A Novel Pentamethoxyflavone Down-Regulates Tumor Cell Survival and Proliferative and Angiogenic Gene Products through Inhibition of I κ B Kinase Activation and Sensitizes Tumor Cells to Apoptosis by Cytokines and Chemotherapeutic Agents

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Received July 13, 2010; accepted October 7, 2010

ABSTRACT

Most anticancer drugs have their origin in traditional medicines and plants. We describe here a flavone, 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone (PMF), from the leaves of the Thai plant *Gardenia obtusifolia*, that has anti-inflammatory and anticancer potential. Because the nuclear factor- κ B (NF- κ B) pathway is linked to inflammation and tumorigenesis, we investigated the effect of PMF on this pathway. We found that PMF suppressed NF- κ B 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- κ B proteins to bind to DNA, it inhibited I κ B α (inhibitory subunit of NF- κ B) kinase, leading to suppression of phosphorylation and degradation of I κ B α and suppressed consequent p65 nuclear translocation, thus attenuating NF- κ B-dependent reporter gene expression.

Suppression of the NF- κ B cell signaling pathway by the flavone led to the inhibition of expression of NF- κ B-regulated gene products that mediate inflammation (cyclooxygenase-2), survival (XIAP, survivin), and proliferation (cyclin D1), 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- α and the chemotherapeutic agents cisplatin, paclitaxel, and 5-fluorouracil. Overall, our results indicate that PMF suppresses the activation of NF- κ B and NF- κ B-regulated gene expression, leading to the enhancement of apoptosis. This is the first report to demonstrate that this novel flavone has anti-inflammatory and anticancer effects by targeting the I κ B complex.

Introduction

More than 80% of the world's population cannot afford modern medicine. Thus, safe, effective, and effective new

treatments 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 (also known as 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 et

This work was supported by the National Institutes of Health National Cancer Institute [Grants CA16672, CA16678], the Center for Targeted Therapy of M. D. Anderson Cancer Center, and the Royal Golden Jubilee Scholarship for the Ph.D. program [Grant 880].

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.110.067512.

ABBREVIATIONS: PMF, pentamethoxyflavone (5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone); NF- κ B, nuclear factor- κ B; I κ B α , inhibitor of nuclear factor- κ B α ; LPS, lipopolysaccharide; OA, okadaic acid; TNF, tumor necrosis factor; COX-2, cyclooxygenase-2; XIAP, X-linked inhibitor of apoptosis protein; cFLIP, caspase-8 (FLICE)-like inhibitory protein; MMP-9, matrix metalloproteinase 9; VEGF, vascular endothelial growth factor; IMDM, Iscove's modified Dulbecco's medium; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; IKK, I κ B kinase; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; TNFR, TNF receptor; TRADD, TNFR-associated death domain; TRAF2, TNFR-associated factor 2; NIK, NF- κ B-inducing kinase; TAK, transforming growth factor- β -activated kinase; TAB1, TAK-1 binding protein-1; SEAP, secretory alkaline phosphatase; RIP, receptor interacting protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; CSC, cigarette smoke condensate; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMA, phorbol 12-myristate 13-acetate; MDR, multidrug resistance protein.

al., 1991). A pentamethoxyflavone isolated from this plant, 5,3'-dihydroxy-3,6,7,8,4'-pentamethoxyflavone (PMF), has been shown to exhibit activity against 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- κ B in tumorigenesis and HIV replication, we hypothesize that this pathway plays a major role in the action of this flavone.

NF- κ B represents a group of five proteins: c-Rel, RelA (p65), RelB, NF- κ B1 (p50 and p105), and NF- κ B2 (Ghosh et al., 1998). In an inactive state, NF- κ B is sequestered in the cytoplasm as a heterotrimer consisting of p50, p65, and I κ B subunits. Upon activation, I κ B α 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 transcription. Many 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- κ B. NF- κ B has been shown to regulate the expression of several genes the products of which are involved in tumorigenesis. These include proapoptotic (X χ P, survivin, Bcl-xL, Bcl-2, cFLIP), proliferative (cyclin D), 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- κ B pathway in the antiproliferative action of PMF, we studied the effect of PMF on the NF- κ B pathway and measured the anticellular and chemosensitizing effects of PMF and their relationship to the NF- κ B pathway. We found that PMF inhibited the activation of NF- κ B through inhibition of I κ B kinase and subsequently of I κ B α phosphorylation and degradation and nuclear translocation. The suppression of NF- κ B activity by this compound led to the down-regulation of genes that promote survival, proliferation, invasion, and angiogenesis of tumor cells. Furthermore, this flavone potentiated the cytotoxicity induced by TNF- α and chemotherapeutic agents.

Materials and Methods

Reagents. PMF was purified from the leaves of *G. obtusifolia* according to the procedure outlined in our report (Phromnoi et al., 2010). Bacteria-derived human recombinant TNF- α , purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium, Iscove's modified Dulbecco's medium, and Dulbecco's modified Eagle's medium were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was supplied by Atlanta Biologicals (Lawrenceville, GA). Antibodies against p65, p50, I κ B α , cyclin D1, cyclooxygenase-2, MMP-9, poly(ADP-ribose) polymerase (PARP), caspase-3, -8, -9, Bcl-2, Bcl-xL, 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 Inc. (Cambridge, MA). An anti-vascular endothelial growth factor (VEGF)

antibody was purchased from ThermoFisher Scientific (Waltham, MA). Phosphospecific anti-I κ B α (Ser32 and Ser36) 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 donated by Ingenex (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 American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium with 15% FBS. HL-60 and H1299 cells were cultured in RPMI 1640 medium and A293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Culture media were supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin.

DNA Binding Assay for NF- κ B. To assess NF- κ B activation, nuclear extracts were prepared, and electrophoretic mobility shift assay (EMSA) was performed as described previously (Chaturvedi et al., 2000). In brief, nuclear extracts prepared from treated cells (10^6 /ml) were incubated with 32 P end-labeled 48-nt double-stranded NF- κ B oligonucleotides (10 μ M) and protein with 10 μ M of DNA from the HIV long terminal repeat (5'-TTGTTACAGG-GACTTTCGCTGGGGACTTTCGGGACCTGG-3' (bold indicates NF- κ B binding sites), for 30 min at 30°C and the DNA-protein complex formed was separated from free oligonucleotide on 4% native polyacrylamide gels. The DNA-protein complex was visualized, and the radioactive bands were quantitated with the use of a Storm 820 PhosphorImager and ImageQuant software (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Western Blot Analysis. To determine the levels of protein expression in whole cells, cytoplasmic 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 enhanced chemiluminescence reagent (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK).

I κ B Kinase Assay. The IKK assay was performed by a method described previously (Takada and Aggarwal, 2004). In brief, the IKK complex from whole-cell extracts was precipitated with antibody against IKK- β , followed by treatment with protein A/G-agarose beads (Pierce and Warriner, 2003). 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 μ Ci of γ - 32 P]ATP, 10 μ M unlabeled ATP, and 2 μ g of substrate glutathione transferase-I κ B α (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.

Immunocytochemistry for NF- κ B p65 localization. Immunocytochemistry was used to examine the effect of PMF on the nuclear translocation of p65 (Takada and Aggarwal, 2004). In brief, treated cells were plated on a poly-L-lysine-coated glass slide by centrifugation (Cytospin 4; Thermo Fisher Scientific), air-dried, and fixed with 4% paraformaldehyde. After being washed in phosphate-buffered saline, 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°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, Tokyo, Japan). Pictures were captured using a Photometrics CoolSnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (GE Healthcare).

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

Immunoprecipitation Assay. To assess the impact of PMF on TNF- α -induced formation of protein complexes associated with the TNF- α receptor TNFR1, protein A/G-agarose beads were first incubated with TRADD antibody for 2 h, then beads were washed with lysis buffer, and incubated with whole-cell extracts (600 μ g of protein) of treated KBM-5 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 the manufacturer's instructions. In brief, 2×10^5 cells were incubated with PMF and treated with 1 nM TNF- α for up to 24 h at 37°C. Cells were stained with the Live/Dead reagent (5 mM ethidium homodimer and 5 mM calcein AM) and incubated at 37°C for 30 min. Cells were viewed under a fluorescence microscope (Labophot-2; Nikon).

Cytotoxicity Assay. The effects of PMF on the cytotoxicity 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 protocol published previously (Takada and Aggarwal, 2004). In brief, 5×10^4 cells were treated with PMF in triplicate in a 96-well plate, then treated with 1 nM TNF- α , 10 μ g/ml cisplatin, 5 nM paclitaxel (Taxol), and 0.1 μ M 5-fluorouracil for 24 h at 37°C. An MTT solution was added to each well and incubated for 2 h at 37°C. After reaction buffer (20% SDS and 50% dimethylformamide) was added, the cells were incubated overnight at 37°C. Then, the absorbance was measured at 570 nm using a 96-well microplate reader (Dynex Technologies; MRX Revelation).

Annexin Assay. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine to the cytoplasmic interface of the membrane on the cellular surface. The loss of membrane asymmetry can be detected using the binding properties of Annexin V, which is conjugated with Annexin V antibody to a FITC fluorescence dye. In brief, 10^5 cells were treated with PMF, treated with TNF- α for 24 h at 37°C, and subjected to Annexin V staining. The cells were washed in phosphate-buffered saline, resuspended in 100 μ l of binding buffer containing a FITC-conjugated anti-Annexin V antibody, and then analyzed with a flow cytometer (FACS Calibur; BD Biosciences).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay. We also examined cytotoxicity by using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method as an in situ cell death detection reagent (Roche Molecular Biochemicals).

Statistical analysis. The statistical analysis was done by one-way analysis of variance using SPSS v15.0 (SPSS, Inc., Chicago, IL). Quantification of Western blots was performed with Image J software (<http://rsbweb.nih.gov/ij/>).

Results

We investigated the effect of PMF on inducible NF- κ B activated by various carcinogens and inflammatory stimuli, on NF- κ B-regulated gene expression, and on apoptosis induced by cytokines and chemotherapeutic agents. We exam-

ined the effect of PMF on the TNF- α -induced NF- κ B activation in detail because the NF- κ B 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 retention factor (R_f) values, melting point, ultraviolet absorption, infrared absorption, nuclear magnetic resonance, and mass spectra in comparison with previously published data. PMF was obtained as yellow crystals. The electron ionization mass spectrum of PMF exhibited a molecular ion peak at m/z 404, suggesting the molecular formula of $C_{20}H_{12}O_6$. The IR spectrum showed strong absorption bands of C=O (3100–3700 cm^{-1} , broad), C=O (1600–1705 cm^{-1} , strong), C=C (1600–1450 cm^{-1} , strong), and C–O (1200–1400 cm^{-1} , strong). The UV spectrum consisted of two absorption maxima at 340 nm (band I) and 278 nm (band II). Inspection of the signals in the ^1H NMR and carbon signals in the ^{13}C NMR spectrum allowed us to deduce the structure of PMF. The spectral data were in agreement with those obtained from the reference compound reported in the literature (Liang et al., 1994; Shi et al., 1995; Achinda et al., 2006) (Fig. 1).

PMF Inhibits TNF- α -Dependent NF- κ B 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- κ B activation in KBM-5 cells. EMSA showed that PMF alone had no effect on basal NF- κ B activation but inhibited TNF- α -mediated NF- κ B activation in a dose- (Fig. 1B, left) and time- (Fig. 1B, right) dependent manner, respectively, and that 16-h exposure to 100 μ M PMF was sufficient to suppress almost 80% of NF- κ B activation.

PMF Inhibits NF- κ B Activation Induced by Carcinogens and Inflammatory Stimuli. TNF- α , CSC, PMA, LPS, and OA are well known potent activators of NF- κ B, but they activate through different mechanisms (Garg and Aggarwal, 2002). We examined the effect of PMF on the activation of NF- κ B by these agents using EMSA. TNF- α , CSC, PMA, LPS, and OA induced NF- κ B, and PMF suppressed activation of NF- κ B to variable degrees in KBM-5 cells: TNF- α , 52%; CSC, 80%; PMA, 51%; LPS, 28%; and OA, 80% (Fig. 1C).

Suppression of TNF- α -Dependent NF- κ B Activation by PMF Is Not Cell Type-Specific. To rule out the possibility of differences among cell types in NF- κ B activation, we tested the effect of PMF on TNF- α -induced NF- κ B activation in A293, HL-60, and H1299 cells. EMSA showed that PMF inhibited TNF- α -activated NF- κ B in these cell types (Fig. 1D) and slightly down-regulated basal NF- κ B levels in A293 cells (Fig. 1D, left). These results suggest that inhibition of NF- κ B activation by PMF was not cell type-specific.

PMF Does Not Interfere with the Binding of NF- κ B to DNA. Some NF- κ B 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- κ B to DNA. We determined whether PMF mediates suppression of NF- κ B activation through a similar mechanism. PMF did not modify the DNA-binding ability of NF- κ B proteins (Fig. 2A). These results suggest that PMF inhibits NF- κ B activation at a step upstream of its DNA binding.

PMF Prevents TNF- α -Dependent I κ B α Degradation and Phosphorylation. The translocation of NF- κ B to the

nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of I κ B α (Ghosh et al., 1998; Aggarwal, 2004). To determine whether Inhibition of TNF- α -induced NF- κ B activation was due to inhibition of I κ B α 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- κ B in the nucleus by EMSA and for I κ B α degradation in the cytoplasm by Western blot analysis. As shown in Fig. 2B, TNF- α activated NF- κ B 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 I κ B α degradation in only 5 min, correlating TNF- α -induced I κ B α degradation to TNF- α -induced NF- κ B DNA binding activation, whereas PMF prevented this degradation, although not completely (Fig. 2C, top left). These results indicate that PMF suppressed both TNF- α -induced I κ B α degradation and NF- κ B activation.

PMF Inhibits Phosphorylation of I κ B α by TNF- α and Leads to Inhibition of Ubiquitination and Degradation of I κ B α . The proteolytic degradation of I κ B α is known to require phosphorylation at Ser32 and Ser36 residues (Ghosh et al., 1998). To determine the effects of PMF on TNF- α -induced I κ B α phosphorylation, we next assay

induced phosphorylated form of I κ B α by Western blot analysis, using an antibody that recognizes the serine-phosphorylated form of I κ B α . TNF- α -induced I κ B α phosphorylation, and this phosphorylation was suppressed by PMF (Fig. 2C, bottom left). Because TNF- α -induced phosphorylation of I κ B α leads to its rapid degradation, we blocked degradation of I κ B α by using the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN). Western blot analysis showed that 1) TNF plus ALLN co-treatment induced phosphorylation of I κ B α at serine 32 and 36 and ubiquitination and 2) that PMF pretreatment decreased phosphorylation of I κ B α and ubiquitination of I κ B α in KBM-5 cells (Fig. 2C, right). This indicates that inhibition of I κ B α phosphorylation by PMF leads to inhibition of I κ B α ubiquitination.

PMF Inhibits TNF-Induced IKK Activation. It has been reported that IKK is required for TNF- α -induced phosphorylation of I κ B α (Ghosh and Karin, 2001). Because PMF inhibits the phosphorylation of I κ B α , 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 I κ B α or β proteins.

Fig. 1. A, the chemical structure of PMF. B, dose- and time-dependent effect of PMF on NF- κ B 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 nM TNF- α for 30 min. The nuclear extracts were assayed for NF- κ B activation by EMSA. Right, KBM-5 cells were preincubated with 100 μ M PMF for the indicated times and then treated with 0.1 nM TNF- α for 30 min. The nuclear extracts were prepared and assayed for NF- κ B activation by EMSA. C, PMF blocks NF- κ B activation induced by TNF- α , CSC, PMA, and OA. KBM-5 cells were preincubated with 100 μ M PMF for 16 h and then treated with 0.1 nM TNF- α for 30 min, 10 μ g/ml CSC for 1 h, 25 ng/ml PMA for 1 h, 100 ng/ml LPS for 2 h, or 500 nM OA for 4 h. Nuclear extracts were analyzed for NF- κ B activation. D, effect of PMF on activation of NF- κ B induced by TNF- α in human embryonic kidney (A293), human leukemic (HL-60), and lung carcinoma (H1299) cells incubated at 37°C with 100 μ M PMF for 16 h and then stimulated with 0.1 nM TNF- α for 30 min. After these treatments, nuclear extracts were prepared and then assayed for NF- κ B by EMSA. The results shown are representative of three independent experiments.

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 blot analysis (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- κ B-Dependent Reporter Gene Expression. Because DNA binding alone does not always correlate with NF- κ B-dependent gene transcription (Nasuhara et al., 1999), there must be additional regulatory steps. We transiently transfected the cells with NF- κ B-regulated SEAP reporter construct and measured 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 I κ B α , indicating specificity. When the cells were pretreated with PMF, TNF- α -induced NF- κ B-dependent SEAP expression was inhibited in a dose-dependent

Fig. 2. A, in vitro effect of PMF on DNA binding of NF- κ B protein. Nuclear extracts (NE) were prepared from 0.1 nM TNF- α -treated KBM-5 cells; 15 μ g/sample NE protein was treated with the indicated concentrations of PMF for 30 min at 37°C and then assayed for NF- κ B by EMSA. B, PMF inhibits TNF- α -induced activation of NF- κ B. KBM-5 cells were incubated with 100 μ M PMF for 16 h, treated with 0.1 nM TNF- α for the indicated times, and nuclear extracts were prepared and analyzed for NF- κ B activation by EMSA. C, top left, effect of PMF on TNF- α -induced degradation of I κ B α . KBM-5 cells were incubated with 100 μ M PMF for 16 h, treated with 0.1 nM TNF- α for the indicated times, and cytoplasmic extracts were prepared and analyzed by Western blotting using antibody against I κ B α . Equal protein loading was evaluated by β -actin. Bottom left, effect of PMF on TNF- α -induced phosphorylation of I κ B α . Cytoplasmic extracts were prepared and analyzed by Western blotting using phospho-specific I κ B α antibody. Equal protein loading was evaluated by β -actin. Top right, PMF decreases TNF- α -induced phosphorylation and ubiquitination of I κ B α in the presence of ALLN. Cells were preincubated with 100 μ M PMF for 16 h, incubated with 50 μ g/ml of ALLN for 30 min, and then treated with 0.1 nM TNF- α for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blotting using phospho-specific I κ B α antibody. Bottom right, the same membrane was reblotted with I κ B α antibody and β -actin. D, effect of PMF on the TNF- α -induced activation of IKK. KBM-5 cells were preincubated with 100 μ M PMF for 16 h and then treated with 1 nM 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.

manner (Fig. 3C). These results indicate that PMF inhibits NF- κ B-dependent reporter gene expression induced by TNF- α .

TNF- α -induced NF- κ B activation is mediated through sequential interaction of the TNFR with TRADD, TRAF2, NIK, TAK1/TAB1, and IKK- β , resulting in phosphorylation of I κ B α , which leads to degradation of I κ B α 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- κ B activation, cells were transiently transfected with TNFR1, TRADD, TRAF2, NIK, TAK1/TAB1, and IKK- β , and then

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

Whether PMF modulates TNF- α -induced formation of protein complexes between adaptor proteins TRADD, TRAF2, and RIP with the TNF receptor was examined by coimmunoprecipitation experiment (Fig. 3E). For this,

Fig. 3. A, PMF inhibits TNF- α -induced nuclear translocation and phosphorylation of p65. KBM-5 cells were either untreated or pretreated with 100 μ M PMF for 16 h and then stimulated with 0.1 nM TNF- α for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using antibodies against p65 (top), phospho-specific p65 (Ser536) (middle). For loading control of nuclear protein, the membrane was probed with anti-PARP antibody. B, immunocytochemical analysis of p65 localization. KBM-5 cells were first treated with 100 μ M PMF for 16 h and then stimulated with 1 nM TNF- α for 10 min. After cytospin, immunocytochemical analysis was performed. The results shown are representative of three independent experiments. C, PMF inhibited TNF- α -induced NF- κ B-dependent reporter gene (SEAP) expression. A293 cells treated with the indicated concentrations of PMF were transiently transfected with a NF- κ B-containing plasmid linked to the SEAP gene. After 24 h in culture with 1 nM 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- α -treated cells. The results were the mean of experiments performed in triplicate. D, PMF inhibited NF- κ B-dependent reporter gene expression induced by TNF- α , TNFR-1, TRADD, TRAF2, NIK, TAK1/TAB1, and IKK- β . A293 cells were pretreated with 100 μ M PMF and transiently transfected with the indicated plasmids along with a NF- κ B-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. KBM-5 cells (3×10^6 /ml) were pretreated with PMF (100 μ M) for 16 h and then incubated with TNF- α (1 nM) 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-TRADD antibody was used as a loading control.

KBM-5 cells were first pretreated with 100 μ M PMF for 16 h and then stimulated with TNF for 10 min. As indicated in Fig. 3E, TNF- α -induced the association of TNFR1 with adaptor proteins TRADD, TRAF2, and RIP; treatment of cells with PMF did not interfere with the formation of this complex. This result indicates that PMF must inhibit the NF- κ B signaling pathway without affecting the recruitment of various adaptor proteins to the TNFR1.

PMF Represses the Expression of TNF- α -Induced NF- κ B-Dependent Antiapoptotic, Proliferation, Invasion, and Angiogenic Gene Products. Because NF- κ B 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 down-regulated 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- κ B-regulated gene products involved in the proliferation of tumor cells. TNF- α has been shown to induce cyclin D1 (Gallagher et al., 1999) and COX-2 (Yamamoto et al., 1995). Therefore, we investigated whether 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- κ B-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 are regulated by NF- κ B (Estève 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- κ B has been shown to inhibit apoptosis induced by TNF- α and chemotherapeutic agents (Chen 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 , epirubicin, paclitaxel, and 5-fluorouracil (Fig. 5A).

The caspase-staining method (also called Live/Dead assay) showed that PMF up-regulated TNF- α -induced apoptosis (Fig. 5B). AnnexinV/propidium iodide (Fig. 5C, left) and TUNEL (Fig. 5C, right) likewise showed that PMF up-regulated TNF- α -induced early and late events of apoptosis. Caspase-3 and caspase-9 cleavage (Fig. 5D, left) and caspase-mediated PARP cleavage (Fig. 5D, right) showed that PMF substantially enhanced the apoptotic effect of TNF- α . These results together indicate that PMF potentiated the apoptotic effect of TNF- α and chemotherapeutic agents.

Discussion

Although 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. 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 (Lichin et al., 1994; Shi et al., 1995; Tuchinda et al., 2002).

In the present report, we found that PMF suppressed NF- κ B activation induced by inflammatory stimuli and carcinogens and that this suppression was cell type-specific. The inhibition of NF- κ B activation involved the suppression of IKK activation, leading to suppression of phosphorylation and degradation of I κ B and consequent p65 nuclear translocation. PMF also inhibited TNF- κ B-dependent reporter gene expression activated by TNF signaling elements. TNF- α -induced NF- κ B-regulated gene products involved in the regulation of apoptosis, proliferation, and invasion were down-regulated by PMF. PMF also potentiated the apoptosis induced by TNF- α and various chemotherapeutic agents.

This is the first report to suggest that PMF can suppress NF- κ B activation induced by TNF- α , okadaic acid, PMA, and OA. Although several flavones have been shown to exhibit anti-inflammatory activities, little is known about the mechanism. How PMF suppresses NF- κ B activation was investigated in detail. To our knowledge, ours is the first study to demonstrate that PMF can suppress NF- κ B activation induced by inflammatory stimuli such as TNF and carcinogens (such as okadaic acid, PMA, and cigarette smoke). It is noteworthy however, that the effect of PMF on LPS-induced NF- κ B activation was less pronounced. This suggests that PMF may have a site common to TNF- α , okadaic acid, PMA, and cigarette smoke but different from LPS-induced NF- κ B signaling. Although LPS-induced NF- κ B activation is triggered by TLR-like receptors, interacting with TRAF6 and TAK1 and then activating IKK (Takaesu et al., 2000), TNF- α -induced NF- κ B 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- α -induced NF- κ B activation upstream of TAK1. In addition, our transfection (SEAP) experiments, which show that PMF suppressed TNFR1-, TRADD-, TRAF2-, and NIK-induced NF- κ B reporter gene expression, but not that of TAK1/TAB1 and IKK β , suggest that PMF inhibits TNF-induced NF- κ B activation upstream of TAK1. In addition, coimmunoprecipitation experiments excluded the possibility 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- κ B signaling pathway downstream of the TNFR1 complex and upstream of IKK.

Upstream activators of the IKK complex remain undefined; 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 (Lee et al., 1997), Cot/TPK2 (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 potent activator of IKK (Malinin et al., 1997; Régnier et al., 1997; Woronicz et al., 1997), some 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 (Takanaga et al., 2000; Hirata et al., 2009; Sun et al., 2009; Xiao et al., 2009; Zheng et al., 2009; Dong et al., 2010), including nobiletin, tangeretin, artemetin (Ahmed et al., 1988; Sertié et al., 1990), and sinensetin (Choi et al., 2002). Besides citrus, PMF analogs 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 the PMF used in our studies is also metabolized, and whether this PMF metabolite is responsible for the suppression of the TNF short-term activation of NF- κ B signaling responses, is not clear. However, it is true that a relatively long duration of PMF incubation (16 h) is required for NF- κ B inhibition. Therefore, it is possible either the PMF gradually accumu-

Fig. 4 PMF inhibits the expression of TNF- α -induced antiapoptotic proteins. KBM-5 cells were incubated with 100 μ M PMF for 16 h and then treated with 1 nM 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 μ M PMF for 16 h and then treated with 1 nM 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 μ M PMF for 16 h and then treated with 1 nM 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.

Fig. 5. A, PMF enhances TNF- α -induced cytotoxicity. Cells (5×10^6) were seeded in triplicate in 96-well plates. The cells were pretreated with 100 μ M PMF for 16 h and then incubated with chemotherapeutic agents for 24 h. Cell viability was then analyzed by the MTT method. a, $P < 0.01$; b, $P < 0.001$. The results shown are representative of three independent experiments. B, PMF enhances TNF- α -induced cytotoxicity. KBM-5 cells were pretreated with 100 μ M PMF for 16 h and then incubated with 1 nM 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. C, PMF enhances TNF- α -induced apoptosis. Cells were pretreated with 100 μ M PMF for 16 h and then incubated with 1 nM 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 μ M PMF for 16 h and then incubated with 1 nM 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 μ M PMF for 16 h and then incubated with 1 nM 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.

lates in the cells due to a slow uptake or that a metabolite of PMF is responsible for NF- κ B inhibition. The second hypothesis has been reported for nobiletin, where metabolites of this flavone have higher potential to suppress NF- κ B transcriptional activities than nobiletin itself (Eguchi et al., 2007).

In addition, 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 (Sertié et al., 1990). It is possible that down-modulation of NF- κ B, 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 analogs of PMF are cytotoxic to various tumor cells (Lichius et al., 1994; Shi et al., 1995; Sergeev et al., 2006). Little is known, however, about the mechanism by which PMFs exhibit anticellular effects. Our studies provide an insight into one possible mechanism, the down-regulation of NF- κ B-regulated gene products that resist apoptosis and promote proliferation. Tangeretin, for example, has been shown to induce G₁ cell cycle arrest in colorectal cancer cells through up-regulation of p21 and p53 (Pan et al., 2002).

We found through down-regulation of NF- κ B that PMF also down-regulates 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 down-modulated by tangeretin (Chen et al., 2007). Although not examined in detail, tangeretin's down-modulation of COX-2 expression was assigned to suppression of NF- κ B activation. Besides suppression of COX-2, anti-inflammatory activity of PMF may also be due to its ability to suppress NO production (Fushia et al., 1999). The enzyme that mediates NO production, inducible nitric-oxide synthase, is also regulated by NF- κ B.

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

Finally, we found that PMF potentiated the apoptosis induced by TNF and the chemotherapeutic agents including cisplatin, paclitaxel, and 5-fluorouracil. Like TNF, all chemotherapeutic agents have been shown to activate NF- κ B and mediate chemoresistance (Giri and Aggarwal, 1998; Li and Sethi, 2010). Thus, it is possible that down-regulation of NF- κ B and NF- κ B-regulated survival gene products are involved in chemosensitization. In addition, the suppression of NF- κ B-regulated multidrug resistance protein reported previously (Takanaga et al., 2000; Choi et al., 2002; Patanasethanon et al., 2007) may also contribute to chemosensitization. Some reports have suggested that certain PMF analogs exhibit chemopreventive potential in vivo in Apc (Min) mouse model of colon cancer (Cai et al., 2009; Sale et al., 2009). Because NF- κ B 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 antiangiogenic effects of PMF may result from the suppression of NF- κ B and NF- κ B-regulated gene products. These results may provide the molecular basis for using these flavones to prevent and even to treat different cancers.

Acknowledgments

We thank Walter Pagel for carefully proof-reading the manuscript and providing valuable comments.

Authorship Contributions

Participated in study design: Phromnoi, Reuter, Sung, Channamasathien, Limtrakul, and Aggarwal.

Conducted experiments: Phromnoi, Reuter, Sung, Prapantapan, Channamasathien, and Limtrakul.

Contributed new reagents or analytic tools: Phromnoi, Channamasathien, Limtrakul, and Aggarwal.

Performed data analysis: Phromnoi, Reuter, Limtrakul, and Aggarwal.

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

References

- Aggarwal BB (2004) Nuclear factor- κ B: the link between inflammation and cancer. *Cancer Cell* **6**:203–208.
- Ahmed D, Choudhary ML, Singh S, and Sen B (1988) Chemical constituents of *Buxus sempervirens*. *Planta Med* **54**:174–177.
- Cai H, Sale S, Schmid R, Britton RG, Bhat K, and Ward WP, and Gescher AJ (2009) Flavones as colorectal cancer chemopreventive agents—phenol-o-methylation enhances efficacy. *Cancer Prev Res (Phila)* **2**:743–750.
- Chaturvedi MM, Choudhary A, and Aggarwal BB (2000) Assay for redox-sensitive transcription factor. *Methods Enzymol* **319**:585–602.
- Chen KH, Wu MS, and Lin JK (2007) Tangeretin suppresses IL-1 β -induced cyclooxygenase (COX-2) expression through inhibition of p38 MAPK, JNK, and AKT activation in human lung carcinoma cells. *Biochem Pharmacol* **73**:215–227.
- Choi CM, Sun H, CS, Lee JC, Hahn KS, Lee IH, Sohng JK, and Kim YC (2002) Inhibition of P-glycoprotein-mediated multidrug resistance by 5,6,7,3',4'-methoxyflavone (Sinenetin). *Biochem Biophys Res Commun* **295**:832–840.
- Chung P, Qiu Y, Li S, Ho CT, McClements DJ, and Xiao H (2010) Simultaneous determination of four 5-hydroxy polymethoxyflavones by reversed-phase high performance liquid chromatography with electrochemical detection. *J Chromatogr A* **1217**:642–647.
- Ea C, Song L, Xia ZP, Pineda G, and Chen ZJ (2006) Activation of IKK by TNF α requires site-specific ubiquitination of RIP1 and polyubiquitin binding. *NEMO. Mol Cell* **22**:245–257.
- Feng J, Mays DC, Huntoon CJ, Bell MP, Pike MG, Sandborn WJ, Lipsky JJ, and Mearns DJ (1999) Inhibition of interleukin-1-stimulated NF- κ B RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity. *J Biol Chem* **274**:26448–26453.
- Eguchi A, Murakami A, Li S, Ho CT, and Ohigashi H (2007) Suppressive effects of demethylated metabolites of nobiletin on phorbol ester-induced expression of scavenger receptor genes in THP-1 human monocytic cells. *Biofactors* **31**:107–116.
- Estève PO, Chicoine E, Robledo O, Aoudjit F, Descoteaux A, Potworowski EF, and St-Pierre Y (2002) Protein kinase C-zeta regulates transcription of the matrix metalloproteinase-9 gene induced by IL-1 and TNF- α in glioma cells via NF- κ B. *J Biol Chem* **277**:35150–35155.
- Fushia S, Kishi Y, Hattori K, Batkhui J, Takano F, Singab AN, and Okuyama T (1999) Flavonoids from *Cleome droserifolia* suppress NO production in activated macrophages in vitro. *Planta Med* **65**:404–407.
- Garg A and Aggarwal BB (2002) Nuclear transcription factor- κ B as a target for cancer drug development. *Leukemia* **16**:1053–1068.
- Ghosh S and Karin M (2002) Missing pieces in the NF- κ B puzzle. *Cell* **109** (Suppl):S81–S96.
- Ghosh S, May MJ, and Kopp EB (1998) NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**:225–260.
- Giri DK and Aggarwal BB (1998) Constitutive activation of NF- κ B causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. Autocrine role of tumor necrosis factor and reactive oxygen intermediates. *J Biol Chem* **273**:14008–14014.
- Guttridge DC, Albanese C, Reuther JY, Pestell RG, and Baldwin AS Jr (1999) NF- κ B controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol* **19**:5785–5799.
- Haldar S, Chintapalli J, and Croce CM (1996) Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res* **56**:1253–1255.
- Hirata T, Fujii M, Akita K, Yanaka N, Ogawa K, Kuroyanagi M, and Hongo D (2009) Identification and physiological evaluation of the components from citrus fruits as potential drugs for anti-cancer and anticancer. *Bioorg Med Chem* **17**:25–28.
- Hsu H, Shu HB, Pan MG, and Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* **84**:299–308.

- Hussain MM, Sokomba EN, and Shok M (1991) Pharmacological effects of *Gardenia erubescens* in mice, rats and cats. *Int J Pharmacogn* **29**:94–100.
- Laurens A, Mboup S, Tignokpa M, Sylla O, and Masquelier J (1985) [Antimicrobial activity of some medicinal species from the Dakar markets]. *Pharmazie* **40**:482–484.
- Lee FS, Hagler J, Chen ZJ, and Maniatis T (1997) Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* **88**:213–222.
- Li F and Sethi G (2010) Targeting transcription factor NF-kappaB to overcome chemoresistance and radioresistance in cancer therapy. *Biochim Biophys Acta* **1805**:167–180.
- Lichius JJ, Thoison O, Montagnac A, Pais M, Guéritte-Voegelein F, Sévenet T, Cosson JP, and Hadi AH (1994) Antimitotic and cytotoxic flavonols from *Zieridium pseudobutisifolium* and *Acronychia porteri*. *J Nat Prod* **57**:1012–1016.
- Lin X, Cunningham ET, Jr., Mu Y, Geleziunas R, and Greene WC (1999) The proto-oncogene Cot kinase participates in CD3/CD28 induction of NF-kappaB acting through the NF-kappaB-inducing kinase and IkappaB kinases. *Immunity* **10**:271–280.
- Mahon TM and O'Neill LA (1995) Studies into the effect of the tyrosine kinase inhibitor herbimycin A on NF-kappa B activation in T lymphocytes. Evidence for covalent modification of the p50 subunit. *J Biol Chem* **270**:28557–28564.
- Malinin NL, Boldin MP, Kovalenko AV, and Wallach D (1997) MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature* **385**:540–544.
- Nasuahara Y, Adcock IM, Catley M, Barnes PJ, and Newton R (1999) Differential IkappaB kinase activation and IkappaBalpha degradation by interleukin-1beta and tumor necrosis factor-alpha in human U937 monocytic cells. Evidence for additional regulatory steps in kappaB-dependent transcription. *J Biol Chem* **274**:19965–19972.
- Natarajan K, Singh S, Burke TR Jr, Grunberger D, and Aggarwal BB (1996) Cinnamic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc Natl Acad Sci USA* **93**:9090–9095.
- Pan MH, Chen WJ, Lin-Shiau SY, Ho CT, and Lin JK (2002) Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human cervical carcinoma cells. *Carcinogenesis* **23**:1677–1684.
- Patanasetthanont D, Nagai J, Matsuura C, Fukui K, Sutthanuttharpornchai BO, Yumoto R, and Takano M (2007) Modulation of function of multidrug resistance associated-proteins by *Kaempferia parviflora* extracts and their components. *Eur J Pharmacol* **566**:67–74.
- Phromnoi K, Reuter S, Sung B, Limtrakul P, and Aggarwal BB (2010) 5-Hydroxypentamethoxyflavone from *Gardenia obtusifolia* suppresses proliferation and promotes apoptosis of tumor cells through modulation of multiple cell signaling pathways. *Anticancer Res* **30**:3599–3606.
- Régnier CH, Song HY, Gao X, Goeddel DV, and Schreiber RD (2005) Identification and characterization of an IkappaB kinase. *Proc Natl Acad Sci USA* **102**:373–378.
- Sale S, Fong IL, de Giovanni C, Lazzari L, Brown J, Steward WP, and Gescher AJ (2009) APC10.1 cells as a model for assessing the efficacy of potential chemopreventive agents in the APC10.1 model in vivo. *J Cancer* **45**:2731–2735.
- Sandur SK, Ichikawa H, Chen G, KKS, and Aggarwal BB (2006) Plumbagin (5-hydroxy-2-methyl-4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and IkappaBalpha kinase activity, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *J Biol Chem* **281**:17023–17033.
- Sanz L, Sanchez J, Lallier C, Diaz-Meco MT, and Moscat J (1999) The interaction of p62 with MAP kinase atypical PKCs to NF-kappaB activation. *EMBO J* **18**:3044–3050.
- Sarkar FH and Bhattacharya SK (2008) NF-kappaB: a potential target for cancer chemoprevention and therapy. *Curr Biochem Sci* **29**:2950–2959.
- Shen Y, Li S, Chen Y, Li CT, and Dushenkov S (2006) Plumbagin, a naphthoquinone flavone, induce Ca²⁺-dependent apoptosis in breast cancer cells. *Life* **50**:253–259.
- Simeoni E, Panizza S, Matida AK, and Zelnik R (1990) Anti-inflammatory activity and acute toxicity of artemetin. *Planta Med* **56**:36–40.
- Song HY, Chen K, Li L, Chang JJ, Autry C, Kozuka M, Konoshima T, Estes JR, and Mel E (1995) Antitumor agents 154. Cytotoxic and anti-inflammatory flavonols from *Genisia dodecandra*. *J Nat Prod* **58**:475–482.
- Simeoni E, Stauber D, Chen G, Hendrichs WA, and Thanos D (1999) Mechanisms by which IkappaB proteins control NF-kappaB activity. *Proc Natl Acad Sci USA* **96**:49–54.
- Smith C, Andreaskos E, Crawley JB, Brennan FM, Feldmann M, and Foxwell BM (2001) NF-kappaB-inducing kinase is dispensable for activation of NF-kappaB in inflammatory settings but essential for lymphotoxin beta receptor activation of NF-kappaB in primary human fibroblasts. *J Immunol* **167**:5895–5903.
- Sun Y, Liu Z, Wang J, Wang Y, Zhu L, and Li L (2009) [Reparative isolation and purification of flavones from *Pericarpium Citri reticulatae* by high-speed counter-current chromatography]. *Se Pu* **27**:24–28.
- Takada Y and Aggarwal BB (2004) Curcumin inhibits NF-kappaB activation induced by various carcinogens and inflammatory cytokines through inhibition of IkappaBalpha kinase and p65 phosphorylation: abrogation of cyclin D1, cyclooxygenase-2, and matrix metalloproteinase-9. *J Biol Chem* **279**:4750–4759.
- Takaesu G, Kishida S, Hiyama T, Yamaguchi T, Shibuya T, Irie K, Ninomiya-Tsuji J, and Matsumoto K (2003) TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol Cell* **9**:659–668.
- Takaesu G, Surebhi R, Irie K, Ninomiya-Tsuji J, Matsumoto K, and Gaynor RB (2003) TAK1 critically mediates IkappaB kinase-mediated activation of NF-kappaB pathway. *J Biol Chem* **278**:115–115.
- Takanashi T, Ohnishi A, Yamaoka S, Iwano H, Morimoto S, Shoyama Y, and Iwano H (2000) Polymethoxylated flavones in orange peel are inhibitors of P-glycoprotein but not cytochrome P-450 3A4. *J Pharmacol Exp Ther* **295**:136–146.
- Takae H, Imai Y, Miura O, Yumioka E, and Watanabe T (2005) Features of anti-ulcer effect of gen-gedoku-to (a traditional Chinese medicine) and its component herb drugs. *J Pharmacol* **49**:301–308.
- Tuchinda P, Pomphim W, Reutrakul V, Pongkajon P, Yoochook C, Yai N, Sophasan S, Sujarit K, Upatham SE, and Aggarwal BB (2002) Cytotoxic and anti-HIV-1 constituents of *Gardenia obtusifolia* and their purified compounds. *Tetrahedron* **58**:773–808.
- Wahl AF, Waldson KL, Fairchild C, Lee J, Foster SA, Duggan GW, and Galloway DA (1998) Loss of normal p53 function confers sensitization to Taxol by increasing cell growth arrest and apoptosis. *Proc Natl Acad Sci USA* **95**:72–75.
- Watanabe J, Shimoto H, and Sushida (2005) Inhibitory effect of flavone derivatives from estragon and thyme on the release of chemical from RBL-2H3 Cells. *Biosci Biotechnol Biochem* **69**:1312–1315.
- Woronicz JD, Gao X, Cao Z, Mothe M, and Goeddel DV (1997) IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science* **278**:866–869.
- Xiao H, Yang CS, Jin H, Ho C, and Patel T (2009) Monodemethylated polymethoxylated sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. *Mol Nutr Food Res* **53**:398–406.
- Xiong HQ, Alizadeh Z, Li J, Wang L, Zheng L, and Xie K (2004) NF-kappaB activity blockade impairs the angiogenic potential of human pancreatic cancer cells. *Int J Cancer* **108**:185–188.
- Yamamoto K, Arizono N, and Yamamoto S (1995) Transcriptional roles of NF-kappaB and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem* **270**:31320–31320.
- Yoda T, Yoshida Y, Yabuki H, Nakazawa T, Ohsawa K, Mimaki Y, and Sashida T (2003) Urinary metabolites of nobletin orally administered to rats. *Chem Pharm Bull (Tokyo)* **51**:1426–1428.
- Yin L, Wesche H, Arthur CD, White JM, Goeddel DV, and Schreiber RD (2001) Ineffective lymphotoxin-beta receptor-induced NF-kappaB transcriptional activity in IKK-deficient mice. *Science* **291**:2162–2165.
- Yang G, Yang D, Wang D, Zhou F, Yang X, and Jiang L (2009) Simultaneous determination of five bioactive flavonoids in pericarpium *Citri reticulatae* from china by high-performance liquid chromatography with dual wavelength detection. *J Agric Food Chem* **57**:6552–6557.

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