A Novel Pentamethoxyflavone Downregulates Tumor Cell Survival, Proliferative, and Angiogenic Gene Products Through Inhibition of Activation of IκB Kinase, and Sensitizes Tumor Cells to Apoptosis by Cytokines and Chemotherapeutic Agents

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Running title: PMF potentiates apoptosis via inhibition of NF-κB pathway

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Abbreviations: COX-2, cyclooxygenase-2; IKK, IκB kinase; MMP-9, matrix metallopeptidase-9; NF-κB, nuclear factor-kappaB; NIK, NF-κB-inducing kinase; PMF, pentamethoxyflavone; TAB1, TAK1-binding protein; TAK1, transforming growth factor (TGF)-β-activated kinase-1; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated death domain; TRAF2, TNFR-associated factor-2; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein

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Abstract

Most anticancer drugs have their origin in traditional medicinal plants. We describe here a flavone from the leaves of a Thai plant, *Gardenia obtusifolia,* 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone (PMF), that has anti-inflammatory and anticancer potential. Because the nuclear factor-kappaB (NF-κB) pathway is linked to inflammation and tumorigenesis, we investigated the effect of PMF on this pathway. We found that PMF suppressed NF-κB activation induced by inflammatory agents, tumor promoters, and carcinogens. This suppression was not specific to the cell type. Although PMF did not directly modify the ability of NF-κB proteins to bind to DNA, it inhibited IκBα (inhibitory subunit of NF-κB) kinase, leading to suppression of phosphorylation and degradation of IκBα, suppressed consequent p65 nuclear translocation, thus abrogating NF-κB-dependent reporter gene expression. Suppression of the NF-κB cell signaling pathway by the flavone led to the inhibition of expression of NF-κB-regulated gene products, that mediate inflammation (cyclooxygenase-2), survival (XIAP, survivin, Bcl-xL, and cFLIP), proliferation (cyclinD1), invasion (matrix metalloproteinase-9) and angiogenesis (vascular endothelial growth factor). Suppression of antiapoptotic gene products by PMF correlated with the enhancement of apoptosis induced by tumor necrosis factor (TNF)-α and the chemotherapeutic agents cisplatin, paclitaxel, and 5-flurouracil. Overall, our results indicate that PMF suppresses the activation of NF-κB and NF-κB-regulated gene expression, leading to the enhancement of apoptosis. This is the first report to demonstrate that this novel flavone has anti-inflammatory and anti-cancer effects by targeting the IKK complex.

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Introduction

More than 80% of the world's population cannot afford modern medicine. Thus new treatments that are safe, inexpensive, and effective are needed. One source of candidates for the new pharmacopeia is a long list of traditional medicines. Unfortunately, in most cases neither the chemical entity, that is the basis for the treatment's efficacy, nor the molecular mechanism of action is well defined.

In the study reported here, we set out to establish these aspects of *Gardenia obtusifolia* Roxb. ex Kurz or Khammok noi, a medicinal plant that is used as an antiulcer (Takase et al., 1989), antibacterial (Laurens et al., 1985), analgesic, antidiuretic, and hypotensive agent (Hussain, 1991). A pentamethoxyflavone (PMF) isolated from this plant has been shown to exhibit activity against human immunodeficiency virus (HIV) and against various mammalian tumor cell lines (Tuchinda et al., 2002). Similar flavones isolated from other medicinal plants have exhibited antiproliferative activity against various tumor cell lines in vitro including non-small cell lung cancer, ovarian cancer, colon cancer, renal cancer, melanoma, and leukemia cell lines (Lichius et al., 1994; Shi et al., 1995). The exact mechanism by which this PMF exhibits antiproliferative and anti-HIV activity is not understood. Because of the critical role of NF-κB in tumorigenesis and HIV replication, we hypothesize that this pathway plays a major role in the action of this flavone.

NF-κB represents a group of five proteins, c-Rel, RelA (p65), RelB, NF-κB1 (p50 and p105), and NF-κB2 (Ghosh et al., 1998). In an inactive state, NF-κB is sequestered in the cytoplasm as a heterotrimer consisting of p50, p65, and IκB subunits. Upon activation, IκBα undergoes phosphorylation and ubiquitination-dependent degradation leading to p65 nuclear translocation and binding to a specific consensus sequence in the DNA, which results in gene

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transcription. Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, lipopolysaccharide (LPS), okadaic acid (OA), and TNF-α, have been shown to activate NF-κB. NF-κB has been shown to regulate the expression of several genes whose products are involved in tumorigenesis. These include antiapoptotic (XIAP, survivin, Bcl-xL, Bcl-2, cFLIP), proliferative (cyclin D1), proinflammatory (COX-2), invasion (matrix metalloproteinase 9, MMP-9), and angiogenic (VEGF) genes (Aggarwal, 2004).

To test the hypothesis of the involvement of the NF-κB pathway in the antiproliferative action of PMF, we studied the effect of PMF on the NF-κB pathway and measured the anticellular and chemosensitizing effects of PMF and their relationship to the NF-κB pathway. We found that PMF inhibited the activation of NF-κB through inhibition of IκBα kinase, and subsequently of IκBα phosphorylation and degradation and p65 nuclear translocation. The suppression of NF-κB by this flavone led to the down-regulation of gene products that promote survival, proliferation, invasion, and angiogenesis of tumor cells. Furthermore, this flavone potentiated apoptosis induced by TNF-α and chemotherapeutic agents.

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Materials and methods

Reagents. Bacteria-derived human recombinant TNF, purified to homogeneity with a specific activity of 5 X 10^7 U/mg, was provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI1640, IMDM and DMEM were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was supplied by Atlanta Biologicals (Lawrenceville, GA). Antibodies against p65, p50, IκBα, cyclin D1, cyclooxygenase-2, matrix mellatoproteinase- 9 (MMP-9), poly (ADP-ribose) polymerase (PARP), caspase-3, -8, -9, Bcl-2, Bcl-xL, and intercellular adhesion molecule-1 and the annexin V staining kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-XIAP antibody was obtained from BD biosciences (San Jose, CA). For immunocytochemistry, an antibody against p65 was obtained from Abcam (Cambridge, MA). An anti–vascular endothelial growth factor (VEGF) antibody was purchased from ThermoScientific (Fremont, CA). Phosphospecific anti-IκBα (serine 32 and 36) and phosphospecific anti-p65 (Ser536) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti- IKK-α, anti–IKK-β and anti-FLIP antibodies were kindly provided by Imgenex (San Diego, CA).

Cell lines. The cell lines KBM-5 (human chronic myeloid leukemia), HL-60 (human promyelocytic leukemia), A293 (human embryonic kidney carcinoma), and H1299 (human lung adenocarcinoma) were obtained from the ATCC (Manassas, VA). KBM-5 cells were cultured in IMDM with 15% FBS; HL-60 and H1299 cells were cultured in RPMI 1640; and A293 cells were cultured in DMEM supplemented with 10% FBS. Culture media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin.

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Extraction and isolation of PMF. The leaves of *Gardenia obtusifolia* were collected from the Doi Suthep-Pui National Park, Chiang Mai, Thailand. Voucher herbarium specimen (No.18749) of the plant was identified by J.F. Maxwell, and deposited in the Chiang Mai University Herbarium, Chiang Mai, Thailand. The samples were washed, air-dried, and chopped into small pieces**.** They were oven-dried at temperature below 50°C and ground to powder. The dried powder was macerated with 95% ethanol. The ethanolic solutions were combined and evaporated at 50°C under reduced pressure to give a dark-brown residue. A portion of the crude extract was separated based on liquid-liquid partition procedure. These chloroform extracts exhibited the highest cytotoxic activity. Based on the bioassay-guide isolation, the crude chloroform extract was subjected to further isolation with column chromatography (CC) on SiO_2. Gradient elution was performed with different compositions of a mobile phase as a gradient of increasing polarity. Separated fractions were evaluated by thin layer chromatography (TLC). Repeated separations were performed using $CHCl_3$/ ethyl acetate with increasing polarity up to a ratio of 5:5 to yield a pure fraction of 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone (PMF). The purity and the structure of these yellow crystals was measured and identified by TLC, HPLC, MS and NMR analysis

DNA binding assay for NF-κB. To assess NF-κB activation, nuclear extracts were prepared and electrophoretic mobility shift assay (EMSA) was done as previously described (Chaturvedi et al., 2000). Briefly, nuclear extracts prepared from TNF-treated cells (2×10^6/mL) were incubated with ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide (10 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5'TTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**CAGGGAG- GCGTGG-3' (bold

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indicates NF-κB binding sites), for 30 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. The dried gels were visualized, and the radioactive bands were quantitated with a Storm 820 and Image quant software (GE Healthcare).

Western Blot Analysis. To determine the levels of protein expression in whole cells, cytoplasm, and nuclear extracts, we prepared each extract (Takada and Aggarwal, 2004) from treated cells and fractionated each by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by ECL reagent (GE Healthcare).

IκBα Kinase Assay. The IKK assay was performed by a method described previously (Takada and Aggarwal, 2004). Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKK-β, followed by treatment with protein A/G-agarose beads (Pierce). After a 2 h incubation, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 μCi of [γ-^{32}P]ATP, 10 μM unlabeled ATP, and 2 μg of substrate glutathione S-transferase-IκBα [amino acid 1–54]. After the immunocomplex was incubated at 30 °C for 30 min, it was boiled with SDS sample buffer for 5 min. Finally, the protein was resolved on 10%SDS-PAGE, the gel was dried, and the radioactive bands were visualized by PhosphorImager. To determine the total amounts of IKK-α and -β in each sample, the IKK immunoprecipitate was resolved on 7.5%SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibodies.

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Immunocytochemistry for NF-κB p65 localization. Immunocytochemistry was used to examine the effect of PMF on the nuclear translocation of p65 (Takada and Aggarwal, 2004). Briefly, treated cells were plated on a poly-L-lysine–coated glass slide by centrifugation (Cytospin 4; Thermoshendon), air dried, and fixed with 4% paraformaldehyde. After being washed in PBS, the slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 at a 1/200 dilution. After overnight incubation at 4^{o}C, the slides were washed, incubated with goat anti-rabbit IgG-Alexa Fluor 594 (Invitrogen) at a 1/200 dilution for 1 h, and counterstained for nuclei with Hoechst 33342 (50 ng/mL) for 5 min. Stained slides were mounted with mounting medium purchased from Sigma-Aldrich and analyzed under a fluorescence microscope (Labophot-2; Nikon). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (GE Healthcare).

NF-κB–dependent reporter gene expression assay. NF-κB–dependent reporter gene expression was assayed as described previously (Takada and Aggarwal, 2004). The effect of PMF on TNF-α-, TNF receptor (TNFR)-, TNFR-associated death domain (TRADD)-, TNFR-associated factor 2 (TRAF2)-, NF-κB-inducing kinase (NIK)-, transforming growth factor (TGF)-β-activated kinase (TAK)-1/TAK-1 binding protein-1 (TAK1/TAB1)-, and IKKβ-induced NF-κB-dependent reporter gene transcription was analyzed by the secretory alkaline phosphatase (SEAP) assay.

Immunoprecipitation assay. To assess the impact of PMF on TNFα-induced formation of protein complexes associated with the TNFα receptor TNFR1, protein A/G-agarose beads were first incubated with TRADD antibody for 2h, then beads were washed with lysis buffer, and

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incubated with whole-cell extracts (600 μg protein) of treated KBM5 cells for overnight (4°C). The following day, beads were washed with lysis buffer and boiled with SDS sample buffer for 5 min. Finally, the supernatant was analyzed on 10% SDS-PAGE with TNFR1, RIP, and TRAF2 antibodies. To determine the total amounts of TRADD proteins in each sample, samples were blotted with anti-TRADD antibody.

Live/dead assay. To measure apoptosis, we also used the Live/Dead assay (Invitrogen), which determines intracellular esterase activity and plasma membrane integrity, following manufactures'instruction. Briefly, 2×10^5 cells were incubated with PMF and treated with 1 nmol/L TNF-α for up to 24 h at 37°C. Cells were stained with the Live/ Dead reagent (5 mmol/L ethidium homodimer and 5 mmol/L calcein-AM) and incubated at 37 °C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

Cytotoxicity assay. The effects of PMF on the cytotoxic effects of TNF-α and other chemotherapeutic agents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method, following the previously published protocol (Takada and Aggarwal, 2004). Briefly, 5,000 cells were incubated with PMF in triplicate in a 96-well plate and then treated with the 1 nmol/L TNF-α, 10 μg/mL cisplatin, 5 nmol/L palitaxel, and 0.1 μmol/L 5-fluorouracil for 24 h at 37 °C. An MTT solution was added to each well and incubated for 2 h at 37°C. An extraction buffer (20% SDS and 50% dimethylformamide) was added, and the cells were incubated overnight at 37 °C. Then, the absorbance was measured at 570 nm using a 96-well multiscanner (Dynex Technologies; MRX Revelation).

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Annexin V assay. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of membrane to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of Annexin V. We conjugated Annexin V antibody to a FITC fluorescence dye. Briefly, 1×10^6 cells were pretreated with PMF, treated with TNF-α for 24 h at 37^{o}C, and subjected to Annexin V staining. The cells were washed in PBS, resuspended in 100 μL of binding buffer containing a FITC-conjugated anti-Annexin V antibody, and then analyzed with a flow cytometer (FACS Calibur).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. We also determined cytotoxicity using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method using an in situ cell death detection reagent (Roche Molecular Biochemicals).

Statistical analysis. The statistical analysis was done by one way ANOVA test using SPSS v15.0. Quantification of Western blots was performed with Image J.

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Results

We investigated the effect of PMF on inducible NF-κB activated by various carcinogens and inflammatory stimuli, on NF-κB-regulated gene expression, and on apoptosis induced by cytokines and chemotherapeutic agents. We examined the effect of PMF on the TNF-α–induced NF-κB activation in detail because the NF-κB activation pathway induced by this agent is relatively well-established.

Identification of the active compound

Bioassay-directed fractionation identified a flavone core structure, PMF, as the most active compound. The isolated compound was identified through analysis of its R_f values, melting point, ultraviolet absorption, infrared absorption, nuclear magnetic resonance, and mass spectra in comparison with previously published data. 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone (PMF) was obtained as yellow crystals. The electron impact mass spectrum (EI-MS) of PMF exhibited a molecular ion peak at m/z 404, supporting the molecular formula of $C_{20}H_{20}O_9$. The IR spectrum showed strong absorption bands of OH (3100-3700 cm^{-1}, broad), C=O (1650-1705 cm^{-1}, medium), C=C (1600-1500 cm^{-1}, strong), C-O (1200-1400 cm^{-1}, strong). The UV spectrum consisted of two absorption maxima (λmax) at 348 nm (band I) and 260-278 nm (band II). Inspection of the signals in the ^{1}H-NMR and carbon signals in the ^{13}C-NMR spectrum allowed us to deduce the structure of PMF that is 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone. Its spectral data were in agreement with those obtained from the reference compound reported in the literature (Lichius et al., 1994; Shi et al., 1995; Tuchinda et al., 2002). (Fig.1A).

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PMF inhibits TNF-α-dependent NF-κB activation in a dose and time dependent manner

We first determined the dose and time of exposure to PMF required to suppress TNF-α-induced NF-κB activation in KBM-5 cells. EMSA showed that PMF alone had no effect on basal NF-κB activation, but it inhibited TNF-α–mediated NF-κB activation in a dose- (Fig.1B, *left*) and time- (Fig.1B, *right*) dependent manner, respectively, and that 16 h exposure to 100 μmol/L PMF was sufficient to suppress almost 80% of NF-κB activation.

PMF inhibits NF-κB activation induced by carcinogens and inflammatory stimuli

TNF-α, CSC, PMA, LPS and, OA are well-known potent activators of NF-κB, but they act by different mechanisms (Garg and Aggarwal, 2002). We examined the effect of PMF on the activation of NF-κB by these agents using EMSA. TNF-α, CSC, PMA, LPS and, OA induced NF-κB, and PMF suppressed activation of NF-κB to variable degrees by TNF-α (52%), CSC (80%), PMA (51%), LPS (28%), and OA (80%) in KBM-5 cells (Fig. 1C).

Suppression of TNF-α-dependent NF-κB activation by PMF is not cell type specific

To rule out the possibility of differences in NF-κB activation between cell types, we tested the effect of PMF on TNF-α-induced NF-κB activation in A293, HL-60 and H1299 cells. EMSA showed that PMF inhibited TNF-α-activated NF-κB in these cell types (Fig. 1D), and slightly down-regulated basal NF-κB levels in A293 cells (Fig. 1D, *left*). These results suggest that inhibition of NF-κB activation by PMF was not cell type–specific.

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PMF does not interfere with the binding of NF-κB to the DNA

Some NF-κB inhibitors, such as caffeic acid phenethyl ester (Natarajan et al., 1996), plumbagin (Sandur et al., 2006), and herbimycin (Mahon and O'Neill, 1995), directly suppress binding of NF-κB to DNA. We determined whether PMF mediates suppression of NF-κB activation through a similar mechanism. PMF did not modify the DNA-binding ability of NF-κB proteins (Fig. 2A). These results suggest that PMF inhibits NF-κB activation at a step upstream to its DNA binding.

PMF prevents TNF-α–dependent IκBα degradation and phosphorylation

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα (Aggarwal, 2004; Ghosh et al., 1998). To determine whether inhibition of TNF-α-induced NF-κB activation was due to inhibition of IκBα degradation, we pretreated KBM-5 cells with PMF and then exposed them to TNF-α for various time periods. We then examined the cells for NF-κB in the nucleus by EMSA and for IκBα degradation in the cytoplasm by Western blot analysis. As shown in Fig. 2B, TNF-α activated NF-κB in the control cells. The earliest activation occurred within 5 min after TNF-α addition. However, the activation was decreased in PMF-pretreated cells. Moreover, TNF-α induced IκBα degradation in only 5 min, correlating TNF-α-induced IκBα degradation to TNF-α-induced NF-κB DNA binding activation, whereas PMF prevented this degradation, although not completely (Fig. 2C, *upper left*). These results indicate that PMF suppressed both TNF-α-induced IκBα degradation and NF-κB activation.

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PMF inhibits phosphorylation of IκBα by TNF-α and leads to inhibition of ubiquitination and degradation of IκBα

The proteolytic degradation of IκBα is known to require phosphorylation at Ser32 and Ser36 residues (Ghosh et al., 1998). To determine the effects of PMF on TNF-induced IκBα phosphorylation, we next assayed the TNF-induced phosphorylated form of IκBα by western blot analysis, using an antibody that recognizes the serine-phosphorylated form of IκBα. TNF induced IκBα phosphorylation, and this phosphorylation was suppressed by PMF (Fig. 2C, *lower left*). Because TNF-induced phosphorylation of IκBα leads to its rapid degradation, we blocked degradation of IκBα by using the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN). Western blot analysis showed that TNF plus ALLN co-treatment induced phosphorylation of IκBα at serine 32 and 36, and ubiquitination, and that PMF pre-treatment decreased phosphorylation of IκBα and ubiquitination of IκBα in KBM5 cells (Fig. 2C, *right panel*). This indicates that inhibition of IκBα phosphorylation by PMF leads to inhibition of IκBα ubiquitination.

PMF Inhibits TNF-α-induced IKK Activation

It has been shown that IKK is required for TNF-α-induced phosphorylation of IκBα (Ghosh and Karin, 2002). Because PMF inhibits the phosphorylation of IκBα, we determined the effect of PMF on TNF-α -induced IKK activation. As shown in Fig. 2D, in immune complex kinase assays, TNF-α activated IKK as early as 2 min after TNF-α treatment. PMF treatment suppressed the TNF-α-induced activation of IKK. Neither TNF-α nor PMF had any effect on the expression of IKK-α or -β proteins.

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PMF inhibits TNF-α-induced phosphorylation and nuclear translocation of p65

We also investigated the effect of PMF on TNF-α-induced phosphorylation of p65 because phosphorylation is also required for its transcriptional activity (Egan et al., 1999). In the nuclear fraction from the TNF-α-treated cells, PMF suppressed the phosphorylated (Ser536) form of p65 (Fig. 3A, *middle*). We further showed that PMF decreased TNF-α-induced nuclear translocation of p65, as measured by western blotting (Fig. 3A, *top*). An immunocytochemistry assay also confirmed that PMF suppressed TNF-α-induced translocation of p65 to the nucleus (Fig. 3B).

PMF represses NF-κB–dependent reporter gene expression

Because DNA binding alone does not always correlate with NF-κB–dependent gene transcription (Nasuhara et al., 1999), there must be additional regulatory steps. We transiently transfected the cells with NF-κB–regulated SEAP reporter construct and pretreated them with PMF or left them untreated and then stimulated the cells with TNF-α. A 5-fold increase in SEAP activity was noted after stimulation with TNF-α, and that was abolished by dominant-negative IκBα, indicating the specificity. When the cells were pretreated with PMF, TNF-α-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner (Fig. 3C). These results indicate that PMF inhibits NF-κB–dependent reporter gene expression induced by TNF-α.

TNF-α-induced NF-κB activation is mediated through sequential interaction of the TNFR with TRADD, TRAF2, NIK, TAK1/TAB1 and IKK-β, resulting in phosphorylation of IκBα, which leads to degradation of IκBα and p65 nuclear translocation (Hsu et al., 1996). To delineate the site of action of PMF in the TNF-α-signaling pathway leading to NF-κB activation, cells were transiently transfected with TNFR1, TRADD, TRAF2, NIK, TAK1/TAB1 and IKK-β, and

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then NF-κB-dependent SEAP expression was monitored with or without PMF treatment. As shown in Fig. 3D, PMF suppressed NF-κB-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, and NIK plasmids. However, PMF had less effect on NF-κB-dependent reporter gene expression induced by TAK1/TAB1 and IKK-β, thus indicating that PMF may act at a site upstream of TAK1.

Whether PMF modulates TNF-induced formation of protein complexes between the adaptor proteins TRADD, TRAF2, and RIP, with the TNFR1 receptor, was examined by co-immunoprecipitation experiments (Fig. 3E). For this, KBM5 cells were first pre-treated with 100 μM PMF for 16h, and then stimulated with TNF for 10 min. As indicated in Figure 3E, TNF induced the association of TNFR1 with adaptor proteins TRADD, TRAF2, and RIP; and treatment of cells with PMF did not interfere with the formation of this complex. This result indicates that PMF must inhibit the NF-κB signaling pathway without affecting the recruitment of various adaptor proteins to the TNFR1.

PMF represses the expression of TNF-α–induced NF-κB–dependent antiapoptotic, proliferation, invasion and angiogenic gene products

Because NF-κB regulates the expression of the antiapoptotic proteins XIAP, survivin, Bcl-2, Bcl-xL, and cFLIP (Aggarwal, 2004), we investigated whether PMF modulates TNF-α-induced expression of these antiapoptotic genes. We found that PMF downregulated TNF-α-induced expression of XIAP, survivin, Bcl-xL, and cFLIP, but not Bcl-2 (Fig. 4A).

We also investigated whether PMF can modulate NF-κB-regulated gene products involved in the proliferation of tumor cells. TNF-α has been shown to induce cyclin D1 (Guttridge et al., 1999) and COX-2 (Yamamoto et al., 1995). Thus, we investigated whether

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PMF inhibits the TNF-α-induced expression of these proteins by Western blot analysis using specific antibodies. We found that PMF abolished TNF-α–induced expression of cyclin D1 and COX-2 (Fig. 4B).

Whether PMF modulates TNF-α-induced NF-κB-dependent gene products involved in the invasion and angiogenesis of tumor cells was also examined. It has been established already that MMP-9 and VEGF is regulated by NF-κB (Esteve et al., 2002; Xiong et al., 2004). We found that PMF abolished TNF-α–induced expression of MMP-9 and VEGF (Fig. 4C).

PMF potentiates apoptosis induced by TNF-α and chemotherapeutic agents

Because the activation of NF-κB has been shown to inhibit apoptosis induced by TNF-α and chemotherapeutic agents (Giri and Aggarwal, 1998), we investigated whether PMF affects TNF-α and chemotherapeutic agent-induced apoptosis. MTT assay showed that PMF enhanced cytotoxicity induced by TNF-α, cisplatin, pacitaxel (taxol), and 5-fluorouracil (5-FU) (Fig. 5A).

The esterase-staining method (also called Live/Dead assay) showed that PMF up-regulated TNF-α–induced apoptosis from 5% to 45% (Fig. 5B). AnnexinV/PI (Fig. 5C, *left*) and terminal deoxynucleotidyltransferase–mediated dUTP nick-end labeling (TUNEL; Fig. 5C, *right*) likewise showed that PMF up-regulated TNF-α-induced early and late events in apoptosis. Caspase-3 and caspase-8 cleavage (Fig. 5D, *left*) and caspase-mediated PARP cleavage (Fig. 5D, *right*) showed that PMF enhanced the apoptotic effect of TNF-α substantially. These results together indicate that PMF potentiated the apoptotic effects of TNF-α and chemotherapeutic agents.

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Discussion

While many anticancer agents have been developed and used, side effects and resistance to anticancer drugs are serious problems to be overcome in the treatment of cancer (Haldar et al., 1996; Wahl et al., 1996). Therefore, the research and development of safer and better therapeutic drugs have become necessary. Recently, there has been growing interest in the use of plant materials for the treatment of various human diseases including cancer. Most anticancer agents are traditionally derived from natural products. In the present report we identify a flavone as the active ingredient from a traditionally used species of Gardenia in Thailand that exhibits anti-inflammatory and anticancer properties. The same flavone**,** when isolated from other medicinal plants, was found to exhibit anti-HIV and cytotoxic effects against various tumor cell lines (Lichius et al., 1994; Shi et al., 1995; Tuchinda et al., 2002).

In the present report, we found that this PMF suppressed NF-κB activation induced by inflammatory stimuli and carcinogens and that the suppression was not cell type specific. The inhibition of NF-κB activation involved the suppression of IKK activation, leading to suppression of phosphorylation and degradation of IκBα and consequent p65 nuclear translocation. PMF also inhibited NF-κB–dependent reporter gene expression activated by TNF signaling elements. TNF-induced NF-κB–regulated gene products involved in the regulation of apoptosis, proliferation, and invasion were all down-regulated by PMF. PMF also potentiated the apoptosis induced by TNF and various chemotherapeutic agents.

This is the first report to suggest that PMF can suppress NF-κB activation induced by TNF, CSC, PMA, and OA. Although several flavones have been shown to exhibit anti-inflammatory activities, very little is known about the mechanism. How PMF suppresses NF-κB

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activation was investigated in detail. To our knowledge, ours is the first study to demonstrate that PMF can suppress NF-κB activation induced by inflammatory stimuli, such as TNF, and carcinogens, such as okadaic acid, PMA, and cigarette smoke. However, interestingly the effect of PMF on LPS-induced NF-κB activation was less pronounced. This suggests that PMF may act at a step common to TNF, okadaic acid, PMA, and cigarette smoke, but different from LPS-induced NF-κB signaling. While LPS-induced NF-κB activation is triggered by Toll-like receptors, interacting with TRAF6 and TAK1, and then activating IKK (Takaesu et al., 2000), TNF-induced NF-κB activation requires sequential activation of TNFR1, TRADD, TRAF2, RIP-1 and TAK1, before it activates IKK (Hsu et al., 1996; Simeonidis et al., 1999). Therefore, it cannot be ruled out that PMF inhibits TNF-induced NF-κB activation upstream of TAK1. Also, our transfection (SEAP) experiments, which show that PMF suppressed TNFR1-, TRADD-, TRAF 2-, and NIK-induced NF-κB reporter gene expression, but not that of TAK1/TAB1 and IKKβ, suggest that PMF inhibits TNF-induced NF-κB activation upstream of TAK1. In addition, co-immunoprecipitation experiments ruled out that PMF modulates complex formation between the TNFR1 and its adaptor proteins TRADD, TRAF2, and RIP. This result indicates that PMF must inhibit the NF-κB signaling pathway downstream of the TNFR1 complex and upstream of IKK.

Upstream activators of the IKK complex remain undefined and therefore we cannot conclude on the direct target of PMF upstream of IKK. Several studies have suggested that different kinases can activate the IKK complex, such as atypical protein kinase C (Sanz et al., 1999), mitogen-activated protein kinase kinase kinase 1 (MEKK1) (Lee et al., 1997), Cot/TPL2 (Lin et al., 1999), NIK (Malinin et al., 1997), TAK1 (Takaesu et al., 2003) and RIP-1 (Ea et al., 2006). Although overexpression of NIK is inhibited by PMF and was long believed to be the most

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potent activator of IKK (Malinin et al., 1997; Regnier et al., 1997; Woronicz et al., 1997), recent NIK$^{-/-}$ experiments, however, question its involvement in IKK activation by TNF (Smith et al., 2001; Yin et al., 2001).

Numerous hydroxylated polymethoxyflavones have been isolated primarily from orange juice, citrus peel and dried tangerine peels (Dong et al.; Hirata et al., 2009; Sun et al., 2009; Takanaga et al., 2000; Xiao et al., 2009; Zheng et al., 2009), including nobiletin, tangeretin, artemetin (Ahmed et al., 1988; Sertie et al., 1990), and sinensetin (Choi et al., 2002). Besides citrus, PMF analogues have also been identified from the spice thyme and estragon (Watanabe et al., 2005). The compound isolated from Gardenia species and used in our studies is 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone. The compound isolated from tangerine peel is 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone. Nobiletin, a hexamethoxyflavone, when orally administered to rats is metabolized to pentamethoxyflavone (Yasuda et al., 2003). Whether PMF used in our studies is also metabolized, and if this PMF metabolite is responsible for the suppression of the TNF short-term activation of the NF-κB signaling responses is not clear. However, it is true that a relatively long time of PMF incubation (16 h) is required for NF-κB inhibition. Therefore, it is possible that either PMF gradually accumulates in the cells due to a slow uptake or that a metabolite of PMF is responsible for NF-κB inhibition. The second hypothesis has been reported for nobiletin, where metabolites of this flavone have higher potential to suppress NF-κB transcriptional activities than nobiletin itself (Eguchi et al., 2007).

Also, whether flavones that are hydroxylated and methoxylated at different positions exhibit similar activities is not known. Artemetin has been shown to exhibit anti-inflammatory activity, but the mechanism is not understood (Sertie et al., 1990). It is possible that

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downmodulation of NF-κB, as described here, plays a role in the anti-inflammatory effects of artemetin.

On the other hand, we found that the expression of several gene products involved in the survival and proliferation of tumor cells was suppressed by PMF. These include XIAP, survivin, Bcl-xL, cFLIP, and cyclin D1. Numerous reports have shown that different analogues of PMF are cytotoxic to various tumor cells (Lichius et al., 1994; Sergeev et al., 2006; Shi et al., 1995). Little is known, however, about the mechanism by which PMFs exhibit anticellular effects. Our studies provide an insight into one possible mechanism, the downregulation of NF-κB-regulated gene products that resist to apoptosis and promote proliferation. Tangeretin, for example, has been shown to induce G1 cell cycle arrest in colorectal cancer cells through upregulation of p21, p27, and p53 (Pan et al., 2002).

We found through downregulation of NF-κB that PMF also downregulates COX-2, a proinflammatory enzyme involved in prostaglandin production. These results are consistent with another report that showed that IL-1-induced COX-2 is downmodulated by tangeretin (Chen et al., 2007). Although not examined in detail, tangeretin's downmodulation of COX-2 expression was assigned to suppression of NF-κB activation. Besides suppression of COX-2, anti-inflammatory activity of PMF may also be due to its ability to suppress NO production (Fushiya et al., 1999). The enzyme that mediates NO production, iNOS, is also regulated by NF-κB.

Our results also demonstrate for the first time that PMF inhibits MMP-9 expression, suggesting that PMF not only blocks primary tumor development, but also malignant progression. Furthermore, we found that PMF inhibited TNF-induced VEGF expression, which is also linked to tumor angiogenesis.

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Finally, we found that PMF potentiated the apoptosis induced by TNF and the chemotherapeutic agents including cisplatin, paclitaxel (taxol) and 5-flurouracil. Like TNF, all chemotherapeutic agents have been shown to activate NF-κB and mediate chemoresistance (Giri and Aggarwal, 1998; Li and Sethi). Thus it is possible that downregulation of NF-κB and NF-κB-regulated survival gene products are involved in chemosensitization. In addition, the suppression of NF-κB-regulated multidrug resistance (MDR) protein reported previously (Choi et al., 2002; Patanasethanont et al., 2007; Takanaga et al., 2000) may also contribute to chemosensitization. Some reports have suggested that certain PMF analogues exhibit chemopreventive potential *in vivo* in Apc (Min) mouse model of colon cancer (Cai et al., 2009; Sale et al., 2009). Since NF-κB is known to play a major role in chemoprevention (Sarkar and Li, 2008), this activity may also be due to its ability to suppress this transcription factor.

Overall, our results suggest that the antiproliferative, proapoptotic, anti-invasive, and anti-angiogenic effects of PMF may result from the suppression of NF-κB and NF-κB-regulated gene products. These results may provide the molecular basis for using these flavones to prevent and even to treat different cancers.

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Phromnoi, Reuter, Sung, Limtrakul, Aggarwal

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Footnotes

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Figure Legends

Figure 1. A, the chemical structure of PMF. **B,** dose-and time-dependent effect of PMF on NF-κB activation induced by TNF-α. *Left,* Human leukemic cells (KBM-5) were incubated with the indicated concentrations of PMF for 16 h and treated with 0.1 nmol/L TNF-α for 30 min. The nuclear extracts were assayed for NF-κB activation by EMSA. *Right,* KBM-5 cells were preincubated with 100 μmol/L PMF for the indicated times and then treated with 0.1 nmol/L TNF-α for 30 min. The nuclear extracts were prepared and assayed for NF-κB activation by EMSA. **C,** PMF blocks NF-κB activation induced by TNF-α, cigarette smoke condensate, phorbol 12-myristate 13-acetate, and okadaic acid. KBM-5 cells were preincubated with 100 μmol/L PMF for 16 h and then treated with 0.1 nmol/L TNF-α for 30 min, 10 μg/mL CSC for 1 h, 25 ng/mL PMA for 1 h, 100 ng/mL LPS for 2 h, or 500 nmol/L OA for 4 h. Nuclear extracts were analyzed for NF-κB activation. **D,** effect of PMF on activation of NF-κB induced by TNF-α in human embryonic kidney (A293), human leukemic (HL-60), and lung carcinoma (H1299) cells incubated at 37^{o}C with 100 μmol/L of PMF for 16 h and then stimulated with 0.1 nmol/L TNF-α for 30 min. After these treatments, nuclear extracts were prepared and then assayed for NF-κB by EMSA. The results shown are representative of three independent experiments.

Figure 2. A, in vitro effect of PMF on DNA binding of NF-κB protein. Nuclear extracts (NE) were prepared from 0.1 nmol/L TNF-α-treated KBM-5 cells; 15 μg/sample NE protein was treated with the indicated concentrations of PMF for 30 min at 37^{o}C and then assayed for NF-κB by EMSA. **B,** PMF inhibits TNF-α-induced activation of NF-κB. KBM-5 cells were incubated with 100 μmol/L PMF for 16 h, treated with 0.1 nmol/L TNF-α for the indicated times, and nuclear extracts were prepared and analyzed for NF-κB activation by EMSA. **C,** *upper left,*

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effect of PMF on TNF-α-induced degradation of IκBα. KBM-5 cells were incubated with 100 μmol/L PMF for 16 h, treated with 0.1 nmol/L TNF-α for the indicated times, and cytoplasmic extracts were prepared and analyzed by Western blotting using antibody against IκBα. Equal protein loading was evaluated by β-actin. *Lower left,* effect of PMF on TNF-α-induced phosphorylation of IκBα. Cytoplasmic extracts were prepared and analyzed by Western blotting using phospho-specific IκBα antibody. Equal protein loading was evaluated by β-actin. *Upper right,* PMF decreases TNF-α-induced phosphorylation and ubiquitination of IκBα in the presence of ALLN. Cells were preincubated with 100 μmol/L PMF for 16 h, incubated with 50 μg/mL of ALLN for 30 min, and then treated with 0.1 nmol/L TNF-α for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blotting using phospho-specific IκBα antibody. *Lower right,* the same membrane was reblotted with IκBα antibody and β-actin. **D,** effect of PMF on the TNF-α–induced activation of IKK. KBM-5 cells were preincubated with 100 μmol/L PMF for 16 h and then treated with 1 nmol/L TNF-α for the indicated times. Whole-cell extracts were immunoprecipitated with antibody against IKK-β and analyzed by an immune complex kinase assay. To examine the effect of PMF on the level of expression of IKK proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti–IKK-α and anti–IKK-β antibodies. The results shown are representative of two or three independent experiments.

Figure 3. A, PMF inhibits TNF-α-induced nuclear translocation and phosphorylation of p65. KBM-5 cells were either untreated or pretreated with 100 μmol/L PMF for 16 h and then treated with 0.1 nmol/L TNF-α for the indicated times. Nuclear extracts were prepared and analyzed by

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Western blotting using antibodies against p65 (*upper panel*) and phospho-specific p65 (Ser536) (*middle panel*). For loading control of nuclear protein, the membrane was reprobed with anti-PARP antibody. **B,** immunocytochemical analysis of p65 localization. KBM-5 cells were first treated with 100 μmol/L PMF for 16 h and then stimulated with 1 nmol/L TNF-α for 15 min. After cytospin, immunocytochemical analysis was performed. The results shown are representative of three independent experiments. **C,** PMF inhibited TNF-α-induced NF-κB-dependent reporter gene (SEAP) expression. A293 cells treated with the indicated concentrations of PMF were transiently transfected with a NF-κB-containing plasmid linked to the SEAP gene. After 24 h in culture with 1 nmol/L TNF-α, cell supernatants were collected and assayed for SEAP activity. Results are expressed as fold activity over the activity of the vector control (Con). $^{a}P < 0.001$; $^{b}P < 0.0001$, compared with TNF-treated cells. The results were the mean of experiments performed in triplicate. **D,** PMF inhibited NF-κB-dependent reporter gene expression induced by TNF-α, TNFR-1, TRADD, TRAF2, NIK, TAK1/TAB1, and IKK-β. A293 cells were pretreated with 100 μmol/L PMF and transiently transfected with the indicated plasmids along with a NF-κB–containing plasmid linked to the SEAP gene. After 24 h, cell supernatants were collected and assayed for SEAP activity. Results are expressed as fold activity over the activity of the vector control (Con). $^{a}P > 0.1$, $^{b}P > 0.01$, $^{c}P < 0.0001$. The results were the mean of experiments performed in triplicate. **E,** PMF does not interfere with the complex formation between the receptor TNFR1 and its adaptor proteins TRADD, TRAF2, and RIP. KBM5 cells ($3x10^{6}$/ml) were pretreated with PMF (100 μmol/L) for 16 h and then incubated with TNF (1 nmol/L) for 10 min. Whole-cell extracts were immunoprecipitated using an antibody against TRADD and then analyzed by Western blot using anti-TNFR1, -RIP, and -TRAF2 antibodies. Anti-TNFR1 antibody was used as a loading control.

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Figure 4. A, PMF inhibits the expression of TNF-α-induced antiapoptotic proteins. KBM-5 cells were incubated with 100 μmol/L PMF for 16 h and then treated with 1 nmol/L TNF-α for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the antibodies to the indicated proteins. Numbers below each panel indicate fold differences after normalization to β-actin. **B,** PMF inhibits TNF-α-induced cyclin D1, and COX-2 expression. KBM-5 cells were incubated with 100 μmol/L PMF for 16 h and then treated with 1 nmol/L TNF-α for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. Numbers below each panel indicate fold differences after normalization to β-actin. **C,** PMF inhibits TNF-α-induced MMP-9 and VEGF expression. KBM-5 cells were incubated with 100 μmol/L PMF for 16 h and then treated with 1 nmol/L TNF-α for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using the relevant antibodies. Numbers below each panel indicate fold differences after normalization to β-actin. The results shown are representative of three independent experiments.

Figure 5. A, PMF enhances TNF-α-, cisplatin-, paclitaxel-, and 5-FU-induced cytotoxicity. $5x10^6$ cells were seeded in triplicate in 96-well plates. The cells were pretreated with 100 μmol/L PMF for 16 h and then incubated with chemotherapeutic agents for 24 h. Cell viability was then analyzed by the MTT method. . $^aP < 0.01$, $^bP < 0.001$. The results shown are representative of three independent experiments. **B,** PMF enhances TNF-α–induced cytotoxicity. KBM-5 cells were pretreated with 100 μmol/L PMF for 16 h and then incubated with 1 nmol/L TNF-α for 24 h. The cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. The results shown are representative of three independent experiments.

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C, PMF enhances TNF-α–induced apoptosis. Cells were pretreated with 100 μmol/L PMF for 16 h and then incubated with 1 nmol/L TNF- α for 24 h. The cells were either stained with FITC-conjugated Annexin V (AnnV FITC), and propidium iodide (PI) (*left*), or TUNEL reagents (*right*), and then analyzed by flow cytometry. The results shown are representative of three independent experiments. **D,** PMF activates caspase-8, caspase-3, and PARP cleavage. *Left*, cells were pretreated with 100 μmol/L PMF for 16 h and then incubated with 1 nmol/L TNF-α for 24 h. Whole-cell extracts were prepared and analyzed by Western blotting using anti-caspase-8 and anti-caspase-3 antibodies. Equal protein loading was evaluated by β-actin. *Right*, effect of PMF on PARP cleavage. Cells were pretreated with 100 μmol/L PMF for 16 h and then incubated with 1 nmol/L TNF-α for the indicated times. Whole-cell extracts were prepared, and analyzed by Western blotting using an anti-PARP antibody. Equal protein loading was evaluated by β-actin. The results shown are representative of three independent experiments.

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Fig. 1

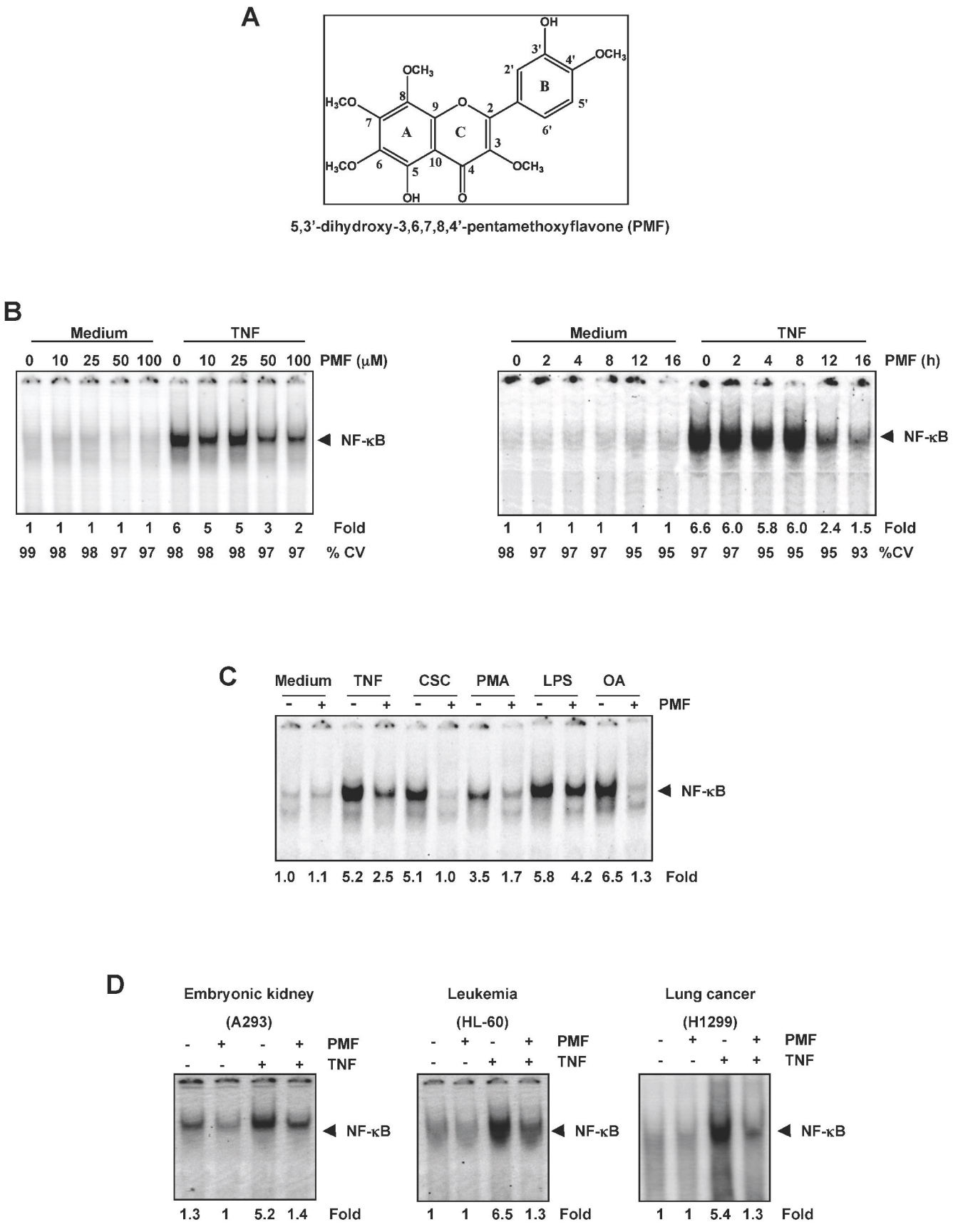

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Fig. 2

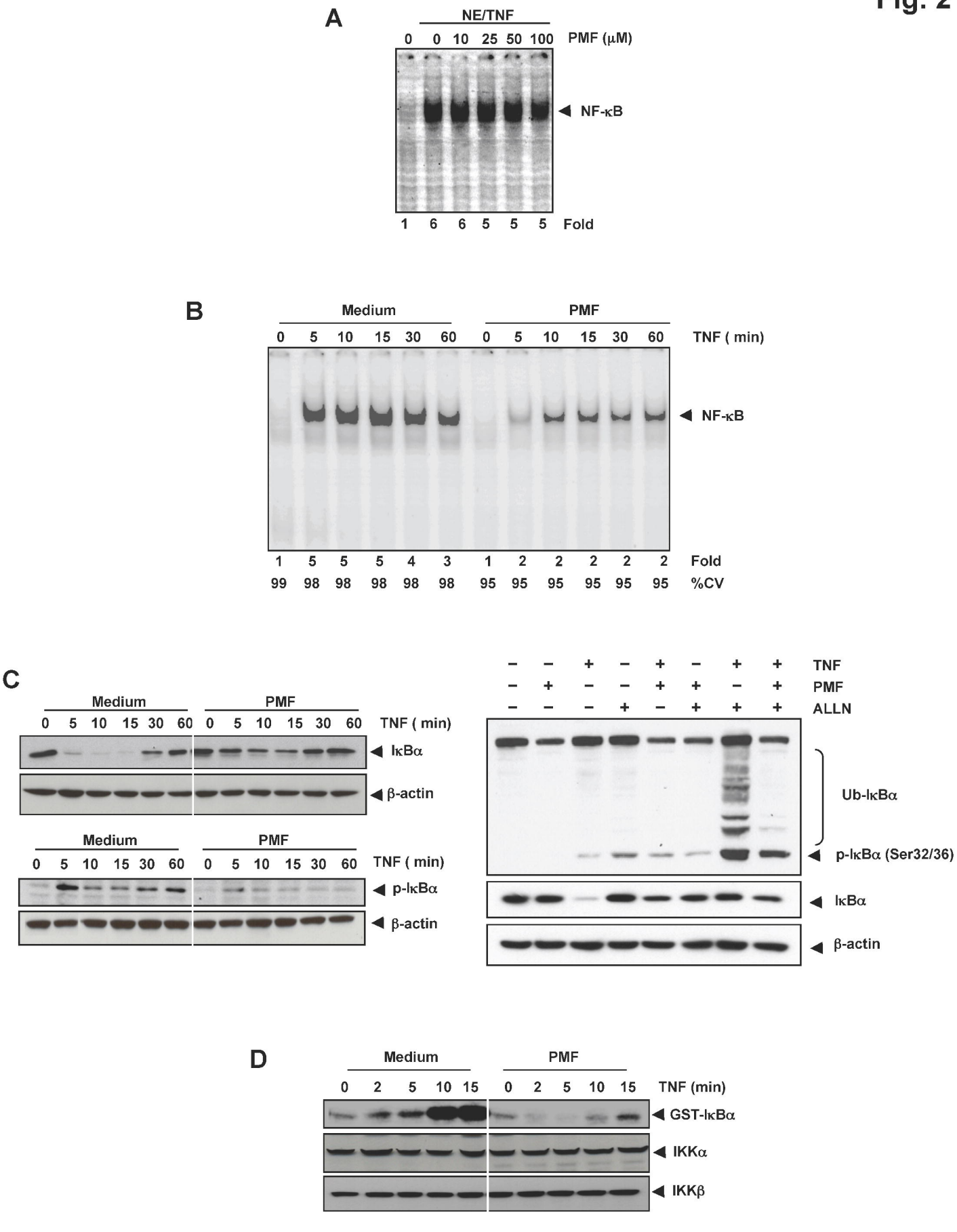

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Fig. 3

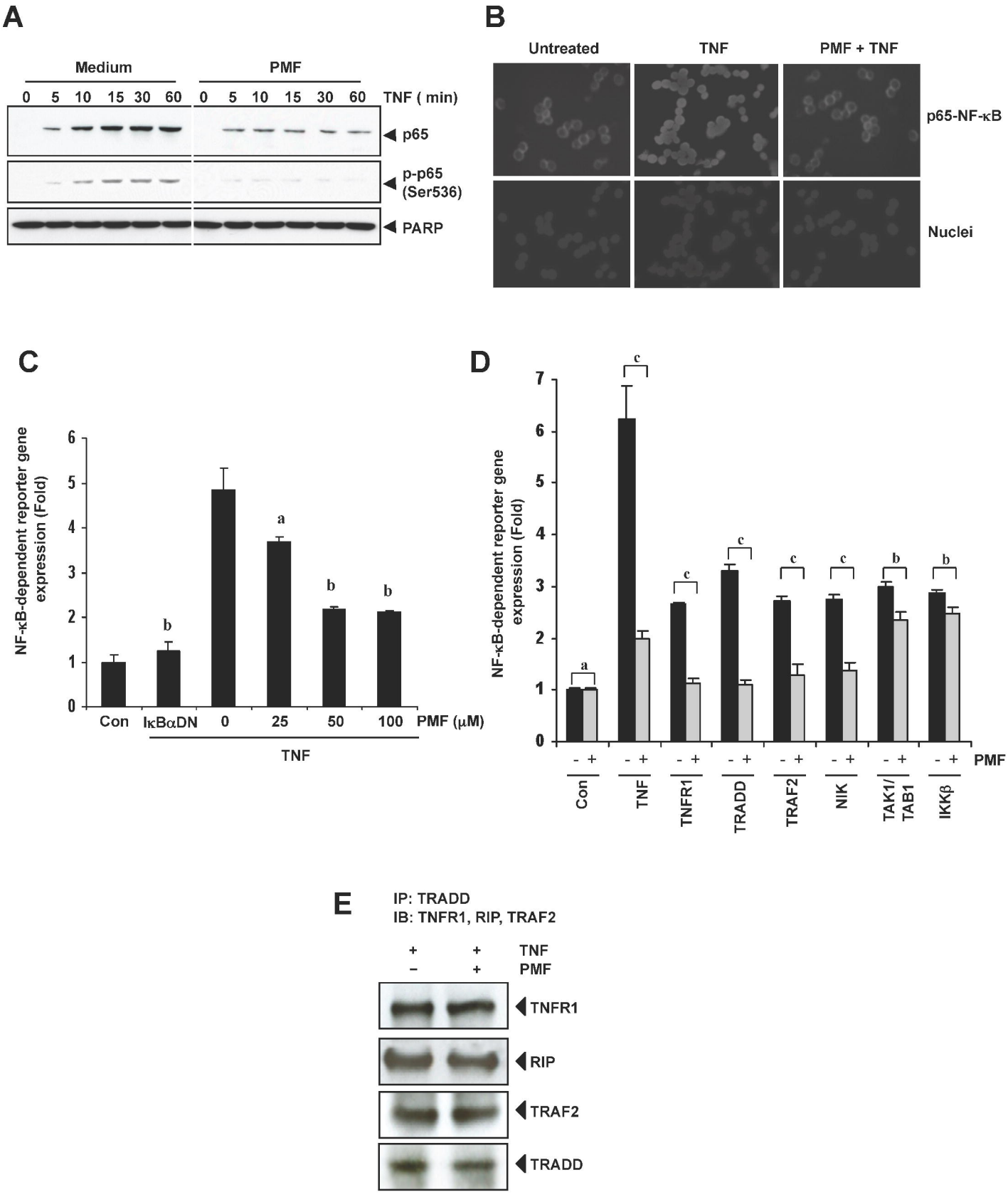

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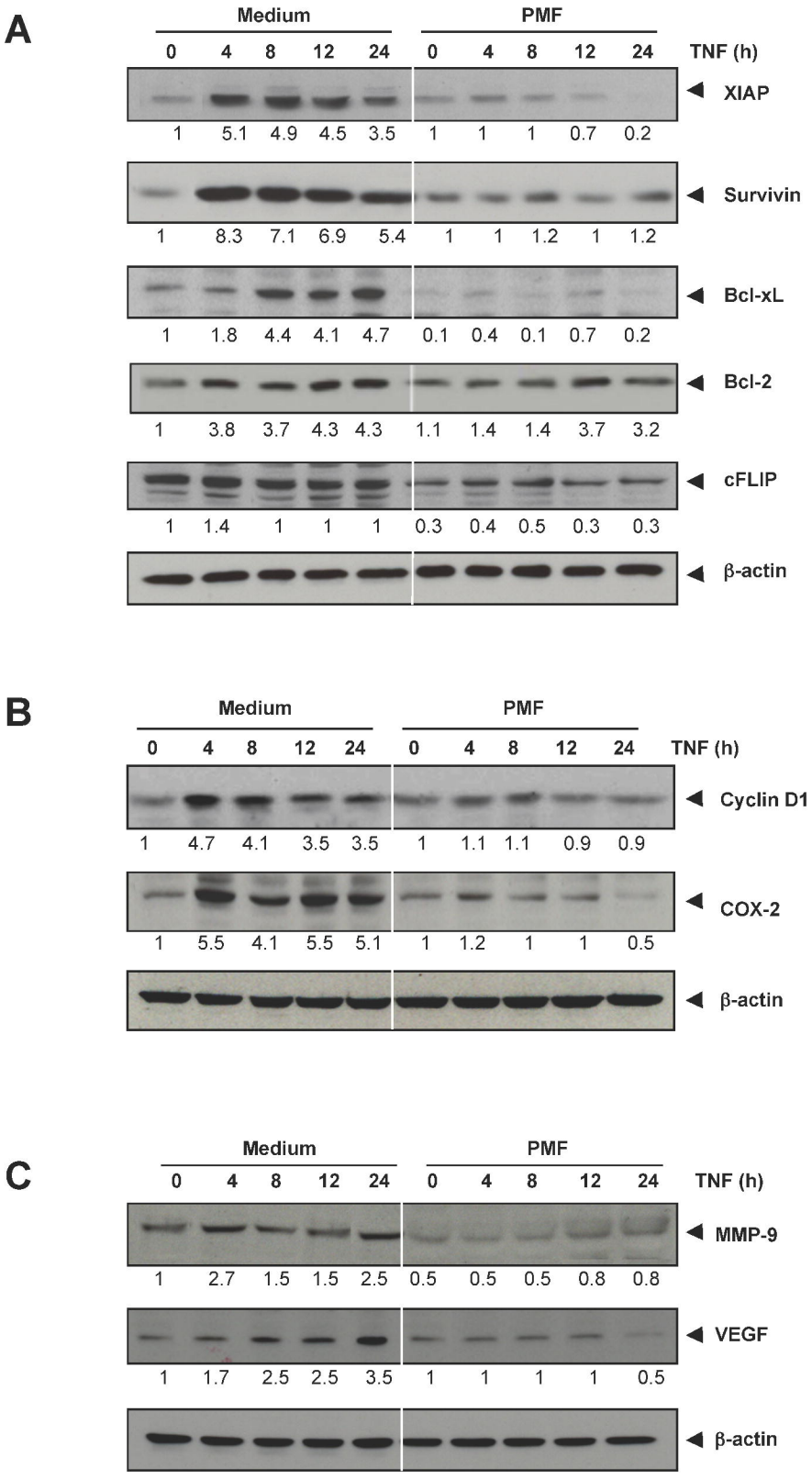
Fig. 4



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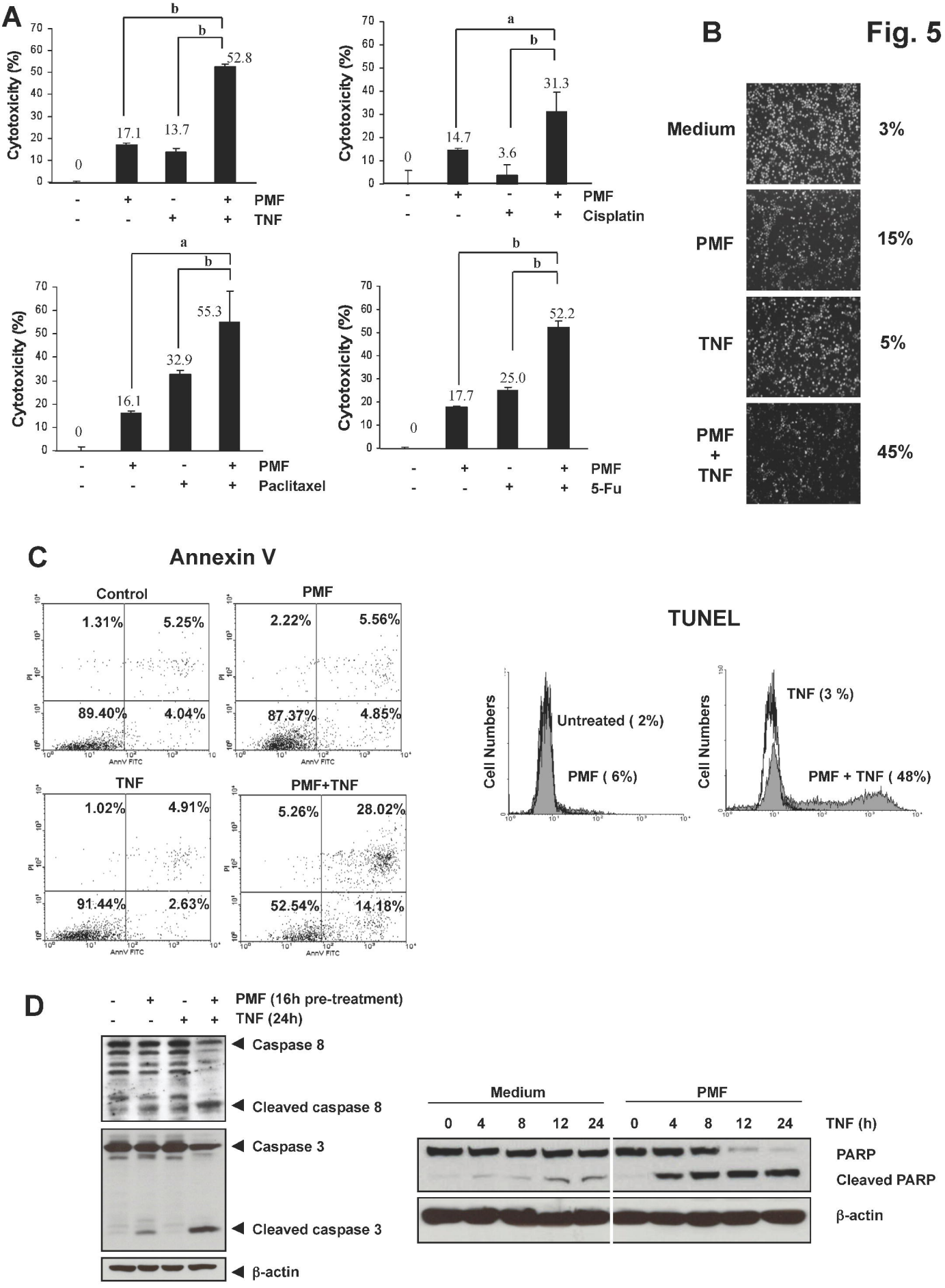

Fig. 5