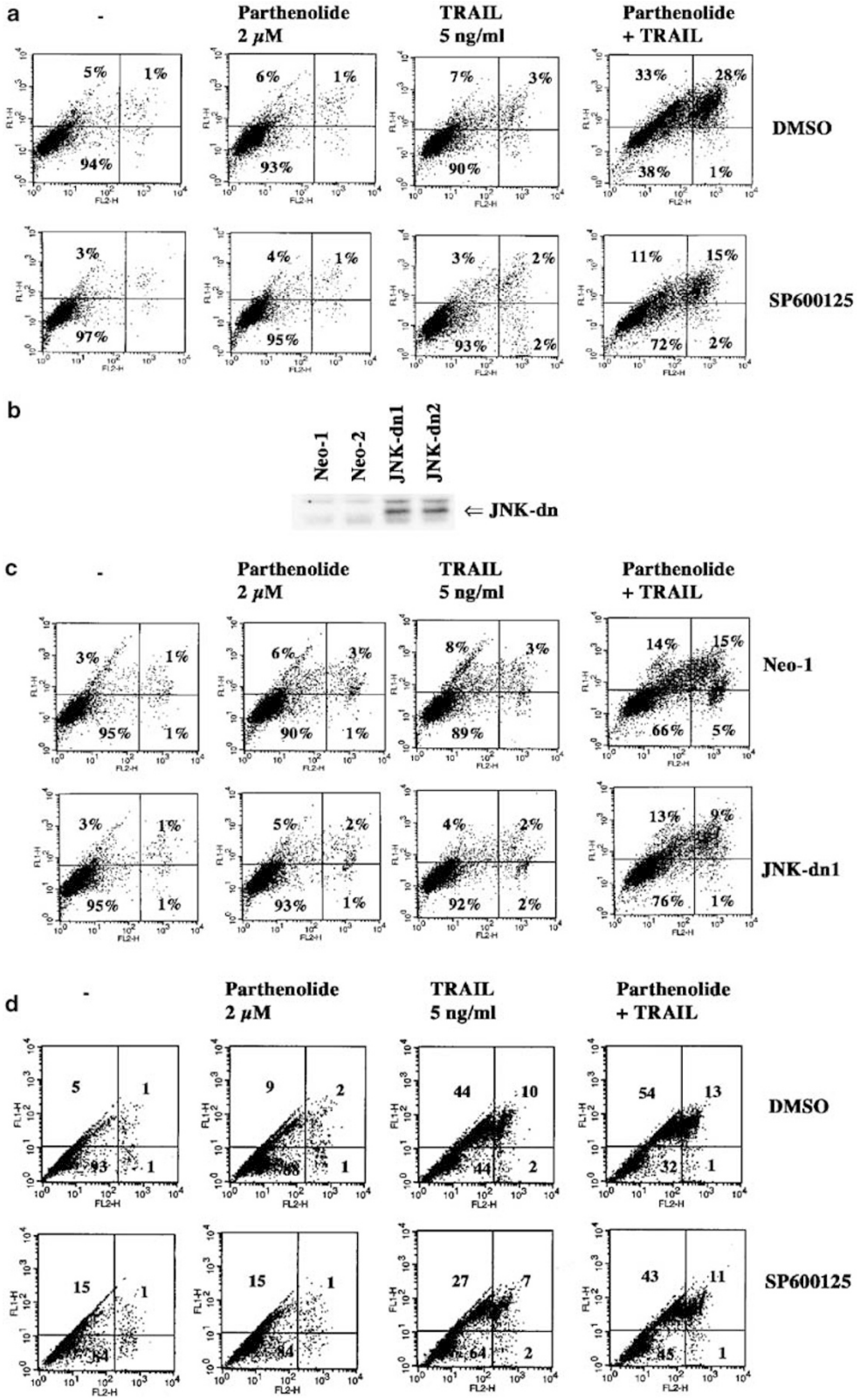


Figure 5 JNK activity is required for parthenolide-induced sensitization to TRAIL. (**a**) SP600125 reduces parthenolide plus TRAIL-induced cell death. Cells were pretreated with SP600125 (5 μM) for 1 h followed by treatment with parthenolide. TRAIL was added 2 h after parthenolide addition. Apoptosis was measured as in Figure 4. (**b**) Generation of MDA-MB-231 cells overexpressing dominant-negative JNK. Overexpression of JNK was confirmed by Western analysis. (**c**) MDA-MB-231 cells overexpressing dominant-negative JNK are less sensitive to parthenolide plus TRAIL. Apoptosis was measured 48 h after drug treatment as in Figure 4. A representative data is presented. (**d**) JNK activity is required for TRAIL and TRAIL plus parthenolide-induced apoptosis of HBL-100 cells. Experiments were performed as in a. A representative data from three experiments is shown

alter sensitivity of cells to parthenolide or TRAIL, sensitivity of these cells to parthenolide plus TRAIL treatment was reproducibly lower compared to control cells with vector alone (231-neo) (Figure 5c). Note that because of extensive culturing required for generating these stable clones, overall sensitivity of 231-neo and JNK-dn cells to parthenolide plus TRAIL was lower than parental MDA-MB-231 cells. Nonetheless, these results suggest that JNK activation is required for parthenolide-induced sensitization of MDA-MB-231 cells to TRAIL.

We next examined the effect of SP600125 on parthenolide and TRAIL-induced apoptosis of HBL-100 cells. Interestingly, TRAIL-induced cell death was substantially lower in HBL-100 cells pretreated with SP600125. There was a corresponding decrease in TRAIL plus parthenolide-induced cell death in cells pretreated with SP600125 (Figure 5d).

Parthenolide induces a post-translational modification of Bid and increases its cleavage by TRAIL

TRAIL-mediated apoptosis involves the activation of caspase 8, cleavage of Bid, release of cytochrome *c*, Smac/DIABLO and/or Omi/HtrA2 from the mitochondria followed by activation of caspase 9 and caspase 3 (Daniel *et al*., 2001; LeBlanc and Ashkenazi, 2003). Insertion of Bid to mitochondria is essential for generating pores that is big enough for the release of Smac/DIABLO but not cytochrome *c* to cytosol (Bratton and Cohen, 2003). To determine whether parthenolide modulates any of these processes, we determined the levels of procaspase 8, active caspase 8 and Bid in cells treated with parthenolide and/or TRAIL. Majority of these studies were conducted in MDA-MB-231 cells because these cells are resistant to TRAIL and become sensitive to TRAIL only after preincubation with parthenolide. Parthenolide did not increase TRAIL-activated active caspase 8 levels because similar levels of active caspase 8 were present in cells treated with TRAIL with or without parthenolide for 8 or 24 h (Figure 6a). Despite caspase 8 activation, TRAIL alone was ineffective in inducing apoptosis, which suggests that antiapoptotic proteins functioning downstream of caspase 8 are blocking apoptosis and parthenolide overcomes the function of these antiapoptotic proteins.

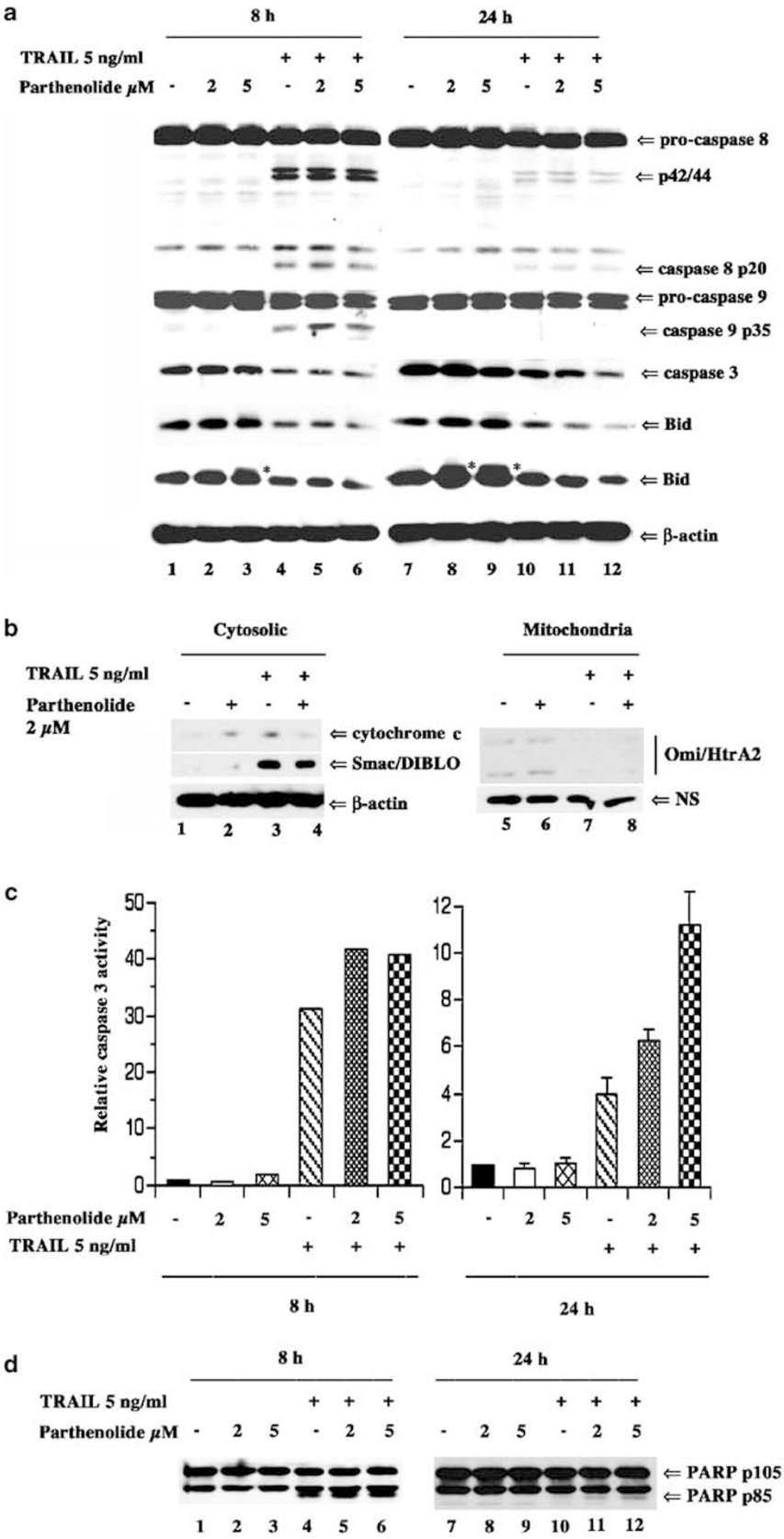
Two distinct species of Bid protein could be detected in parthenolide-treated cells. We believe that the slower migrating form of Bid is the phosphorylated form of the protein because treatment of extracts with alkaline phosphatase reduced the level of the slower migrating Bid (data not shown). Also, the slower migrating Bid was not detected when extracts were prepared in buffer lacking phosphatase inhibitors (data not shown). Slow migrating form of Bid was also observed in HBL-100 and MDA-MB-436 cells treated with parthenolide (data not shown). The level of Bid was substantially lower in cells treated with a combination of parthenolide and TRAIL (Figure 6a). Unfortunately, we were unable to detect cleaved Bid products in these cells using two different antibodies, possibly due to reduced stability of these products. Nonetheless, these results suggest mitochondria as one of the target of parthenolide.

We measured the level of cytochrome *c* and Smac/DIABLO in the cytoplasm to determine whether parthenolide enhances TRAIL-mediated release of these proteins from the mitochondria. Both TRAIL and parthenolide increased the level of cytoplasmic cytochrome *c* modestly after 8 h treatment (Figure 6b). It is not clear why the cytoplasmic cytochrome *c* was lower in cells treated with both parthenolide and TRAIL. It is possible that released cytochrome *c* is less stable in cells treated with both parthenolide and TRAIL because of enhanced apoptosis. Only TRAIL caused the release of Smac/DIABLO, which was not further increased upon parthenolide cotreatment (Figure 6b, lanes 1–4). Our repeated attempts to detect cytoplasmic Omi/HtrA2 were not successful. However, TRAIL treatment resulted in the depletion of Omi/HtrA2 from mitochondria, which was not further enhanced by parthenolide (Figure 6b, lanes 5–8).

There was a modest increase in the levels of active caspase 9 in cells treated with parthenolide plus TRAIL for 8 h compared to cells treated with either drug alone. Interestingly, although there was no increase in the cleavage of caspase 3 in TRAIL plus parthenolide-treated cells compared to cells treated with either drug for 8 h, caspase 3 cleavage was enhanced in cells treated with TRAIL plus parthenolide for 24 h (Figure 6a). There was a corresponding increase in caspase 3 activity in cells treated with TRAIL plus parthenolide (Figure 6c, $P = 0.0005$ TRAIL versus parthenolide 5 μM plus TRAIL at 24 h). Thus, caspase 3 activation appears to be persistent in cells treated with parthenolide plus TRAIL compared to cells treated with either drug alone. Cleavage of PARP, a substrate of caspase 3, was modestly higher in cells treated with TRAIL plus parthenolide compared to cells treated with TRAIL alone for 8 h (Figure 6d). However, at 24 h, cleaved PARP was detected only in cells treated with parthenolide plus TRAIL, which also suggests persistent activation of caspase 3 in cells treated with parthenolide plus TRAIL. Thus, it appears that the inability of TRAIL to induce apoptosis of MDA-MB-231 cells is due to an antiapoptotic activity downstream of mitochondria because TRAIL was able to induce caspase 8 activity and release cytochrome *c*, Smac/DIABLO and Omi/HtrA2 from mitochondria. Parthenolide somehow enables TRAIL to overcome this antiapoptotic activity.

Parthenolide increases TRAIL-induced cleavage of XIAP

The elevated caspase 3 activity in parthenolide plus TRAIL-treated cells suggested that the activity of an inhibitor of caspase 3 is lower in these cells compared to cells treated with either drug. XIAP is an ideal candidate because it is the potent inhibitor of caspase 3 and suppresses TRAIL-induced apoptosis at the caspase 3 activation step without inhibiting caspase 8 activation (Bratton and Cohen, 2003). XIAP can be degraded


a
8 h
24 h
TRAIL 5 ng/ml
Parthenolide μM
⇐ pro-caspase 8
⇐ p42/44
⇐ caspase 8 p20
⇐ pro-caspase 9
⇐ caspase 9 p35
⇐ caspase 3
⇐ Bid
⇐ Bid
⇐ β-actin
1 2 3 4 5 6 7 8 9 10 11 12
b
Cytosolic
Mitochondria
TRAIL 5 ng/ml
Parthenolide 2 μM
⇐ cytochrome c
⇐ Smac/DIBLO
⇐ β-actin
Omi/HtrA2
⇐ NS
1 2 3 4 5 6 7 8
c
Relative caspase 3 activity
Parthenolide μM
TRAIL 5 ng/ml
8 h
24 h
d
8 h
24 h
TRAIL 5 ng/ml
Parthenolide μM
⇐ PARP p105
⇐ PARP p85
1 2 3 4 5 6 7 8 9 10 11 12

proteolytically by Omi/HtrA2 or cleaved by caspases (Deveraux *et al*., 1999; Suzuki *et al*., 2001; Hell *et al*., 2003). Treatment with parthenolide or TRAIL alone did not lead to changes in the levels of XIAP (Figure 7a). In contrast, parthenolide increased XIAP cleavage in TRAIL-treated cells. Caspases and JNK play a role in this XIAP cleavage because the z-VAD-FMK and SP600125 or a JNK inhibitory peptide reduced XIAP cleavage (Figure 7b). Interestingly, parthenolide and/or TRAIL had no effect on the stability of cIAP-2 (data not shown).

Oxidative stress pathway or altered death receptor expression are not responsible for parthenolide and TRAIL synergy

Parthenolide has been shown to induce the apoptosis of SH-J1 cells at a concentration of 10 μM through an oxidative stress mechanism (Wen *et al*., 2002). However, others have failed to detect oxidative stress by parthenolide in Jurkat cells (Hehner *et al*., 1998). To determine whether the parthenolide-induced sensitization to TRAIL involves oxidative stress in MDA-MB-231 cells, we measured the levels of reactive oxygen species using the oxidation-sensitive DCFH-DA dye. Parthenolide either alone or in combination with TRAIL did not increase the oxidative stress level (Figure 8a). In contrast, H_2O_2 increased the oxidative stress. These results suggest that the oxidative stress pathway is not involved in the parthenolide-induced sensitization to TRAIL. Furthermore, the antioxidant *N*-acetyl-L-cysteine (NAC) increased TRAIL-induced atypical apoptosis (Figure 8b). In the presence of NAC, TRAIL with or without parthenolide-induced cell death was shifted from typical apoptosis to atypical apoptosis. Pyrrolidine dithiocarbamate (PDTC), another antioxidant, induced massive necrosis of these cells and parthenolide enhanced the PDTC induced atypical apoptosis as well as necrosis (Figure 8b). In the presence of TRAIL, atypical apoptosis appears to be the predominant form of cell

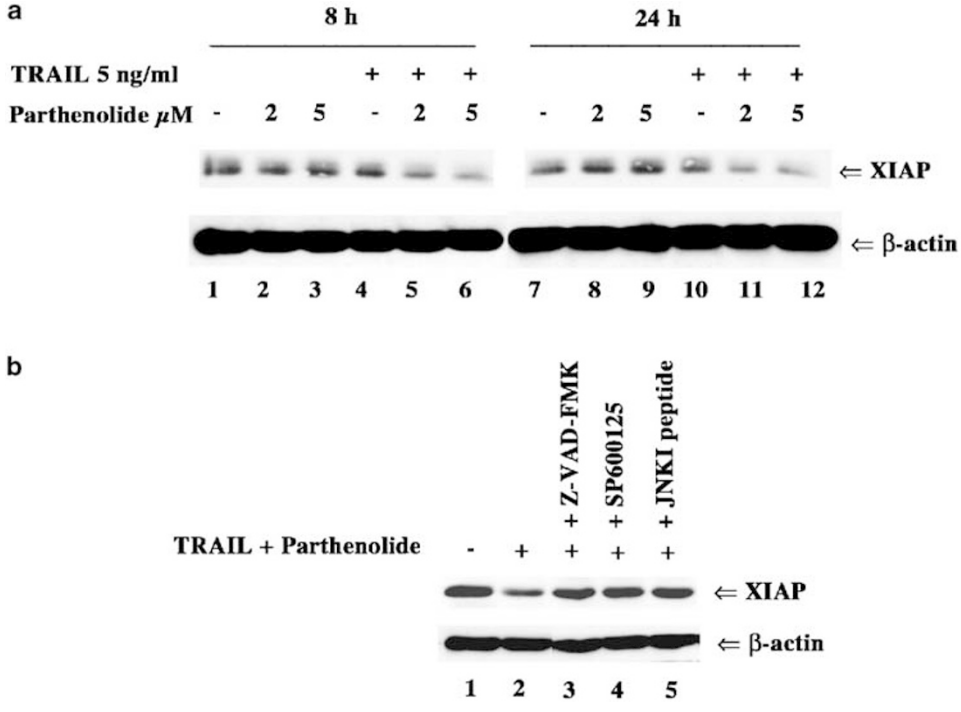


Figure 7 Parthenolide enhances TRAIL-induced XIAP cleavage, which requires JNK activity. (**a**) XIAP cleavage in parthenolide plus TRAIL-treated cells. XIAP cleavage was measured in cells treated for 8 or 24 h. (**b**) XIAP cleavage was reduced in cells pretreated with the pancaspase inhibitor Z-VAD-FMK, SP600125 or a JNK inhibitory peptide. Cells were pretreated with inhibitors for 1 h before adding parthenolide. TRAIL was added 2 h after parthenolide treatment and XIAP levels were measured 8 h after TRAIL addition

Figure 6 Parthenolide and TRAIL-induced changes in the apoptosis machinery. (**a**) Effect of parthenolide and TRAIL treatment on caspase 3, caspase 8, caspase 9 and Bid cleavage. Cells were treated with the indicated drugs for 8 or 24 h and cell lysates were subjected to Western analysis using the indicated antibodies. Note differences in the mobility of Bid in cells treated with parthenolide (indicated by an asterisk). Two exposures of Bid blot are shown. (**b**) Parthenolide and TRAIL-induced changes in cytoplasmic cytochrome *c* and Smac/DIABLO, and mitochondrial Omi/HtrA2. Cytosolic S-100 fraction was subjected to Western analysis using antibodies against Smac/DIABLO or cytochrome *c* (lanes 1–4). Cells were treated with drugs for 8 h. Similar results were obtained with cells treated for 24 h. TRAIL-mediated depletion of Omi/HtrA2 from the mitochondria as analysed by Western analysis (lanes 5–8). NS = nonspecific, which also served as a loading control. (**c**) Caspase 3 activity in cells treated with parthenolide with or without TRAIL for 8 and 24 h. Caspase 3 activity was measured using the caspase 3 substrate DEVD-AMC as described in Materials and methods. (**d**) Parthenolide plus TRAIL-induced PARP cleavage. PARP cleavage was measured by Western analysis

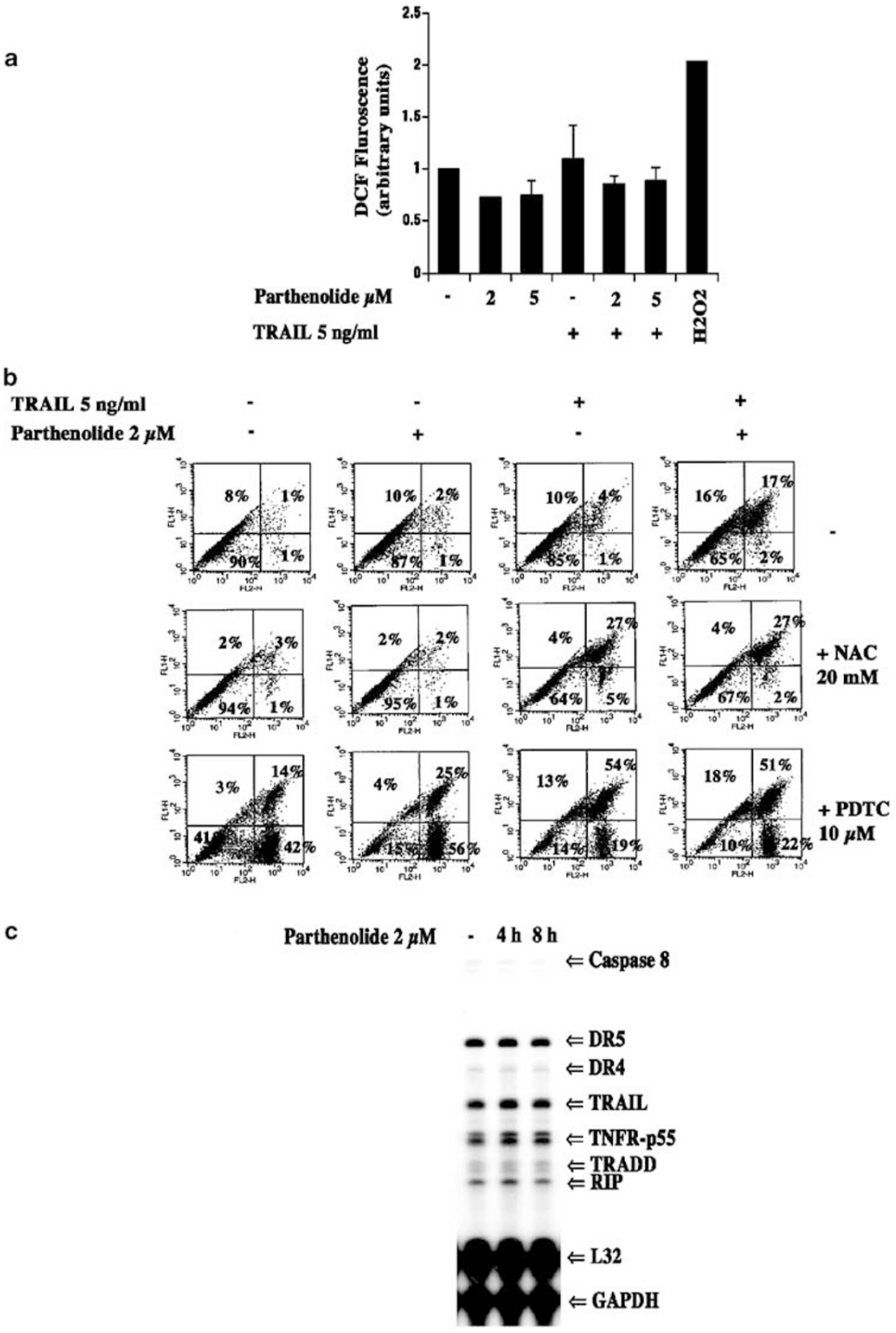


Figure 8 Parthenolide-induced sensitization to TRAIL does not involve oxidative stress or altered death receptor expression. (**a**) Oxidative stress levels in cells treated with parthenolide with or without TRAIL for 3 h. Oxidative stress were measured using the oxidation sensitive dye DCFH-DA. H_2O_2 was used as a positive control. (**b**) The effect of NAC and PDTC on parthenolide or TRAIL-induced cell death. Cells were pretreated with 20 mM NAC or 10 μM PDTC before the addition of parthenolide and/or TRAIL and apoptosis was measured as in Figure 4. (**c**) Parthenolide does not alter the expression levels of death receptors. Cells were treated with 2 μM parthenolide for the indicated time and death receptor levels were measured by RNAse protection assay

death induced by PDTC with or without parthenolide. Taken together, these results suggest that oxidative stress pathway is not required for TRAIL-induced cell death.

To determine whether the parthenolide-induced sensitization to TRAIL involves increased expression of death receptors, an RNAse protection assay with untreated and parthenolide-treated MDA-MB-231 cell

RNA was performed. DR4 and DR5 levels remained unchanged in parthenolide-treated cells compared to untreated cells (Figure 8c). Thus, parthenolide-induced sensitization to TRAIL involves activation of the pre-existing apoptotic machinery.

Discussion

In this study, we investigated the signaling pathways affected by parthenolide in breast cancer cells, which are unrelated to parthenolide-mediated inhibition of NF-κB DNA-binding activity. We show that parthenolide activates JNK and increases sensitivity of breast cancer cells to TRAIL. Parthenolide-activated signaling pathways appear to prolong the activation of executioner caspases because caspase 3 activity as well as PARP cleavage was prolonged in cells exposed to parthenolide plus TRAIL compared to cells treated with TRAIL alone. A comparison of TRAIL-sensitive and resistant melanoma cells have shown a strong correlation between TRAIL sensitivity and caspase 3 activity (Zhang *et al*., 2001). Our results suggest that parthenolide can be used to increase the levels of active caspase 3 in cells treated with TRAIL, which may help in overcoming resistance. In this regard, MDA-MB-231 cells, which are relatively resistant to TRAIL compared to other cell types tested, became sensitive to TRAIL upon preincubation with parthenolide. TRAIL could efficiently initiate intrinsic cell death pathway in MDA-MB-231 cells but fails to execute apoptosis because of a block in apoptosis downstream of mitochondria. The block appears to be at the level of duration of caspase 3 activation (Figure 6c). Prolonged caspase 3 activation in parthenolide plus TRAIL-treated cells could be due to reduced levels of XIAP in these cells. XIAP is a potent inhibitor of caspase 3 activity (Bratton and Cohen, 2003). XIAP has previously been shown to confer resistance to TRAIL (Ng *et al*., 2002; Kim *et al*., 2004).

The proapoptotic protein Bid appears to be another target of parthenolide. We observed altered mobility of Bid in cells treated with parthenolide. This is most likely due to phosphorylation because slow migrating Bid protein was not detected when cell extracts were prepared in the absence of phosphatase inhibitors (data not shown). Interestingly, parthenolide-mediated changes in Bid mobility correlated with the release of cytochrome *c* from the mitochondria. Several putative phosphorylation sites have been identified in mouse Bid. Two of these sites are phosphorylated by casein kinase I (CKI) and casein kinase II (CKII) (Desagher *et al*., 2001). Interestingly, the major casein kinase phosphorylation site S61 is present in mouse but not human Bid. Phosphorylation by CKI and CKII has been shown to inhibit Bid cleavage by caspases but not granzyme B. However, we observed enhanced cleavage of Bid in cells treated with both parthenolide and TRAIL, which suggests that parthenolide-induced phosphorylation of Bid is less likely to have inhibitory effect on cell death. Furthermore, apigenin, an inhibitor of CKI and CKII, failed to reduce parthenolide-induced changes in Bid mobility (data not shown). Phosphorylation is reduced but not lost in Bid with mutated casein kinase phosphorylation sites, which suggests that there are additional amino acids in Bid that are phosphorylated by distinct kinases. Amino acids 66–88 of Bid contain three conserved putative phosphorylation sites and this fragment is phosphorylated *in vivo* (Desagher *et al*., 2001). We propose that parthenolide phosphorylates Bid through an unknown kinase, which increases its ability to function as proapoptotic protein. A Bid cleaved product called jBid has been shown to cause release of Smac/DIABLO but not cytochrome *c* from mitochondria during TNF-induced cell death (Deng *et al*., 2003). Generation of jBid requires JNK activity (Deng *et al*., 2003). Because parthenolide did not increase the cytoplasmic levels of Smac/DIABLO, it is less likely that jBid is generated in cells treated with parthenolide alone. We note that neither parthenolide-induced phosphorylation nor enhanced cleavage of Bid in parthenolide plus TRAIL-treated cells is dependent on JNK because JNK inhibitors failed to reduce parthenolide with or without TRAIL-induced changes in Bid (data not shown).

Although there was an increased cleavage of Bid in parthenolide plus TRAIL-treated cells, the other cleaved Bid product tBid, which initiates loss mitochondrial membrane potential, were not detected in these cells (Luo *et al*., 1998). We were unsuccessful in detecting tBid with two different antibodies. This could be due to unstable nature of tBid. Bid is cleaved by both caspase 8 and caspase 3. Bid cleavage by caspase 8 initiates changes in mitochondrial potential whereas cleavage by caspase 3 may prolong the loss of mitochondrial membrane potential (Daniel *et al*., 2001). Because caspase 3 but not caspase 8 activities were higher in parthenolide plus TRAIL-treated cells compared to cells treated with either drug alone, we suspect that cleaved Bid plays a role in prolonging rather than initiation of loss of mitochondrial potential by TRAIL. It is possible that parthenolide-induced JNK initiates loss of mitochondrial membrane potential, which is prolonged in TRAIL plus parthenolide-treated cells because of enhanced cleavage of Bid. In summation, results presented in this study provide a new avenue to understand the role of post-translation modification of Bid and its cleavage during apoptosis.

The role in JNK in parthenolide-mediated sensitization to TRAIL is not known. The proapoptotic function of JNK is mediated in part by Bim or Bmf. Bim and Bmf are phosphorylated by JNK and phosphorylated Bim and Bmf increases Bax-dependent apoptosis (Lei and Davis, 2003). However, we did not observe a phosphorylation-induced shift in the mobility of Bim in both MDA-MB-231 and HBL-100 cells (data not shown). One of the functions of JNK is to enhance the cleavage of XIAP. How JNK increases XIAP cleavage is not known. Although XIAP has been shown to antagonize JNK function, there is no evidence for direct interaction between XIAP and JNK (Tang *et al*., 2001). It is possible that JNK through Smac/DIABLO and/or Omi/HtrA2 increases cleavage/degradation of XIAP (Bratton

and Cohen, 2003; Chauhan *et al*., 2003). Additional studies are required to test this possibility.

The role of JNK within the context of active NF-κB has been controversial. Several studies have shown that a persistent activation of JNK, which can be attained by inhibiting NF-κB activity, leads to cell death (De Smaele *et al*., 2001; Javelaud and Besancon, 2001; Tang *et al*., 2001, 2002). In contrast, transient JNK activation coupled with NF-κB activation leads to an antiapoptotic response through overexpression of the antiapoptotic cIAP-2 protein (Lamb *et al*., 2003). It was also shown in a recent study that JNK transmits an antiapoptotic signal in the absence of NF-κB (Reuther-Madrid *et al*., 2002). In light of these controversial observations, it is difficult to predict the benefits of the dual function of parthenolide (JNK activation and NF-κB inhibition). Based on the ability of parthenolide to induce JNK in cells lacking the p65 subunit of NF-κB, it is also difficult to propose the mechanisms involved in the parthenolide-mediated activation of JNK. Parthenolide-related sesquiterpene lactones have been shown to affect plasma membrane lipid oxidation (Berman and Adams, 1997). Furthermore, parthenolide contains two reactive centers in the form of an exomethylene group and an epoxide ring that may react with exposed cysteines of proteins (Hehner *et al*., 1999). It is possible that these properties of parthenolide are involved in initiating JNK activation step from the plasma membrane. At least 13 MAP kinase kinase kinases (MAPKKKs) and two MAPKK (MKK4 and MKK7) are involved in JNK activation (Davis, 2000; Pearson *et al*., 2001). Which among these kinases are targeted by parthenolide remains to be investigated. We do not believe that an oxidative stress pathway is involved in JNK activation.

The parthenolide-mediated sensitization to TRAIL is independent of p53 because this phenomenon was observed in MDA-MB-231 cells, which expresses mutant p53 (Bykov *et al*., 2002). Parthenolide-induced sensitization does not involve the altered expression of death receptors. Ionizing radiation has been shown to sensitize breast cancer cells to TRAIL, which is due to p53-dependent increase in death receptor expression (Chinnaiyan *et al*., 2000; Zhou *et al*., 2000). The p53-independent sensitization to TRAIL by parthenolide is particularly important because p53 mutation predicts poor response to systemic therapy in advanced breast cancer (Berns *et al*., 2000).

In summation, in this study, we have identified parthenolide as an agent that can increase the sensitivity of cancer cells to TRAIL. In a xenograft model of breast cancer, we have observed the activation of JNK in tumors by parthenolide at serum concentration that is not sufficient for NF-κB inhibition (H. Nakshatri, unpublished observations). Because JNK activity is required for parthenolide-induced sensitization to TRAIL *in vitro*, we anticipate similar sensitization to TRAIL *in vivo*. Sensitization, in the absence of NF-κB inhibition, may be essential to prevent the toxicity of TRAIL to normal cells *in vivo* because the expression of decoy receptors, which protects normal cells from TRAIL, is regulated by NF-κB (Bernard *et al*., 2001). We have used lower concentration of TRAIL in our studies, which on its own has been shown to be ineffective on MDA-MB-231 cells and is far below the effective serum concentrations observed in *in vivo* studies (Walczak *et al*., 1999). Depending on quality of preparation, TRAIL at higher concentration has been shown to form aggregates, which show nonspecific toxicity to normal hepatocytes (Lawrence *et al*., 2001). By lowering the requirement for TRAIL, parthenolide may overcome the nonspecific toxic effects of TRAIL *in vivo*.

Materials and methods

Breast cancer cell lines

All breast cancer cell lines were purchased from the American Type Tissue Culture Collection (Rockville, MD, USA) and grown as described previously (Bhat-Nakshatri *et al*., 1998). Wild-type fibroblasts and p65−/− fibroblasts were gifts from Drs A Hoffman and D Baltimore and have been described previously (Beg and Baltimore, 1996). The dominant-negative mutant of JNK1 was a gift from Dr M Cobb and has been described previously (Swantek *et al*., 1997). The dominant-negative mutant was cloned into the bicistronic retrovirus vector pQXCIN (BD Biosciences, San Diego, CA, USA) and the retrovirus was packaged using Amphophenix cell line. MDA-MB-231 cells were transduced with control retrovirus (MD-231-neo) or retrovirus encoding the dominant-negative JNK1 (MD231-JNKdn) as described previously (Patel *et al*., 2000).

Electrophoretic mobility shift assays (EMSAs)

Cell extracts were subjected to EMSA with NF-κB and Oct-1 probes (Promega, Madison, WI, USA) as described previously (Newton *et al*., 1999).

Drug treatment, apoptosis, and cell death assays

For cell death assays, 2×10^3 cells grown overnight on a 96-well plate were treated with parthenolide (dissolved in ethanol; Sigma Chemicals, St Louis, MO, USA). Cell death was measured 24 or 48 h after TRAIL treatment using the MTS assay (Promega, Madison, WI, USA). Recombinant TRAIL was purchased from R&D systems (Minneapolis, MN, USA). The carboxyfluorescein FLICA apoptosis detection kit (Immunohistochemistry Technologies, LLC, Bloomington, MN, USA) was used to measure typical apoptosis, atypical apoptosis and necrosis (Pozarowski *et al*., 2003b). Briefly, 2×10^5 cells grown overnight on a 60 mm plate were treated with indicated drugs for 48 h. In a combination treatment, parthenolide was added 2 h before the addition of TRAIL. Both attached and floating cells were collected by trypsinization, incubated with carboxyfluorescein-labeled pan-caspase inhibitor FAM-VAD-FMK for 2 h at 37°C. Labeled cells were rinsed twice in PBS and resuspended in 300 μl of PBS containing 0.3 μg of propidium iodide. Apoptotic cells were identified by FACScan analysis as described previously (Pozarowski *et al*., 2003b). All apoptosis assays were performed 2–5 times and representative data are presented in the text. Synergism in combination treatment was always observed although degree of synergism varied from experiment to experiment, depending on batch of commercial TRAIL preparation. For the clonogenic assay, cells (100 cells/well in six-well plate, assayed in triplicate) were treated with the indicated drugs for 24 h. The drugs were removed, cells were washed and fresh media was added. Number of colonies after

21 days of drug treatment was counted after staining with Giemsa stain.

Western blotting

Cell lysates prepared in radioimmunoassay buffer were analysed by Western blotting as described previously (Newton *et al*., 1999). To detect the activated caspases, MG132 (5 μM) was added to the cells for 1 h just before harvesting in some experiments. MG132 pretreatment was required for detecting cleaved caspase 8 and caspase 9 products but not for detecting caspase 3, Bid and XIAP cleavage in TRAIL $\pm$ parthenolide-treated cells. JNK activation was measured by probing the membranes with antibodies that recognizes c-Jun phosphorylated by JNK (Cell Signaling, Beverly, MA, USA) or phosphorylated JNK (Promega). The same blot was reprobed with both c-Jun, JNK and β-actin antibodies to ensure equal loading (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Sigma Chemicals). Caspase 8, Bid, XIAP, cytochrome *c* and Smac/DIABLO antibodies were purchased from BD Biosciences. The Omi/HtrA2 antibody was generous gift from Dr J Downward (Martins *et al*., 2003). The caspase 3 antibody was purchased from Calbiochem (San Diego, CA, USA) whereas the caspase 9 antibody was purchased from Santa Cruz Biotechnology.

Subcellular fractionation

S-100 fraction, which contains cytosolic proteins without mitochondria from exponentially growing cells treated for 8 h were prepared as described previously (Singh *et al*., 2003). Briefly, cell pellets were resuspended in 5 volumes of ice-cold buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 2 μg/ml of leupeptin, aprotinin and pepstatin). After homogenization with a 22-gauge needle, nuclei were pelleted by centrifugation at 750 *g* for 10 min at 4°C. The supernatant was recentrifuged at 10 000*g* for 25 min to separate cytosolic S-100 fraction (supernatant) and mitochondria (pellet).

Mitochondria were isolated separately for extract preparation as described previously (Suzuki *et al*., 2004). Briefly, cell pellets were resuspended in 5 volumes of mitochondria isolation buffer (MIB) (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 10 mM sodium succinate, 0.2 mM EGTA, 60 mM sucrose, 210 mM mannitol, 1 mM ADP, and 0.5 mM DTT and protease inhibitors). After 10 min on ice, cells were disrupted by 100 strokes of a dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation at 600 *g* for 5 min. Mitochondria in the supernatant were pelleted by centrifugation at 5500*g* for 10 min. The mitochondria were resuspended in MIB with 80 mM KCl.

Caspase 3 activity and oxidative stress measurements

Caspase 3 activity was measured using the flurogenic caspase 3 substrate (DEVD-AMC, Allexis, San Diego, CA, USA). Briefly, 2×10^5 cells grown overnight in 60 mm dishes were treated with the indicated drugs for 8 or 24 h. Both floating and attached cells were collected by trypsinization and lysed in 100 μl of lysis buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$ and 1 mM DTT). After incubation on ice for 1 h, the lysate was collected by centrifugation. A volume of 50 μl of cell lysate was incubated with 2 $\times$ reaction buffer (20 mM HEPES, pH 7.5, 20% sucrose, 0.2% CHAPS, 0.2 mg/ml BSA and 20 mM DTT) and 5 μl of 1 mM caspase 3 substrate for 1 h at 37°C. The reaction was stopped by adding 750 μl of water and placing the reaction mixture on ice. Caspase 3 activity was measured at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Caspase 3 activities were normalized to protein concentration and basal activity in untreated cells was set as one unit. Oxidative stress was measured by using the dye DCFH-DA (Molecular Probes, Eugene, OR, USA). Briefly, cells were treated with the indicated drugs for 3 h in phenol red-free media and collected by trypsinization. The cells were resuspended in 100 μl of PBS with 50 μM DCFH-DA and incubated for 1 h at 37°C. After two gentle washes in PBS, the cells were resuspended in 1 ml of phenol red-free media and incubated for another 1 h at 37°C. Fluorescence intensity of the cell suspension was measured using a fluorescence spectrophotometer with excitation wavelength at 488 nm and an emission wavelength at 525 nm.

Statistical analysis

Data were analysed using GraphPad software (Graphpad.com). Analysis of variance was employed to determine *P*-values between mean measurements. A P-value < 0.05 was deemed significant.

Abbreviations

TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; JNK, c-Jun N-terminal kinase; PARP, poly(ADP-ribose) polymerase; Z-VAD-FMK, *N-tert*-butoxy-carbonyl-Val-Ala-Asp-fluromethyl-ketone; NF-κB, nuclear factor kappaB; EMSA, electrophoretic mobility shift assay; XIAP, X-linked inhibitor of apoptosis protein; Smac, second mitochondria-derived activator of caspases; MTS, (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

Acknowledgements

We thank MP Cobb for the dominant-negative JNK vector and J Downward for Omi/HtrA2 antibody. We thank Hui Lin Chua and Colin Crean for critical reading of this manuscript. This work is supported by grants from the American Institute for Cancer Research (03A069-REN) and National Cancer Institute (R01-CA89153).

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