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# Suppressed NF-κB and sustained JNK activation contribute to the sensitization effect of parthenolide to TNF-α-induced apoptosis in human cancer cells

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**Parthenolide (PN) is the main sesquiterpene lactone found in feverfew with potent anti-inflammatory function. The anticancer property of PN has been demonstrated in both *in vitro* cell culture and *in vivo* animal model, while the molecular mechanisms remain to be further elucidated. In the present study, we evaluated the involvement of nuclear transcription factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK) in the anticancer activity of PN by examining the sensitization effect of PN on tumor necrosis factor (TNF)-α-induced apoptosis in human cancer cells. Pretreatment with PN greatly sensitized various human cancer cells to TNF-α-induced apoptosis. Such sensitization is closely associated with the inhibitory effect of PN on TNF-α-mediated NF-κB activation. Our study revealed a new mechanism that PN inhibits TNF-α-mediated NF-κB activation via disrupting the recruitment of the IκB kinases (IKK) complex to TNF receptor, which then blocked the subsequent signaling events including IKK kinase activation, IκBα degradation, p65 nuclear translocation, DNA binding and transactivation. Moreover, PN also markedly enhanced and sustained TNF-α-mediated JNK activation. A specific JNK inhibitor (SP600125), as well as overexpression of dominant-negative forms of JNK1 and JNK2 abolished the sensitization effect of PN on TNF-α-induced apoptosis. It is thus believed that suppressed NF-κB activation and sustained JNK activation contribute to the sensitization effect of PN to TNF-α-mediated cell death in human cancer cells.**

## Introduction

Tumor necrosis factor (TNF) is a proinflammatory cytokine with a wide spectrum of functions in many biological processes, including cell growth and cell death, development, oncogenesis, immunity, inflammatory and stress responses (1). The bioactivities of TNF are elicited by two TNF receptors (TNFR1 and TNFR2), which mediate distinct signaling pathways, including the activation of nuclear transcription factor-κB (NF-κB), c-Jun N-terminal kinase (JNK) and a caspase cascade (2). NF-κB is a ubiquitous nuclear transcription factor that governs the expression of various important genes and one key biological function is the regulation of apoptosis (3,4). In the TNF signaling pathway, NF-κB acts as a cell survival mechanism through its regulatory role over the expression of an array of anti-apoptotic genes, including Bcl-2 family proteins, inhibitors of apoptosis proteins, Mn-superoxide dismutase, cyclooxygenase-2, etc. (3,5). On the other hand, the exact role of JNK in TNF-α-induced apoptosis remains inconclusive, although it is known to be required for the apoptotic process triggered by some stress factors such as UV and oxidative stress (6). Various reports coexist showing JNK activation promotes, suppresses or has no role in TNF-α-induced apoptosis (7–10). Recent emerging evidence tends to suggest that JNK is a contextual modulator of TNF-α-induced apoptosis (11). One of the important factors affecting the role of JNK in TNF-α-mediated apoptosis is NF-κB: inhibition of transcriptional activity of NF-κB leads to a prolonged JNK activation, which then plays a pro-apoptotic role in TNF-α-induced apoptosis (8,12,13).

Parthenolide (PN) is the principal component of sesquiterpene lactones contained in feverfew (*Tanacetum parthanium*), a common herbal plant, which has been documented for centuries in Europe for treatment of numerous ailments including fever, arthritis and migraine (14). PN contains an α-methylene-γ-lactone ring and an epoxide, which are able to interact readily with nucleophilic sites of biological molecules (15). Previous studies demonstrated that NF-κB signaling molecules are the main molecular targets for PN, and the inhibition of the NF-κB signaling pathway is the underlying mechanism responsible for its remarkable anti-inflammatory property (16). PN can specifically suppress the activity of the IκB kinases (IKK) complex and the subsequent degradation of the NF-κB inhibitory proteins (IκBα and IκBβ) (17,18) or directly modify the p65 protein (19). In comparison with its well-established anti-inflammatory activity, the anticancer effect of PN is relatively less documented. Preliminary studies have shown that PN is capable of inhibiting DNA synthesis and cell proliferation in human cancer cells (20,21). Recently, PN has been found to induce apoptosis in a number of human cancer cells by depleting intracellular thiols and activation of caspase cascades (22,23), or to prevent UVB-induced skin cancer in hairless mice (24). In the present study, we further evaluated the anticancer property of PN by assessing the sensitization effect of PN on TNF-α-induced apoptosis in human cancer cells. More importantly, the implication of various TNF signaling pathways including NF-κB and JNK in the sensitization process was also examined. Findings from the present study provide novel insights into the molecular mechanisms of PN and suggest the potential value of PN as an anticancer agent.

**Abbreviations:** DAPI, 4′,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; IκB, NF-κB inhibitory protein; IKK, IκB kinases; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; PMSF, phenylmethylsulfonyl fluoride; PN, parthenolide; RIP, receptor-interacting protein; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; TUNEL, TdT-mediated dUTP nick end labeling.

The first two authors contributed equally to this work.

## Materials and methods

*Chemicals, reagents and plasmids*

All common chemicals and reagents including PN were purchased from Sigma (St Louis, MO) unless stated otherwise. Bay 11-7085 (a specific NF-κB inhibitor) and SP600125 (a specific JNK inhibitor) were from Biomol (Plymouth Meeting, PA) and Calbiochem (San Diego, CA), respectively. 4′,6-Diamidino-2-phenylindole (DAPI) was from Molecular Probes (Eugene, OR). T4 polynucleotide kinase and Lipofectamine transfection reagent were from Invitrogen (Carlsbad, CA). Anti-NF-κB (p65), anti-IκBα, anti-TRAF2, anti-JNK1 and anti-IKKα antibody were from Santa Cruz (Santa Cruz, CA). Recombinant human TNF-α, anti-human TNFR1 antibody and anti-IKKβ antibody were from R&D systems (Minneapolis, MN). Anti-receptor interacting protein (RIP) antibody was from BD Transduction Laboratories (Los Angeles, CA). Anti-phospho-cJun(ser63) was from Cell Signaling (Beverly, MA). The secondary antibodies (horseradish peroxidase conjugated goat anti-mouse IgG and rabbit anti-goat IgG) and the enhanced chemiluminescence substrate were from Pierce (Rockford, IL). NF-κB and SP-1 consensus oligonucleotides, TransFast$^{TM}$ transfection reagent and luciferase assay kit were all from Promega (Madison, WI). TdT-mediated dUTP nick end labeling (TUNEL) assay kit was from Roche (Mannheim, Germany). The Mercury Pathway Profiling System (containing the pNF-κB-luc, pAP-1-luc and the control vector pTAL-luc) and pDsRed expression vector were obtained from Clontech (Palo Alto, CA). The dominant-negative JNK1 and JNK2 vectors (DN-JNK1, DN-JNK2) were kindly provided by Dr A.G.Porter (25). The CrmA expression vector was kindly provided by Dr Z.G.Liu (NIH).

*Cell culture and treatments*

The human nasopharyngeal carcinoma cell line CNE1 was obtained from Sun Yat-sen University (Guangzhou, China) and human colorectal cancer cell line COLO205, human cervical cancer cell line Hela and human breast cancer cell line MDA-MB-231 were all from American Type Culture Collection (ATCC, Manassas, VA). CNE1 and COLO205 cells were cultured in RPMI-1640 medium. Hela and MDA-MB-231 cells were cultured in DMEM medium. The stock solution of PN (100 mM) was prepared in DMSO and the control group was always balanced with the same concentration of DMSO. In the pre-treatment study, cells were pre-treated with various concentration of PN (5–25 μM) for up to 4 h prior to TNF-α exposure (25 ng/ml). Detailed treatment procedures were described in figure legends.

*Cell viability test and detection of apoptosis*

The general cytotoxicity of PN and TNF-α on cultured CNE1 cells were detected by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) test as described before (26) and the results were presented as the relative cell viability compared with the control group. In the present study, apoptosis was determined by the following assays: (i) nuclear condensations of apoptotic cells stained by DAPI and counted under an inverted fluorescent microscope (Nikon ECLIPSE TE2000-S) and (ii) TUNEL assay coupled with flow cytometry and fluorescent microscopy to evaluate characteristic DNA fragmentation occurred in apoptotic cell death.

*Preparation of cytosolic and nuclear extracts*

Both the nuclear and cytosolic protein extracts were prepared according to published methods with modifications (27,28). After designated treatments, all cells (both detached and attached) were collected and washed with cold PBS twice. Cells (~3–4 × $10^6$) were then re-suspended in ice-cold Buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin and 1 μg/ml aprotinin]. After incubation on ice for 15 min, Nonidet P-40 (final concentration 0.3%) was added to the cell suspension and mixed gently. The cytosolic extracts were collected after cells were centrifuged at 2000 *g* for 10 min at 4°C. The nuclear pellets were then re-suspended in Buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin), and incubated on ice for 30 min with gentle votex once every 5 min. The nuclear extracts were collected after centrifugation (20 000 *g* for 15 min, 4°C). Protein concentration was quantified using a Bio-Rad protein assay kit.

*Electrophoretic mobility shift assay (EMSA)*

The DNA binding activity of the nuclear protein was tested according to established method with modifications (17). NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and SP-1 consensus oligonucleotides (5′-ATTCGATCGGGGCGGGGCGAGC-3′) were labeled using T4 kinase and purified through a G50 column. Equal amounts of nuclear protein (5 μg) were incubated with 100 000 c.p.m. labeled NF-κB oligonucleotides in 5× reaction buffer (100 mM HEPES/KOH, pH 7.9, 20% glycerol, 1 mM DTT and 300 mM KCl) for 30 min at room temperature, in the presence of 2 μg poly(dI–dC) and 2 μg BSA in a total volume of 20 μl. The DNA–protein complexes were resolved on a 5% polyacrylamide gel using a vertical gel electrophoresis apparatus (Gibco BRL Model v16-2) at 150 V for 1.5 h. Gels were then dried and exposed to X-ray film at −80°C overnight.

*Transient transfections and luciferase reporter gene assay*

The transient transfection of pNF-κB-luc, pAP-1-luc, pTAL-luc and expression vectors (DN-JNK1, DN-JNK2, CrmA, pDsRed) were performed in CNE1 and Hela cells using TransFast$^{TM}$ Transfection Reagent (Promega) or Lipofectamine reagent according to the manufacturers' protocols. The luciferase activity was measured in the cellular extracts using a luciferase assay kit (Promega) based on the protocol provided by the manufacturer. Briefly, following the treatment, the cell lysate was collected from each well after the addition of 1× cell lysis reagent (50 μl/well in 24-well plate). The relative light units were then determined in a luminometer (Lumi-One, Trans Orchid, Tampa, FL) for a total period of 15 s after a 5 s delay time.

*IKK and JNK in vitro kinase assay*

Cells were lysed with M2 cell lysis buffer (20 mM Tris pH 7.0, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate and proteinase inhibitor cocktail). Immunoprecipitation was performed with anti-IKKγ antibody and anti-JNK antibody, respectively. After extensive wash, the harvested protein A–Sepharose beads (Roche) were used for the kinase assay in a complete kinase buffer (20 mM HEPES pH 7.5, 20 mM β-glycerol phosphate, 10 mM $MgCl_2$, 1 mM DTT, 20 μM ATP and 5 μCi [γ-$^{32}$P]ATP) with the presence of various substrates (GST–IκB-α for IKK, GST-c-Jun for JNK). After a 30-min incubation at 30°C, the reaction was stopped by addition of SDS sample buffer. The samples were then separated on a 4–20% gradient SDS–PAGE and visualized by autoradiography.

*Co-immunoprecipitation and western blotting*

Whole cell lysate was prepared with co-immunoprecipitation lysis buffer (50 mM HEPES pH 7.6, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 0.5 mM PMSF and the proteinase inhibitor cocktail). After protein quantification, equal amounts of protein were incubated with the relevant antibodies and protein A–Sepharose beads at 4°C overnight. The beads were extensively washed with co-immunoprecipitation lysis buffer four times. After boiling in SDS-sample buffer, samples were then separated on SDS–PAGE and transferred onto a Hybond-C nitrocellulose membrane (Amersham). The membrane was blocked with 5% non-fat milk in TBST (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) and incubated with various primary antibodies for 2 h at room temperature or overnight at 4°C. After the membrane was exposed to respective secondary antibodies for 1 h, the blots were detected using the enhanced chemiluminescence method (Pierce).

*Statistics*

All numerical data were presented as means ± standard deviation (SD) from at least three independent experiments and analyzed using the Student's *t*-test. A $P$ value $<0.05$ is considered statistically significant.

## Results

### *PN sensitizes cancer cells to TNF-α-mediated apoptosis*

It has been reported that PN treatment increases the sensitivity of human breast cancer cells to a chemotherapeutical drug paclitaxel, probably via its inhibitory effect on constitutively activated NF-κB (29). Here we examined the sensitization of PN on TNF-α-mediated apoptosis in four different human cancer cell lines: human nasopharyngeal carcinoma cell line CNE1, human colorectal cancer cell line COLO205, human cervical cancer cell line Hela and human breast cancer cell line MDA-MB-231. Based on the preliminary data, a non-cytotoxic concentration of PN was used. Apoptotic cell death was examined by: (i) evident nuclear condensation of apoptotic cells using an inverted fluorescence microscope after DAPI staining and (ii) DNA fragmentation determined by TUNEL assay coupled with flow cytometry. As shown in Figure 1, PN (25 μM) or TNF-α (25 ng/ml) treatment alone for 24 h did not cause any significant reduction of cell viability (Figure 1A) and morphological changes (Figure 1B) in CNE1 cells. However, pre-treatment with PN (25 μM × 4 h) greatly

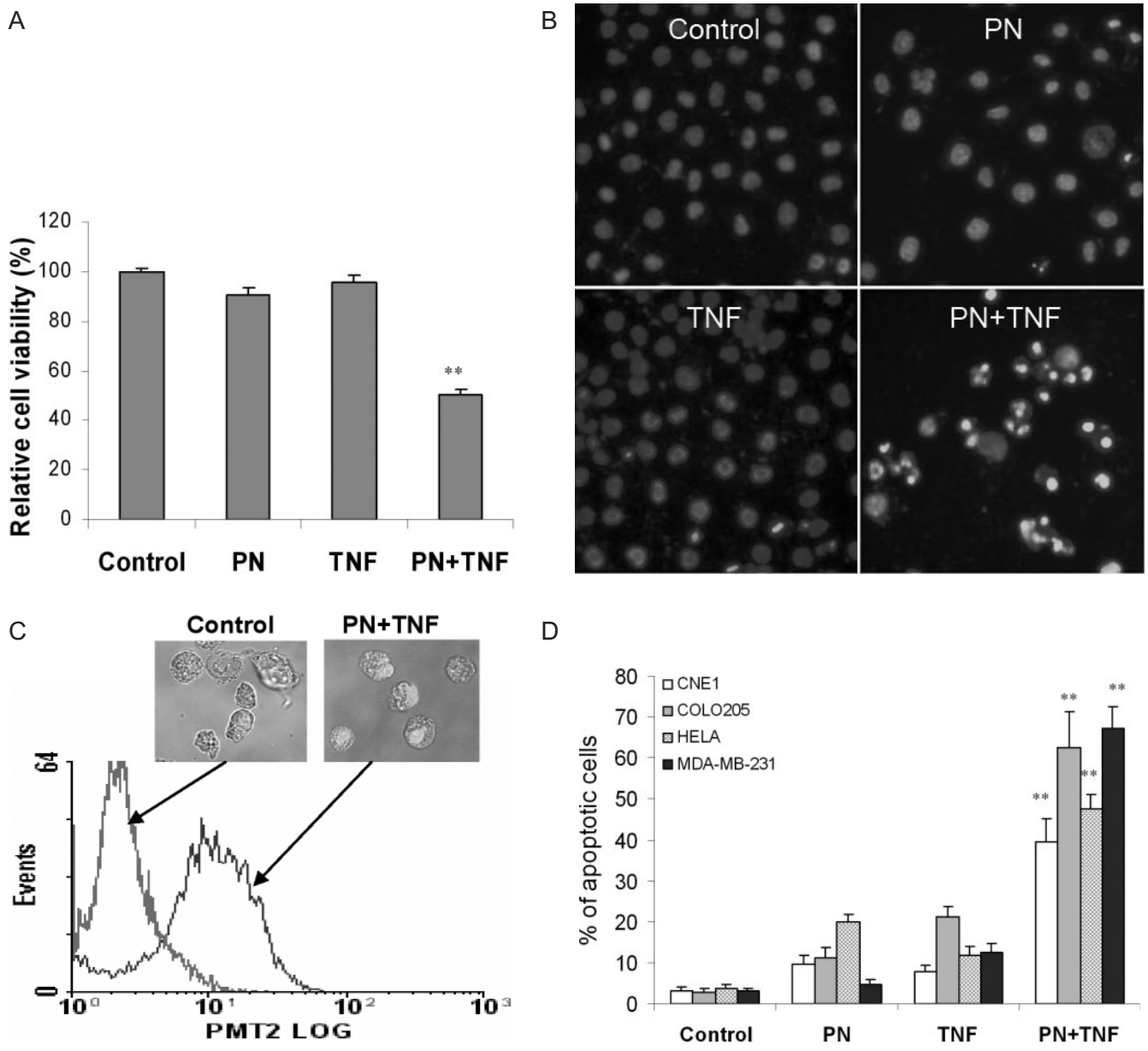


**Fig. 1.** PN sensitizes cancer cells to TNF-α-mediated apoptosis. (**A**) Changes of cell viability detected by MTT test. CNE1 cells were treated with PN (25 μM × 28 h), TNF-α (25 ng/ml × 24 h) individually or treated with PN (25 μM × 4 h) prior to TNF-α exposure (25 ng/ml × 24 h). The changes of relative cell viability were calculated by comparing with the control group (100%). Data were presented as means ± SD ($n = 3$). $^{**}P < 0.01$ compared with control group (Student's *t*-test). (**B**) Nuclear condensation of apoptotic cells detected by DAPI staining. CNE1 cells were treated as described in (A). Cells were stained with DAPI (300 nM) for 5 min then checked under an inverted fluorescence microscope. (**C**) Apoptotic cell death detected by TUNEL assay. CNE1 cells were treated as in (A). Apoptotic cells were identified by increased FITC fluorescence intensity in the histogram from flow cytometry, as well as by green-colored nuclei under a fluorescent microscope. (**D**) Quantification of apoptotic cell death measured by DAPI staining in human cancer cell lines. Human nasopharyngeal cancer cell line CNE1, human colorectal cancer cell line COLO205, human cervical cancer cell line Hela and human breast cancer cell line MDA-MB-231 were treated with PN (25, 10, 20 and 20 μM, respectively) for 4 h prior to TNF-α (25, 10, 25 and 25 ng/ml, respectively) for 24 h. Ten randomly selected fields for a total of 200 cells were examined and the cells with evident nuclear condensation were considered as apoptotic cells. Data were presented as means ± SD ($n = 3$). $^{**}P < 0.01$ compared with the group treated with TNF-α only (Student's *t*-test). See online supplementary material for a color version of this figure.

enhanced TNF-α-induced cell death. The TUNEL assay was used to evaluate the characteristic DNA fragmentation and those apoptotic cells were marked with higher FITC fluorescent intensity in flow cytometry and with green-colored nuclei under a fluorescent microscope (Figure 1C). Furthermore, a similar sensitization effect of PN pre-treatment was also observed in the other three cancer cells (Figure 1D); that apoptosis was quantified by nuclear condensation counting after DAPI staining, suggesting that the sensitization effect on TNF-α-induced apoptosis is not cell type specific.

### *PN inhibits NF-κB activation*

As NF-κB is known to be the principle anti-apoptotic factor in TNF signaling and a confirmed molecular target for PN (17), we next examined the effects of PN on NF-κB activation induced by TNF-α. As demonstrated in Figure 2A, PN pre-treatment dose-dependently inhibited NF-κB luciferase activity induced by TNF-α in CNE1 cells. At 25 μM, PN pre-treatment almost completely abolished TNF-α-induced NF-κB transactivation. In contrast, no obvious effect was observed with PN post-treatment (25 μM, 1 h before harvest)

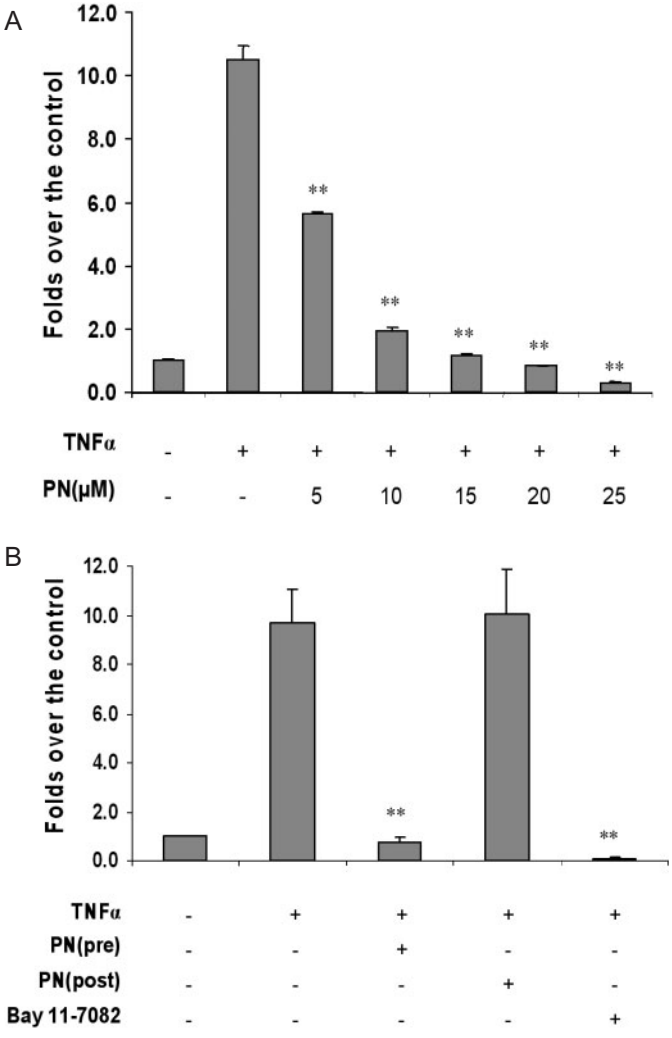


**Fig. 2.** PN inhibits transcriptional activity of NF-κB determined by luciferase reporter gene assay. (**A**) Dose-dependent inhibition of NF-κB transcription activity by PN. CNE1 cells were first transiently transfected with pNF-κB-luc vector for 24 h, followed by pre-treatment with different concentration of PN for 4 h and TNF-α exposure (25 ng/ml × 24 h). (**B**) Comparison with a known NF-κB inhibitor (Bay 11-7082, 5 μM × 1 h). The post-treatment of PN was done by adding PN 1 h before cell collection. Data were presented as the folds of changes over the non-treatment control group after being normalized with β-gal activity (means ± SD, $n = 3$). $^{**}P < 0.01$ compared with the group treated with TNF-α only (Student's *t*-test).

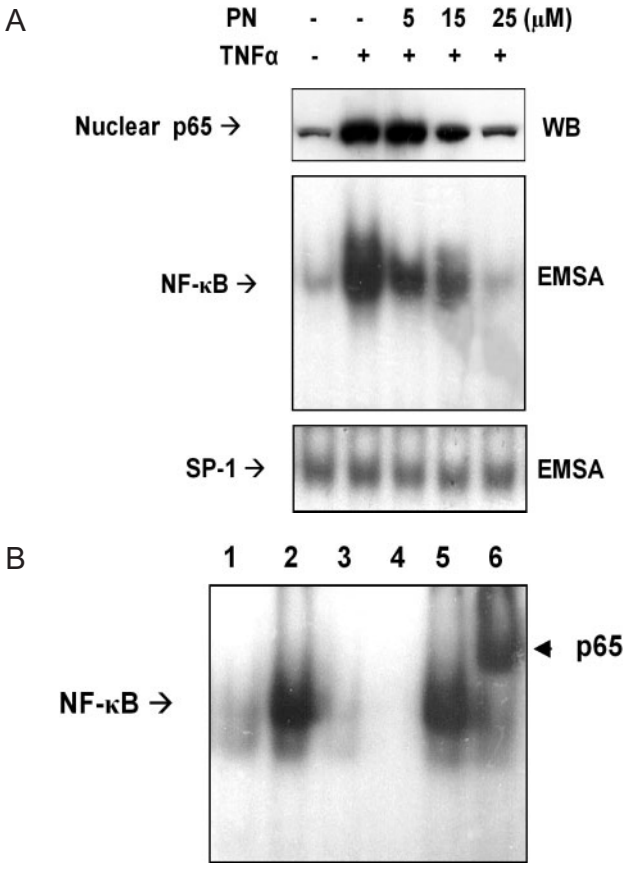


**Fig. 3.** PN inhibits p65 nuclear translocation and DNA binding. (**A**) CNE1 cells were treated with PN (25 μM) up to 4 h before TNF-α treatment (25 ng/ml × 30 min). At the end of treatment, cells were collected and both the nuclear and cytosolic extracts were prepared. Nuclear p65 translocation and NF-κB–DNA binding were then detected by western blot for p65 and EMSA using NF-κB probe, respectively. (**B**) Competition and super-shift assay: lane 1, control cells; lane 2, TNF-α (25 ng/ml × 30 min); lane 3, PN only (25 μM × 4.5 h); lane 4, nuclear protein as in lane 2 incubated with 50-fold excess amount of unlabeled cold NF-κB probe; lane 5, nuclear protein as in lane 2 incubated with 50-fold excess amount of unlabeled cold SP-1 probe; and lane 6, super-shift with anti-p65 antibody. The preparation of nuclear extract and EMSA were carried out as described in detail in the Materials and methods.

(Figure 2B), thus excluding the possibility that the reduction of luciferase activity is due to the direct inhibitory effect of PN on luciferase enzyme activity. Bay 11-7082, a known NF-κB specific inhibitor (30) was also highly effective for the inhibition of NF-κB transcriptional activity (Figure 2B).

We next examined the effects of PN on p65 nuclear translocation and DNA binding activity. As shown in Figure 3A, PN dose-dependently inhibited the p65 nuclear translocation (upper panel, western blot) as well as its DNA binding capability (middle panel, EMSA), while another nuclear transcription factor SP-1 was spared (low panel, EMSA). The specificity of the EMSA used in our test was confirmed by the complete inhibition of NF-κB DNA binding by excess amount of unlabeled NF-κB cold probe (Figure 3B, lane 4), while a similar amount of non-specific cold probe (SP-1) failed to affect the binding activity (Figure 3B, lane 5). Moreover, the majority of NF-κB is found to be p65 as shown by the super-shift assay (Figure 3B, lane 6).

The inhibitory effect of PN pre-treatment on TNF-α-induced IκBα protein degradation and IKK kinase activation were studied. As shown in Figure 4A, PN pre-treatment completely prevented the IκBα degradation induced by TNF-α. Meanwhile, the TNF-α-induced IKK kinase activity was also greatly suppressed by PN pre-treatment (Figure 4B).

### *PN prevents recruitment of the IKK complex to TNFR1*

It has been reported that PN is a specific IKK inhibitor and one of the mechanisms is the direct binding of PN to IKK proteins (19). As the recruitment of the IKK complex to TNFR1 is an indispensable step in TNF-α-induced NF-κB activation (31,32), here we attempted to assess the possible effect of PN on recruitment of the IKK complex to TNFR1, using endogenous co-immunoprecipitation experiments. After cells were treated with TNF-α for 5 min, as shown in Figure 5A, a quick recruitment of both IKKα and IKKβ to TNFR1 was detected. Pre-treatment of PN significantly suppressed the recruitments

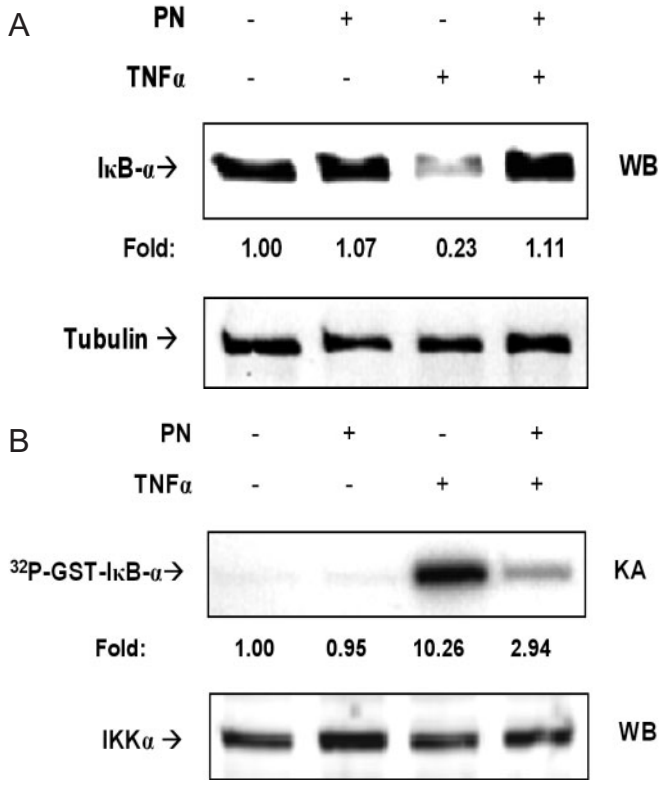


**Fig. 4.** PN inhibits TNF-α-induced IKK activation and IκB degradation. CNE1 cells with or without PN (25 μM × 4 h) pre-treatment were treated with TNF-α (25 ng/ml) for 30 min and lysed with M2 lysis buffer. (**A**) Western blot was performed for detecting the degradation of IκB-α proteins. (**B**) The same cell lysate from (A) was also subject to IKK *in vitro* kinase assay by using GST−IκB-α substrate and [γ-$^{32}$P]ATP (upper panel). Equal amount of the IKKα protein expression was detected by IKKα western blot (lower panel).

of both IKKα and IKKβ to TNFR1, suggesting that PN is capable of inhibiting NF-κB activation via disrupting the recruitment of the IKK complex to TNFR1.

As the recruitment of IKKα and IKKβ requires some intermediate adaptor proteins such as RIP and anti-TNF-α receptor associated factor 2 (TRAF2), we next studied whether PN also interferes with the interaction of those proteins with the IKK complex. As shown in Figure 5A, the recruitment of RIP to TNFR1 was blocked by PN pre-treatment. When co-immunoprecipitation experiments were performed with the TRAF2 antibody, similar trends were observed: TNF-α induces a rapid association of the IKK complex with TRAF2, which can be substantially prevented by pre-treating cells with PN (Figure 5B). As both RIP and TRAF2 are important mediators in IKK phosphorylation and NK-κB activation (31−33), these results provide evidence that PN acts on upstream signaling molecules controlling IKK activation.

*Pre-treatment of PN leads to a sustained JNK activation in TNF-α-treated cells*

One of the important components in TNFR1 signaling is JNK activation (34). Here we examined the effect of PN pre-treatment on TNF-α-induced JNK activation. As shown in Figure 6A, JNK kinase activity increased rapidly and transiently in response to TNF-α treatment; reached the peak level at 15 min and returned to its basal level at 60 min. In contrast, in cells pre-treated with PN (25 μM), a sustained and prolonged JNK activation was observed (Figure 6B), which is consistent with previous observations that inhibition of NF-κB leads to prolonged JNK activation (8,12).

*Sustained JNK activation plays an important role in the sensitization effect of PN to TNF-α-mediated apoptosis*

It has been reported that sustained JNK activation contributes to TNF-α-induced apoptosis (13). Here we further examined whether the prolonged JNK activation is involved in the sensitization effect of PN to TNF-α-mediated apoptosis. We used both pharmacological and genetic approaches to block JNK activation. We first used SP600125, a specific JNK chemical inhibitor (35), and found that it effectively suppressed TNF-α-mediated JNK activation (Figure 7A) and significantly subdued apoptotic cell death in cells treated with PN and TNF-α (Figure 7B). Next we transiently transfected CNE1 cells with dominant-negative JNK1 and JNK2 vectors (DN-JNK1 and DN-JNK2) together with pDsRed fluorescence protein expression vector as a transfection marker. The expression vector for CrmA, a known specific caspase 8 inhibitor (36) was also used as a positive control. In our system, the effectiveness of DN-JNK1 and DN-JNK2 was confirmed by significantly reduced c-Jun phosphorylation induced by TNF-α (Figure 8A). As shown in Figure 8B, the successfully transfected cells were marked by the strong red fluorescence. Following combined treatments with PN and TNF-α, most of the cells transfected with pcDNA died. Over-expression of CrmA offered a significant protection against apoptotic cell death induced by PN and TNF-α, suggesting that such apoptosis is mediated by caspase 8 activation. More importantly, ectopic expression of DN-JNK1 and DN-JNK2 markedly reduced the extent of cell death caused by PN and TNF-α. Figure 8C provided a quantitative analysis, which was performed by counting the percentage of cell death among those transfected cells in 10 randomly selected fields.

## Discussion

In the present study we attempted to further address the anticancer potential of PN by assessing the sensitization effect of PN on TNF-α-mediated apoptotic cell death and the molecular mechanisms involved. Here we provided convincing evidence demonstrating the strong inhibitory activity of PN on TNF-α-induced NF-κB activation (Figures 2−4). These findings are generally consistent with previous studies showing that PN is a potent NF-κB inhibitor in a number of cells including Jurkat cells, Hela cells and L929 fibroblasts stimulated with TNF-α, phorbol 12-myristate 13-acetate, $H_2O_2$ or CD3/CD28 ligation (15,17,27). The main explanation for the inhibitory effect of PN on NF-κB is based on the observation that PN targets the IKK complex (17,18). The IKK complex consists of IKKα, IKKβ and IKKγ, which are the upstream kinases of IκB proteins and the point of convergence for most NF-κB activating stimuli (37,38). Recently, it was reported that the recruitment of the IKK complex to TNFR1 is an indispensable step in TNF-α-induced NF-κB activation (31,32). In the present study, by using endogenous co-immunoprecipitation experiments, we for the first time demonstrated a new mechanism for the inhibitory activity of PN on the NF-κB signaling pathway: PN disrupts the recruitment of the IKK complex to TNFR1 (Figure 5A). Furthermore, the recruitment of IKKα and IKKβ to TRAF2, an important adaptor protein in the TNFR1 signaling complex, was also inhibited by PN. Although PN is known

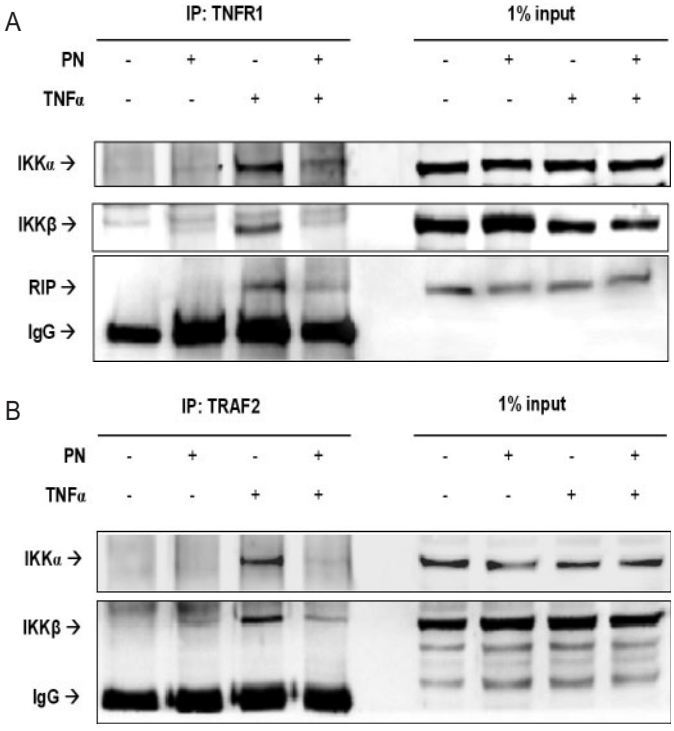


**Fig. 5.** PN interrupts the recruitment of IKKs to TNFR1 and TRAF2. (**A**) Endogenous co-immunoprecipitation experiments were performed with cell extracts prepared from CNE1 cells treated with or without TNF-α (25 ng/ml) for 5 min. In some groups, cells were pre-treated with PN (25 μM) for 4 h before TNF-α treatment. Cell extracts were immunoprecipitated with anti-TNFR1 antibody overnight. The immunoprecipitants were resolved by SDS−PAGE and probed with anti-RIP, anti-IKKα and anti-IKKβ antibodies sequentially. Cell extracts (1%) were used as protein input control. (**B**) CNE1 cells were treated as in (A) and followed by co-immunoprecipitation with anti-TRAF2 antibody.

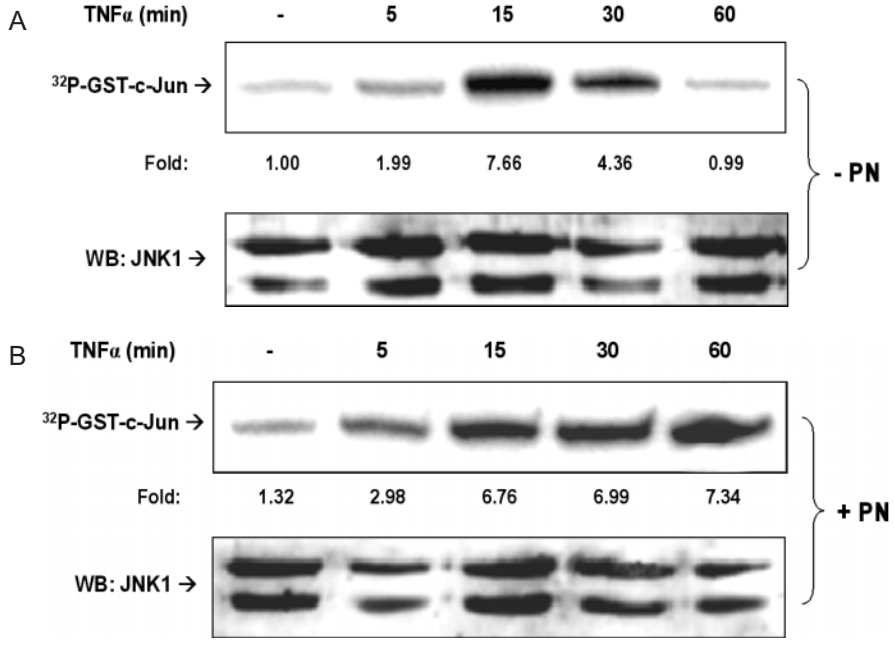


**Fig. 6.** PN pre-treatment induces a sustained JNK activation after TNF-α stimulation. (**A**) TNF-α-induced JNK activation in CNE1 cells without PN pre-treatment. Cells were treated with TNF-α (25 ng/ml) for different times then lysed with M2 buffer and subjected to JNK kinase assay as described in the Materials and methods. (**B**) TNF-α-induced JNK activation in CNE1 cells pre-treated with PN (25 μM × 4 h). Cells were pre-treated with PN before the TNF-α (25 ng/ml) exposure. The same JNK kinase assay was performed as in (A).

to be a specific IKK inhibitor (17), the exact mechanisms responsible for such inhibition have not been fully understood. One possibility is that PN may bind to and directly modify p65 or IKK proteins (18,19). Therefore, the decreased recruitment of IKKα and IKKβ to TNFR1 could be the result of the structural modification of IKK proteins. On the other hand, we also found that PN treatment blocked the interaction of RIP with TNFR1. As RIP plays an essential role in bringing MEK

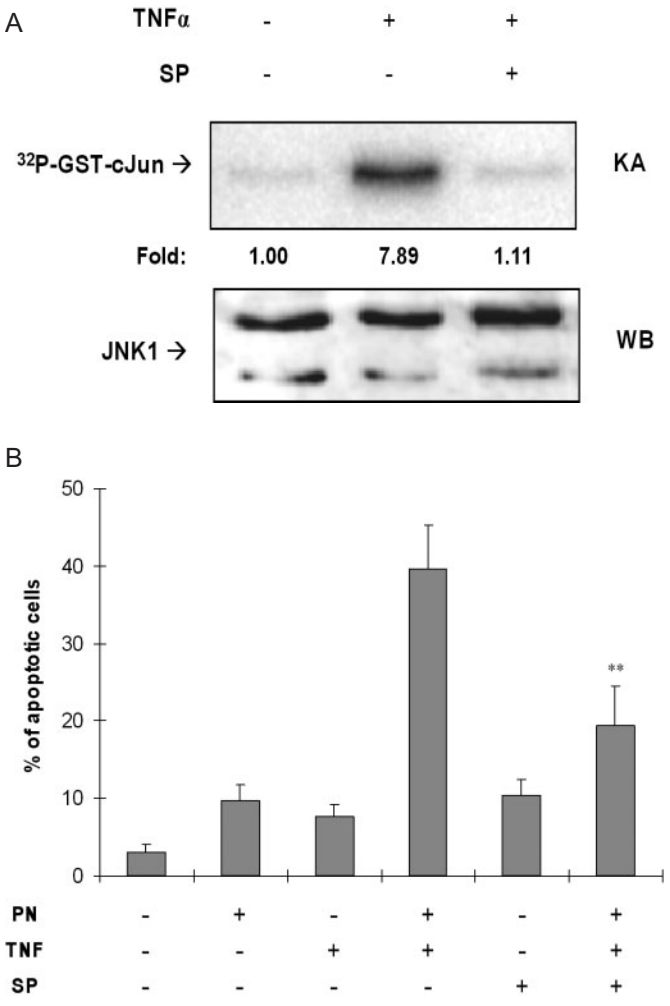


**Fig. 7.** JNK inhibitor SP600125 prevents the sensitization effect of PN to TNF-α-mediated apoptosis. (**A**) SP600125 effectively blocks TNF-α-induced JNK activation detected by JNK kinase assay. CNE1 cells were treated with SP600125 (20 μM) 30 min prior to TNF-α exposure (25 ng/ml × 15 min). (**B**) SP600125 suppresses the apoptotic cell death induced by combined treatment of TNF-α and PN pre-treatment. CNE1 cells were pre-treated with SP600125 (20 μM) for 30 min followed by PN pre-treatment (25 μM × 4 h) and then TNF-α (25 ng/ml) stimulation for another 24 h. At the end of experiments, the cells were stained by DAPI and checked under UV fluorescence microscope. Ten randomly selected fields were examined. Percentage of apoptotic cell death was evaluated by calculating the cells with nuclear condensation. Data were presented as means ± SD ($n = 3$). $^{**}P < 0.01$ compared with the group treated with TNF-α only (Student's *t*-test).

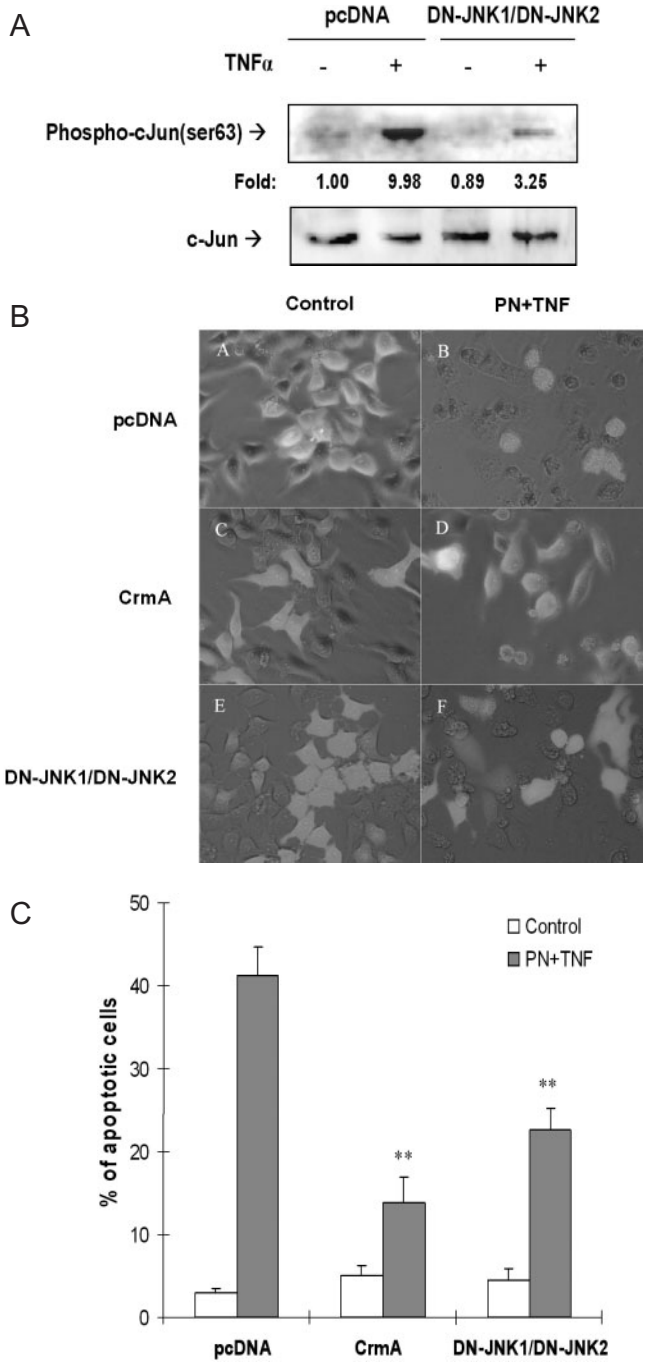


**Fig. 8.** Over-expression of DN-JNK1, DN-JNK2 suppresses PN's sensitization effects to TNF-α-mediated apoptosis. (**A**) Ectopic expression of DN-JNK1 and DN-JNK2 effectively blocks TNF-α-mediated JNK activation. CNE1 cells were transiently co-transfected with DN-JNK1 and DN-JNK2 expression vectors. The cells transfected with pcDNA3.1 vectors were used as a negative control. Cells were treated with TNF-α (25 ng/ml) for 15 min then subjected to western blot for phosphorylation of c-Jun. (**B**) Over-expression of DN-JNK1, DN-JNK2 as well as CrmA suppresses cell death induced by combined treatment with PN and TNF-α. pDsRed vector was used as a transfection marker. Forty-eight hours after transfection, cells were treated with PN (25 μM × 4 h) followed by TNF-α (25 ng/ml) stimulation for another 24 h. The morphological changes of apoptotic cell death were examined under an inverted fluorescence microscope. (**C**) Quantification of apoptotic cell death in cells with various transient transfections. Cells were treated as in (**B**). Apoptotic cell death was counted in 10 randomly selected fields under an inverted fluorescent microscope after DAPI staining for a total of 200 successfully transfected cells. $^{**}P < 0.01$ compared with the pcDNA transfected group (Student's *t*-test). See online supplementary material for a color version of this figure.

kinase 3 close to IKK and activates the latter (33), it is thus believed that PN may also inhibit the NF-κB signaling pathway by targeting other key signaling molecules such as TNFR1 and RIP. It has been well established that NF-κB generally acts as a potent anti-apoptosis regulator by the transactivation of an array of anti-apoptosis genes such as Bcl-xL, c-IAP and XIAP (3,4). As a result, inhibition of NF-κB is an effective strategy in sensitizing human cancer cells to apoptosis (39). Data from this study also support an earlier report (29) that PN enhances breast cancer cells to paclitaxel toxicity by blocking the constitutively activated NF-κB in those cancer cells, indicating the chemopreventive and chemotherapeutical value of PN.

One of the important components in TNF-α signaling via TNFR1 is JNK activation. The involvement of JNK in various

forms of cell death has been documented extensively (6). It is generally believed that JNK is required for apoptotic cell death elicited by environmental stress stimuli such as oxidative stress and UV. For instance, JNK1−/− and JNK2−/− null cells are resistant to UV-induced apoptosis (40). Although the exact role of JNK in TNF-α-mediated apoptosis is still controversial, recent evidence tends to support the hypothesis that JNK plays a pro-apoptotic role especially when the anti-apoptotic NF-κB signaling pathway is inhibited (11). In this study, PN was found to block TNF-α-mediated NF-κB signaling pathway, which prompted us to postulate that JNK is an important contributing factor in the sensitization effect of PN on TNF-α-mediated apoptosis. The duration of JNK activation appears to be a critical factor determining the pro- or anti-apoptotic role of JNK after initiation of apoptosis (41,42). Here we first noticed that PN markedly prolonged the duration of JNK activation in TNF-α-exposed cells. Next, we found the sensitization effect of PN was significantly diminished by a specific JNK inhibitor SP600125 (Figure 7) or by ectopic expression of two dominant-negative JNKs (DN-JNK1 and DN-JNK2) (Figure 8). Meanwhile, the cells become more sensitive once transfected with a constitutively active JNKK2−JNK1 fusion protein, which induces a sustained activation of JNK (data not shown). Taken together, data from this study point out that sustained JNK activation contributes to the sensitization effect of PN to TNF-α-mediated apoptosis.

It is known that the TRAF2 is indispensable in TNF-α-induced JNK activation (43). And a recent study reported that the RIP is also required for TNF-α-mediated JNK activation (44). In this study, PN disrupted the recruitment of RIP to TNFR1, while the effect of PN on the interaction between TRAF2 and TNFR1 is not known due to lack of a suitable TRAF2 antibody. It appears to be contradictory that PN interferes with RIP and TNFR1 interaction, while it enhances TNF-α-elicited JNK activation. There are two possible explanations for such discrepancy: First, the suppressive effect of PN on TNF-α-induced NF-κB activation removes the blockage of JNK activation imposed by NF-κB. Recent studies have provided unambiguous evidence that absence of NF-κB activation leads to sustained or prolonged JNK activation in TNF-α-treated cells (8,11). Secondly, PN itself may also activate or enhance the JNK activation through a TNF receptor independent pathway, based on the observation that PN alone induces a certain degree of JNK activation (Figure 6B). However, the exact mechanism for the observed JNK augmentation by PN remains to be further elucidated.

Another important question that remains to be answered is how sustained JNK activation contributes to the cell death process elicited by PN and TNF-α. In UV-induced apoptosis, JNK activation modulates the mitochondria-dependent cell death pathway (40). In our study, we noticed that over-expression of a caspase 8 inhibitor protein, CrmA, markedly blocked the sensitization effects conferred by PN pre-treatment to TNF-α. Such a finding is consistent with a recent report that TNF-α-activated JNK induces cleavage of Bid, which then causes preferential release of Smac/DIABLO to disrupt the inhibitory complex TRAF2−cIAP1 on caspase 8 activation (10). Nevertheless, the underlying mechanism of JNK's pro-apoptotic role in PN sensitization effect remains to be further defined.

In summary, here we have highlighted a novel function of PN: sensitizes human cancer cells to TNF-α-induced apoptosis via reduced NF-κB and sustained JNK activation. Although the clinical application of TNF-α is rather limited as most of the cancer cells are resistant to TNF-α treatment alone, some recent studies have re-examined the possible application of recombinant TNF-α or TNF-α expression vectors *in vitro* as well as in clinical trials (45,46). Therefore, the strong sensitization effect of PN to TNF-α-mediated apoptosis in human cancer cells suggests the usefulness of PN as a chemopreventive and chemotherapeutic agent against cancer.

## Supplementary material

Supplementary material can be found at: http://www.carcin.oupjournals.org/.

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