

# ***N*-(4-Hydroxyphenyl)Retinamide Inhibits Invasion, Suppresses Osteoclastogenesis, and Potentiates Apoptosis through Down-regulation of I $\kappa$ B $\alpha$ Kinase and Nuclear Factor- $\kappa$ B–Regulated Gene Products**

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## **Abstract**

*N*-(4-hydroxyphenyl) retinamide [4-HPR], a synthetic retinoid, has been shown to inhibit tumor cell growth, invasion, and metastasis by a mechanism that is not fully understood. Because the nuclear factor- $\kappa$ B (NF- $\kappa$ B) has also been shown to regulate proliferation, invasion, and metastasis of tumor cells, we postulated that 4-HPR modulates the activity of NF- $\kappa$ B. To test this postulate, we examined the effect of this retinoid on NF- $\kappa$ B and NF- $\kappa$ B–regulated gene products. We found that 4-HPR potentiated the apoptosis induced by tumor necrosis factor (TNF) and chemotherapeutic agents, suppressed TNF-induced invasion, and inhibited RANKL-induced osteoclastogenesis, all of which are known to require NF- $\kappa$ B activation. We found that 4-HPR suppressed both inducible and constitutive NF- $\kappa$ B activation without interfering with the direct DNA binding of NF- $\kappa$ B. 4-HPR was found to be synergistic with Velcade, a proteasome inhibitor. Further studies showed that 4-HPR blocked the phosphorylation and degradation of I $\kappa$ B $\alpha$  through the inhibition of activation of I $\kappa$ B $\alpha$  kinase (IKK), and this led to suppression of the phosphorylation and nuclear translocation of p65. 4-HPR also inhibited TNF-induced Akt activation linked with IKK activation. NF- $\kappa$ B–dependent reporter gene expression was also suppressed by 4-HPR, as was NF- $\kappa$ B reporter activity induced by TNFR1, TRADD, TRAF2, NIK, and IKK but not that induced by p65 transfection. The expression of NF- $\kappa$ B–regulated gene products involved in antiapoptosis (IAP1, Bfl-1/A1, Bcl-2, cFLIP, and TRAF1), proliferation (cyclin D1 and c-Myc), and angiogenesis (vascular endothelial growth factor, cyclooxygenase-2, and matrix metalloproteinase-9) were also down-regulated by 4-HPR. This correlated with potentiation of apoptosis induced by TNF and chemotherapeutic agents. (Cancer Res 2005; 65(20): 9555–65)

## **Introduction**

A synthetic retinoid, *N*-(4-hydroxyphenyl)retinamide (4-HPR), also called fenretinide, was first reported by Sporn et al. 25 years ago. It was found to be less toxic than retinyl acetate, inhibited *N*-nitroso-*N*-methylurea–induced breast cancer in rats, had superior pharmacology to retinyl acetate, accumulated selectively in breast tissue, and exhibited antiproliferative effects against

mammary epithelium (1). This retinamide has since been shown to suppress the proliferation of a wide variety of tumor cells, including hematopoietic tumor cells (2–6), breast cancer cells (7), glioma (8), neuroblastoma (9, 10), lung cancer (11, 12), squamous cell carcinoma (12–14), melanoma (15), cervical carcinoma (16), uterine leiomyoma (17), epidermoid carcinoma (18), colon adenocarcinoma (19), prostate cancer (20), hepatoma (21), and ovarian carcinoma (22). 4-HPR is also effective in organotypic cultures of ovarian carcinoma (23). Several studies in animals have also shown that 4-HPR has activity against neuroblastoma (24), prostate cancer (25, 26), and Kaposi's sarcoma (27). These preclinical studies have led to clinical testing of the effectiveness of 4-HPR against breast cancer (28–30) and prostate cancer (31). 4-HPR is also quite effective against actinic keratoses, a precursor to squamous cell carcinoma of the skin (32).

Exactly how 4-HPR mediates its anticancer effects is poorly understood. It has been found to induce apoptosis of tumor cells independently of the retinoid receptor pathways retinoic acid receptor (RAR) and retinoid X receptor (RXR). Modulation of the antiapoptotic proteins Bcl-2 (33, 34), cyclooxygenase-2 (COX-2; refs. 35, 36), cyclin D1 (36), and c-Jun NH<sub>2</sub>-terminal kinase (37) has been implicated in 4-HPR-induced apoptosis. Other reports suggest that the mitochondrial pathway plays a special role in apoptosis induced by this retinamide (18, 34, 37). 4-HPR induces G<sub>1</sub>-S arrest in the cells (4), which is most likely mediated through its effects on cyclin D1 and pRb-cyclin-dependent kinase (CDK) regulation (38). *In vivo*, 4-HPR has been shown to exhibit anti-invasive (39), antiangiogenic (40), and antimetastatic (25) activities. Tumor cell proliferation, invasion, angiogenesis, and metastasis have been shown to be regulated by the nuclear transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B; ref. 41).

NF- $\kappa$ B is a family of Rel domain–containing proteins present in the cytoplasm of all cells, where they are kept in an inactive state by a family of anchorin domain–containing proteins, which includes I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , Bcl-3, p105, and p100. Under resting conditions, NF- $\kappa$ B consists of a heterotrimer of p50, p65, and I $\kappa$ B $\alpha$  in the cytoplasm; only when activated and translocated to the nucleus is the sequence of events leading to activation initiated. Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, okadaic acid, H<sub>2</sub>O<sub>2</sub>, and tumor necrosis factor (TNF), have been shown to activate NF- $\kappa$ B. The activation of NF- $\kappa$ B involves the phosphorylation, ubiquitination, and degradation of I $\kappa$ B $\alpha$  and phosphorylation of p65, which in turn leads to the translocation of NF- $\kappa$ B to the nucleus where it binds to specific response elements in the DNA. The phosphorylation of I $\kappa$ B $\alpha$  is catalyzed by I $\kappa$ B $\alpha$  kinase (IKK), which is essential for NF- $\kappa$ B activation by most agents. NF- $\kappa$ B has been shown to regulate the expression of several genes whose

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products are involved in tumorigenesis (41). These include antiapoptotic genes (e.g., *cIAP*, *survivin*, *TRAF*, *cFLIP*, *Bfl-1*, *Bcl-2*, and *Bcl-xL*); *COX-2*, *matrix metalloproteinase-9 (MMP-9)*; *vascular endothelial growth factor (VEGF)*; genes encoding adhesion molecules, chemokines, and inflammatory cytokines; and cell cycle regulatory genes (e.g., *cyclin D1* and *c-myc*).

Because 4-HPR regulates the expression of various gene products that are regulated by NF- $\kappa$ B and suppresses tumor cell proliferation, invasion, angiogenesis, and metastasis, which are also regulated by NF- $\kappa$ B, we postulated that this retinamide must mediate its effects through modulation of NF- $\kappa$ B activation pathway. To test this postulate, we investigated the effect of 4-HPR on NF- $\kappa$ B activation induced by a variety of inflammatory agents and carcinogens in various cell types, including those where NF- $\kappa$ B is constitutively active. We found that 4-HPR inhibited the activation of NF- $\kappa$ B through inhibition of I $\kappa$ B kinase, I $\kappa$ B $\alpha$  phosphorylation and degradation, p65 nuclear translocation, DNA binding, and NF- $\kappa$ B-dependent reporter gene expression. The suppression of NF- $\kappa$ B by 4-HPR inhibited TNF-induced cell invasion and abrogated RANKL-induced osteoclastogenesis. 4-HPR also led to the down-regulation of gene products that prevent apoptosis and promote inflammation and tumor metastasis.

## Materials and Methods

**Materials.** 4-HPR (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO as a 10 mmol/L stock solution and stored at  $-20^{\circ}\text{C}$ . Bacteria-derived human TNF, purified to homogeneity with a specific activity of  $5 \times 10^7$  units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640, fetal bovine serum (FBS), and LipofectAMINE 2000 were obtained from Invitrogen (Grand Island, NY). The following polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-p65, against the epitope corresponding to amino acids mapping within the NH<sub>2</sub>-terminal domain of human NF- $\kappa$ B p65; anti-p50, against a peptide 15 amino acids long mapping at the nuclear localization sequence region of NF- $\kappa$ B p50; anti-I $\kappa$ B $\alpha$ , against amino acids 297 to 317 mapping at the COOH terminus of I $\kappa$ B $\alpha$ /MAD-3; anti-c-Rel; anti-cyclin D1 against amino acids 1 to 295, which represents full-length cyclin D1 of human origin; anti-MMP-9; anti-poly(ADP-ribose) polymerase (PARP); anti-IAP1; anti-Bcl-2; anti-Bfl-1/A1; and anti-TRAF1. Anti-COX-2 and anti-MMP-9 antibodies were obtained from BD Biosciences (San Diego, CA), and phosphospecific anti-I $\kappa$ B $\alpha$  (Ser<sup>32</sup>), phosphorylated Akt, and phosphorylated p65 (Ser<sup>336</sup>) antibody from Cell Signaling (Beverly, MA). Anti-IKK $\alpha$  and anti-IKK $\beta$  antibodies were kindly provided by Imgenex (San Diego, CA). Velcade (PS-341) was obtained from Millennium (Cambridge, MA).

**Cell lines.** The cell lines used in our studies included human non-small cell lung carcinoma cells (H1299), human lung epithelial cell carcinoma cells (1170-I), human leukemia (Jurkat), human myelogenous leukemia (KBM-5), human multiple myeloma cell lines U266 and RPMI 8226, and human head and neck squamous cell carcinoma (HNSCC) cell lines MDA 1986, HN5, and FADU. HNSCC cell lines were obtained from Dr. Gary Clayman (University of Texas, M.D. Anderson Cancer Center, Houston, TX). 1170-I cell lines were obtained from Dr. Klein-Szanto (Fox Chase Cancer Center, Philadelphia, PA). All the other cell lines were obtained from the American Type Culture Collection (Manassas, VA). Jurkat, H1299, and multiple myeloma cells were cultured in RPMI 1640 with 10% FBS, and KBM-5 cells were cultured in Iscove's modified DMEM with 15% FBS. Raw 264.7 cells were cultured in DMEM/F-12. For 1170-I cells, keratinocyte serum-free medium was supplemented with 3% FBS. All HNSCC cell lines were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine (1%), and vitamins (2%). All media were supplemented with 100 units/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin.

**Cytotoxicity assay.** Cytotoxicity was assayed by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (42).

**Poly(ADP-ribose) polymerase cleavage assay.** For detection of cleavage products of PARP, whole-cell extracts were prepared by subjecting 4-HPR-treated cells to lysis in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01  $\mu\text{g}/\text{mL}$  aprotinin, 0.005  $\mu\text{g}/\text{mL}$  leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L NaVO<sub>4</sub>]. Lysates were spun at 14,000 rpm for 10 minutes to remove insoluble material, resolved by 10% SDS-PAGE, and probed with PARP antibodies.

**Live and dead assay.** To measure apoptosis, we used the Live and Dead assay (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity. This assay employs calcein, a polyanionic dye, which is retained within the live cells and provides green fluorescence (42). It also employs the ethidium monomer dye (red fluorescence), which can enter the cells only through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells. Briefly,  $1 \times 10^5$  cells were incubated with 10  $\mu\text{mol}/\text{L}$  4-HPR for 24 hours and then treated with 1 nmol/L TNF for 16 hours at  $37^{\circ}\text{C}$ . Cells are stained with the Live and Dead reagent (5  $\mu\text{mol}/\text{L}$  ethidium homodimer, 5  $\mu\text{mol}/\text{L}$  calcein-AM) and then incubated at  $37^{\circ}\text{C}$  for 30 minutes. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan).

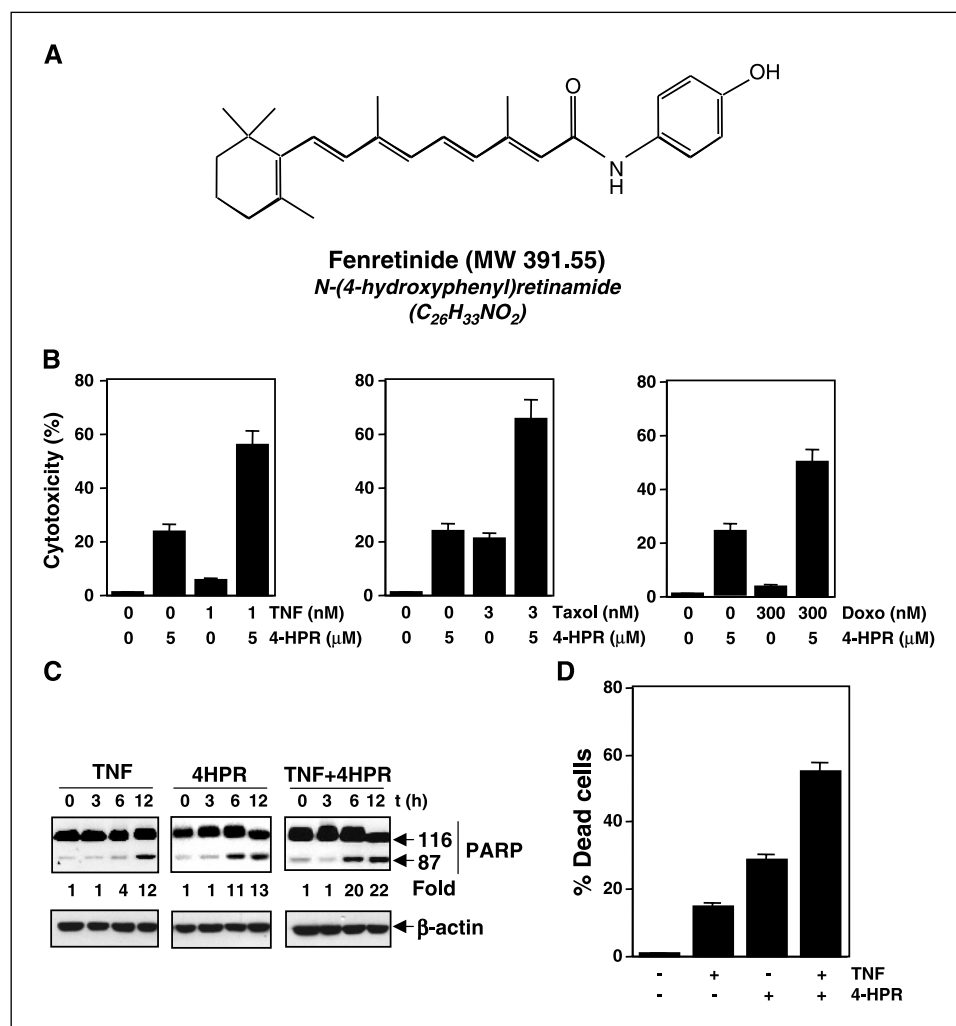
**Invasion assay.** The membrane invasion culture system was used to assess cell invasion because invasion through the extracellular matrix is a crucial step in tumor metastasis. The BD BioCoat Tumor Invasion System is a chamber that has a light-tight polyethylene terephthalate membrane with 8- $\mu\text{m}$ -diameter pores and is coated with a reconstituted basement membrane gel (BD Biosciences). A total of  $2.5 \times 10^4$  H1299 cells were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with 10  $\mu\text{mol}/\text{L}$  4-HPR for 12 hours and then stimulated with 1 nmol/L TNF for a further 24 hours in the presence of 1% FBS and the 4-HPR. The cells that invaded through the Matrigel (i.e., those that migrated to the lower chamber during incubation) were stained with 4  $\mu\text{g}/\text{mL}$  calcein-AM (Molecular Probes) in PBS for 30 minutes at  $37^{\circ}\text{C}$  and scanned for fluorescence with a Victor 3 multiplate reader (Perkin-Elmer Life and Analytical Sciences, Boston, MA); fluorescent cells were counted.

**Osteoclast differentiation assay.** To determine the effect of 4-HPR on RANKL-induced osteoclastogenesis, we cultured RAW 264.7 cells, which can differentiate into osteoclasts by RANKL *in vitro* (42). RAW 264.7 cells were cultured in 24-well dishes at a density of  $1 \times 10^4$  per well and allowed to adhere overnight. The medium was then replaced, and the cells were pretreated with 5  $\mu\text{mol}/\text{L}$  4-HPR for 12 hours and then treated with 5 nmol/L RANKL. At days 4 and 5, the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression as described previously using an acid phosphatase kit (Sigma-Aldrich), and the TRAP-positive multinucleated osteoclasts (>3 nuclei) per well were counted.

**Nuclear factor- $\kappa$ B activation.** To determine NF- $\kappa$ B activation by TNF (which has a well-established role in inflammation, tumor proliferation, promotion, invasion, and metastasis; ref. 43), we carried out electrophoretic mobility shift assay (EMSA) essentially as described previously (44). Briefly, nuclear extracts prepared from TNF-treated cells ( $1 \times 10^6/\text{mL}$ ) were incubated with <sup>32</sup>P-end-labeled 45-mer double-stranded NF- $\kappa$ B oligonucleotide (15  $\mu\text{g}$  protein with 16 fmol DNA) from the HIV long terminal repeat, 5'-TTGTTACAAGGGACTTTCGCTGGGGACTTTCAGGGAGGCGTGG-3' (boldface indicates NF- $\kappa$ B-binding sites), for 30 minutes at  $37^{\circ}\text{C}$ , and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACAACACTCACTTTCGCTGCTCACTTTCAGGGAGGCGTGG-3', was used to examine the specificity of binding of NF- $\kappa$ B to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF- $\kappa$ B for 30 minutes at  $37^{\circ}\text{C}$  before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm820 and radioactive bands were quantitated using ImageQuant software (Amersham Pharmacia Biotechnology, Piscataway, NJ).

**Western blot analysis.** To determine the effect of 4-HPR on TNF-dependent I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, p65 translocation, and

**Figure 1.** A, structure of 4-HPR. B, 4-HPR enhances apoptosis induced by TNF and chemotherapeutic agents. KBM-5 cells ( $5,000/0.1$  mL) were incubated at  $37^{\circ}\text{C}$  with TNF, Taxol, or doxorubicin (Doxo) in the presence and absence of  $5\ \mu\text{mol/L}$  4-HPR, as indicated, for 72-hour duration, and the viable cells were assayed using MTT reagent. Columns, mean cytotoxicity from triplicate cultures; bars, SD. C, 4-HPR enhances TNF-induced PARP cleavage. KBM-5 cells ( $2 \times 10^6/1$  mL) were serum starved for 24 hours and then incubated with TNF ( $1\ \text{nmol/L}$ ) alone or in combination with 4-HPR ( $25\ \mu\text{mol/L}$ ) for the indicated times, and PARP cleavage was determined by Western blot analysis as described in Materials and Methods. Values (bottom) indicate the densitometry analysis of the  $87,000$  Da band. D, 4-HPR enhances TNF-induced cell death. KBM-5 cells ( $2 \times 10^6/\text{mL}$ ) were serum starved for 24 hours and then incubated with TNF ( $1\ \text{nmol/L}$ ) alone or in combination with 4-HPR ( $10\ \mu\text{mol/L}$ ) as indicated for 24 hours. Cell death was determined by calcein-AM-based Live and Dead assay as described in Materials and Methods. Representative experiment of the three independent ones showing similar results.



p65 phosphorylation, cytoplasmic extracts were prepared as described previously (44) from H1299 cells ( $2 \times 10^6/\text{mL}$ ) that had been pretreated with  $25\ \mu\text{mol/L}$  4-HPR for 24 hours and then exposed to  $0.1\ \text{nmol/L}$  TNF for various times. Cytoplasmic protein ( $30\ \mu\text{g}$ ) was resolved on 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with specific antibody against  $\text{I}\kappa\text{B}\alpha$ , phosphorylated  $\text{I}\kappa\text{B}\alpha$ , p65, and phosphorylated p65. To determine the expression of cyclin D1, COX-2, MMP-9, cIAP1, TRAF1, Bcl-2, Bfl-1, and cFLIP in whole-cell extracts of treated cells ( $2 \times 10^6$  cells in 2 mL medium), protein ( $30\text{--}50\ \mu\text{g}$ ) was resolved on SDS-PAGE and probed by Western blot with specific antibodies as per manufacturer's recommended protocol. The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 hour, and finally detected by enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology). The bands were quantitated using a Personal Densitometer Scan version 1.30 using ImageQuant software version 3.3 (Molecular Dynamics).

**$\text{I}\kappa\text{B}\alpha$  kinase assay.** To determine the effect of 4-HPR on TNF-induced IKK activation, we analyzed IKK by a method essentially as described previously (44). Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against  $\text{IKK}\alpha$  and  $\text{IKK}\beta$  and then treated with protein A/G-Sepharose beads (Pierce, Rockford, IL). After 2 hours, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing  $50\ \text{mmol/L}$  HEPES (pH 7.4),  $20\ \text{mmol/L}$   $\text{MgCl}_2$ ,  $2\ \text{mmol/L}$  DTT,  $20\ \mu\text{Ci}$  [ $\gamma\text{-}^{32}\text{P}$ ]ATP,  $10\ \mu\text{mol/L}$  unlabeled ATP, and  $2\ \mu\text{g}$  substrate glutathione S-transferase- $\text{I}\kappa\text{B}\alpha$  (amino acids 1-54). After incubation at  $30^{\circ}\text{C}$  for 30 minutes, the reaction was terminated by boiling with SDS sample buffer for 5 minutes. Finally, the protein was resolved on 10%

SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of  $\text{IKK}\alpha$  and  $\text{IKK}\beta$  in each sample, whole-cell proteins ( $50\ \mu\text{g}$ ) were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti- $\text{IKK}\alpha$  or anti- $\text{IKK}\beta$  antibody.

**Immunolocalization of nuclear factor- $\kappa\text{B}$  p65.** The effect of 4-HPR on TNF-induced nuclear translocation of p65 was examined by an immunocytochemical method using an epifluorescence microscope (Labophot-2; Nikon, Tokyo, Japan) and a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) as described previously (44).

**Nuclear factor- $\kappa\text{B}$ -dependent reporter gene transcription.** The effect of 4-HPR on TNF-induced NF- $\kappa\text{B}$ -dependent reporter gene transcription in H1299 cells was measured as described previously (44).

**Cyclooxygenase-2 promoter-dependent reporter luciferase gene expression.** COX-2 promoter activity was examined as described elsewhere (44). To further determine the effect of 4-HPR on COX-2 promoter, A293 cells were seeded at a concentration of  $1.5 \times 10^5$  cells per well in six-well plates. After overnight culture, the cells in each well were transfected with  $2\ \mu\text{g}$  DNA consisting of COX-2 promoter-luciferase reporter plasmid along with  $6\ \mu\text{L}$  LipofectAMINE 2000 according to the manufacturer's protocol. The COX-2 promoter ( $-375$  to  $+59$ ), which was amplified from human genomic DNA by using the primers  $5\text{'-GAGTCTCTTATTATTATTTT-3\text{'}$  (sense) and  $5\text{'-GCTGCTGAGGAGTTCCTGGACGTGC-3\text{'}$  (antisense), was kindly provided by Dr. Xiao-Chun Xu (M.D. Anderson Cancer Center). After a 6-hour exposure to the transfection mixture, the cells were incubated in medium containing 4-HPR for 12 hours. The cells were exposed to TNF ( $0.1\ \text{nmol/L}$ ) for 24 hours and then harvested. Luciferase activity was

measured by using the LucLite (Perkin-Elmer) luciferase assay system according to the manufacturer's protocol and detected by luminometer (Victor 3, Perkin-Elmer). All experiments were done in triplicate and repeated at least twice to prove their reproducibility.

## Results

The goal of this study was to investigate the effect of a synthetic retinoid, 4-HPR, on the transcription factor NF- $\kappa$ B signaling pathway, on NF- $\kappa$ B-regulated gene products, and on NF- $\kappa$ B-mediated cellular responses. The structure of this retinoid is shown in Fig. 1A. The concentration of 4-HPR used and the duration of exposure had minimal effect on the viability of H1299 cells as determined by trypan blue dye exclusion test. For most studies, human small cell lung carcinoma H1299 cells were used because these cells have been shown to be sensitive to 4-HPR (11, 12). To examine the effect of 4-HPR on the NF- $\kappa$ B activation pathway, most studies employed TNF because the pathway activated by this agent is well understood.

***N*-(4-hydroxyphenyl) retinamide potentiates the apoptotic effects of tumor necrosis factor and chemotherapeutic drugs.** Because NF- $\kappa$ B activation has been shown to suppress the apoptosis induced by various agents (45), whether 4-HPR will modulate the apoptosis induced by TNF and chemotherapeutic agents was investigated. The effect of 4-HPR on TNF and chemotherapeutic agent-induced apoptosis was examined by the MTT assay. We found that 4-HPR enhances the cytotoxic effects of TNF, paclitaxel, and doxorubicin (Fig. 1B). By using caspase-activated PARP cleavage, we show that the enhanced cytotoxicity was due to apoptosis. TNF-induced PARP-cleavage was enhanced in the 4-HPR-treated cells (Fig. 1C). The Live and Dead assay, which measures intracellular esterase activity and plasma membrane integrity, also indicated that 4-HPR up-regulated TNF-induced apoptosis from 18% to 58% (Fig. 1D). The results of all the assays together suggest that 4-HPR enhances the apoptotic effects of TNF and chemotherapeutic agents.

***N*-(4-hydroxyphenyl) retinamide suppresses tumor necrosis factor-induced tumor cell invasion activity.** It is known that NF- $\kappa$ B regulates the expression of gene products (e.g., MMP-9,

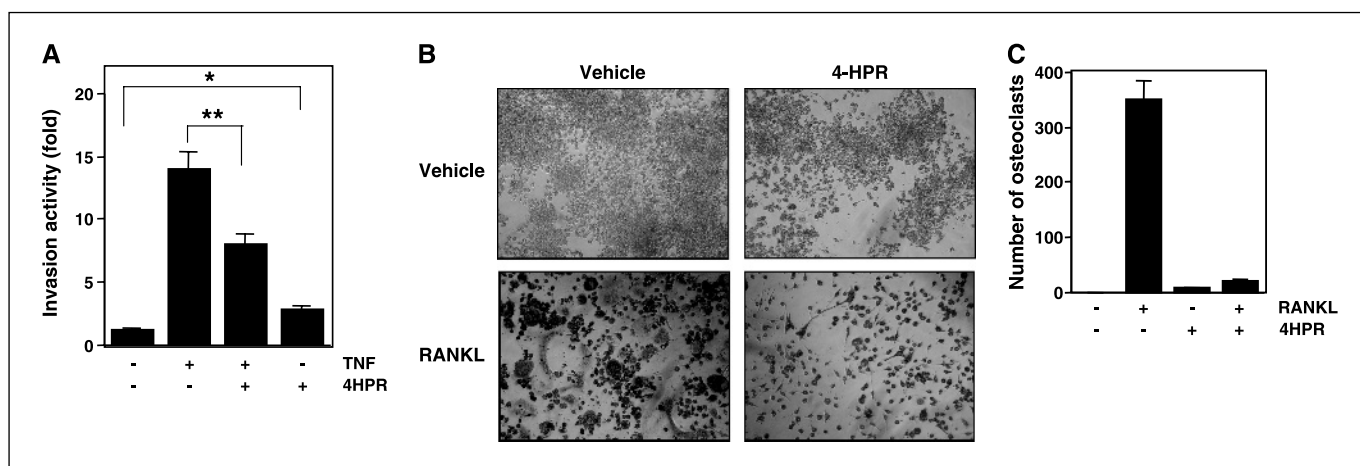
COX-2, and VEGF) that mediate tumor cell invasion (46). Whether 4-HPR can modulate TNF-induced tumor cell invasion activity was investigated *in vitro*. To determine this, tumor cells were seeded to the top chamber of the Matrigel invasion chamber with TNF in the presence or absence of 4-HPR and then examined for invasion. As shown in Fig. 2A, TNF induced tumor cell invasion by ~15-fold and 4-HPR suppressed this activity. 4-HPR alone had no invasion activity.

***N*-(4-hydroxyphenyl) retinamide suppresses RANKL-induced osteoclastogenesis.** Because RANKL, a member of the TNF superfamily, induces osteoclastogenesis through the activation of NF- $\kappa$ B (47), we determined whether 4-HPR can suppress RANKL-induced osteoclastogenesis. We found that RANKL induced osteoclast differentiation, as indicated by the expression of TRAP, and that 4-HPR suppressed it (Fig. 2B and C).

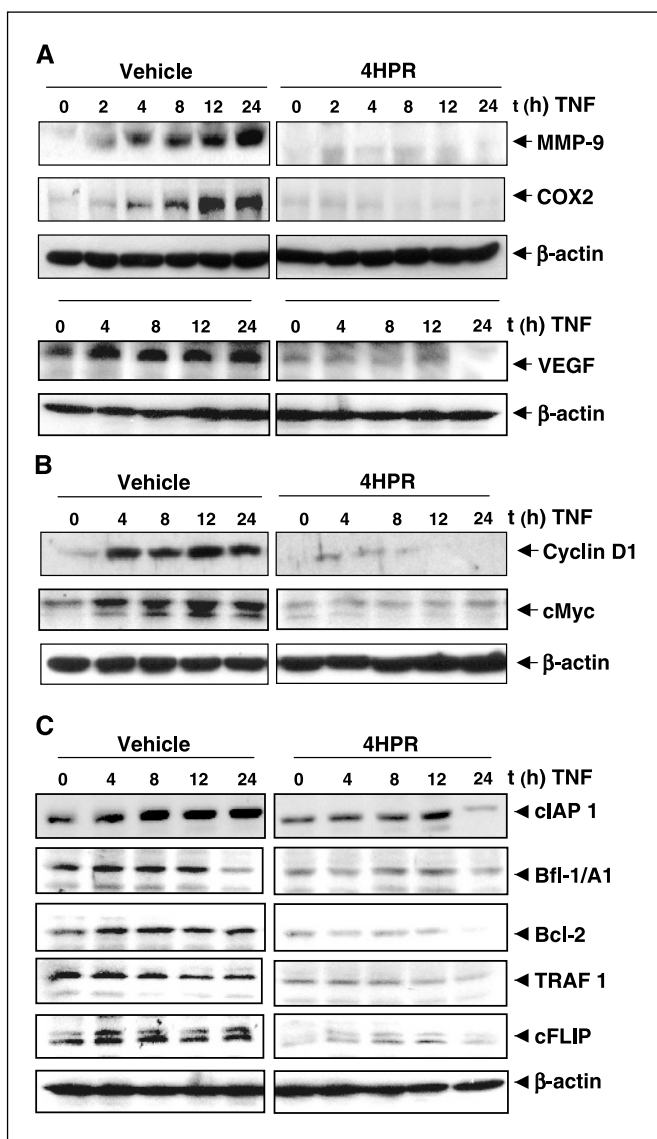
***N*-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-induced cyclooxygenase-2, matrix metalloproteinase-9, and vascular endothelial growth factor expression.** Above results indicate that 4-HPR inhibits TNF-induced tumor cell invasion. Whether these effects of 4-HPR are mediated through the suppression of COX-2, MMP-9, and VEGF gene products was investigated. As shown in Fig. 3A, TNF treatment induced the expression of VEGF, COX-2, and MMP-9 gene products and 4-HPR abolished the expression (Fig. 3A).

***N*-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-induced cyclin D1 and *c-myc* expression.** Both *cyclin D1* and *c-myc* regulate cellular proliferation and are regulated by NF- $\kappa$ B (41). Whether 4-HPR controls the expression of these gene products was also examined. Our results show that 4-HPR abolished, in a time-dependent fashion, the TNF-induced expression of *cyclin D1* and *c-myc* (Fig. 3B).

***N*-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-induced activation of antiapoptotic gene products.** Above results indicate that 4-HPR potentiates the apoptosis induced by TNF. Whether this effect of 4-HPR is through suppression of antiapoptotic gene products was investigated. NF- $\kappa$ B up-regulates the expression of several genes implicated in facilitating tumor cell survival, including *cIAP1*, *Bfl-1*, *Bcl-2*, *TRAF1*,



**Figure 2.** 4-HPR suppresses TNF-induced invasive activity and RANKL-induced osteoclastogenesis. **A**, H1299 cells ( $2.5 \times 10^4$ ) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with 10  $\mu$ M/L 4-HPR for 12 hours, treated with 1 nmol/L TNF for 24 hours in the presence of 1% serum, and then subjected to invasion assay. The value for no 4-HPR and no TNF was set to 1.0. Columns, mean of three experiments; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ . **B**, RAW 264.7 cells ( $1 \times 10^4$ ) were plated overnight, pretreated with 5  $\mu$ M/L 4-HPR for 12 hours, and then treated with 5 nmol/L RANKL. Four and 5 days later, cells were stained for TRAP and evaluated for osteoclastogenesis. Photographs were taken after 5 days of incubation with RANKL. The numbers of TRAP-positive multinucleated osteoclasts (>3 nuclei) per well were counted (**C**).



**Figure 3.** 4-HPR inhibits TNF-induced NF- $\kappa$ B-regulated gene products. **A**, 4-HPR inhibits MMP-9, COX-2, and VEGF expression induced by TNF. H1299 cells ( $2 \times 10^6$ /mL) were left untreated or incubated with 25  $\mu$ mol/L 4-HPR for 24 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared, and whole-cell lysate (80  $\mu$ g) was analyzed by Western blotting using antibodies against VEGF, MMP-9, and COX-2. **B**, 4-HPR inhibits cyclin D1 and *c-myc* expression induced by TNF. H1299 cells ( $2 \times 10^6$ /mL) were left untreated or incubated with 25  $\mu$ mol/L 4-HPR for 24 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared, and whole-cell lysate (80  $\mu$ g) was analyzed by Western blotting using antibodies against cyclin D1 and *c-myc*. Representative experiment of the three independent ones showing similar results. **C**, 4-HPR inhibits the expression of antiapoptotic gene products cIAP1, Bfl-1/A1, Bcl-2, TRAF1, and cFLIP: H1299 cells ( $2 \times 10^6$ /mL) were left untreated or incubated with 25  $\mu$ mol/L 4-HPR for 24 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared and whole-cell lysate (50  $\mu$ g) was analyzed by Western blotting using antibodies against IAP1, Bfl-1/A1, Bcl-2, TRAF1, and cFLIP as indicated. Representative experiment of the three independent ones showing similar results.

and *cFLIP* (41). We found that 4-HPR inhibited the TNF-induced as well as the basal expression of all of these proteins (Fig. 3C).

***N*-(4-hydroxyphenyl) retinamide blocks nuclear factor- $\kappa$ B activation induced by various agents.** We found that the expression of various gene products that are involved in antiapoptosis (IAP1, Bfl-1/A1, Bcl-2, cFLIP, and TRAF1), prolifer-

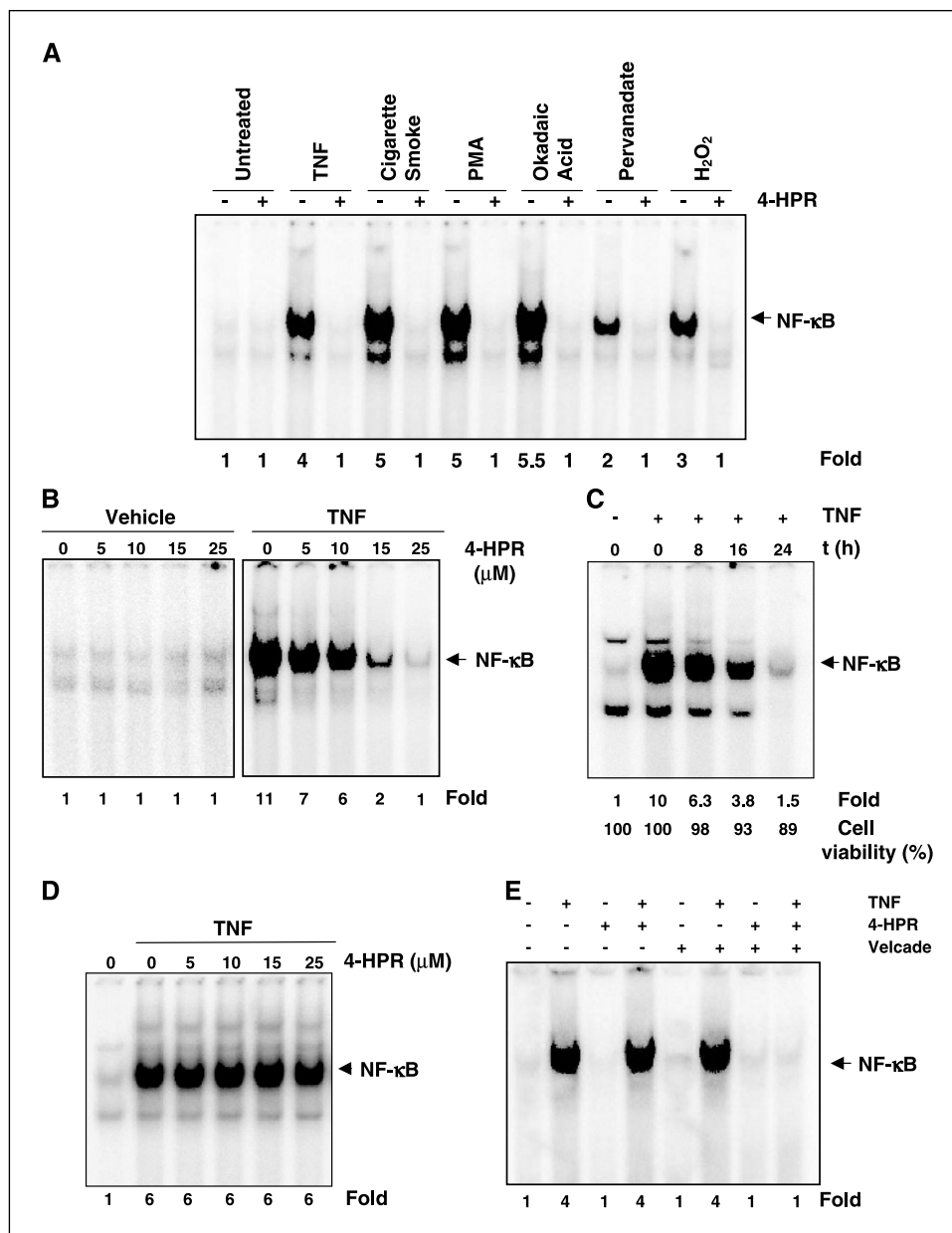
ation (cyclin D1, and *c-myc*), and invasion (VEGF, COX-2, and MMP-9) were down-regulated by 4-HPR. All of these gene products are regulated by NF- $\kappa$ B. Whether 4-HPR modulates NF- $\kappa$ B activation was investigated. We first examined the effect of 4-HPR on the activation of NF- $\kappa$ B induced by various agents, including TNF, pervanadate, phorbol 12-myristate 13-acetate (PMA), okadaic acid, cigarette smoke condensate, and H<sub>2</sub>O<sub>2</sub>. DNA-binding assay (EMSA) showed that 4-HPR suppressed the NF- $\kappa$ B activation induced by all these agents (Fig. 4A). These results suggest that 4-HPR acted at a step in the NF- $\kappa$ B activation pathway that is common to all these agents.

***N*-(4-hydroxyphenyl) retinamide suppresses nuclear factor- $\kappa$ B activation in a dose- and time-dependent manner.** We next determined the dose and time of exposure to 4-HPR required to suppress NF- $\kappa$ B activation. EMSA results showed that 4-HPR alone had no effect on NF- $\kappa$ B activation. However, it inhibited TNF-mediated NF- $\kappa$ B activation in a dose-dependent manner (Fig. 4B). The suppression of NF- $\kappa$ B activation by 4-HPR was also found to be time dependent (Fig. 4C). When nuclear extracts from TNF-activated cells were incubated with antibodies to the p50 (NF- $\kappa$ B1) and the p65 (RelA) subunit of NF- $\kappa$ B, the resulting bands were shifted to higher molecular masses (data not shown), suggesting that the TNF-activated complex consisted of p50 and p65. Preimmune serum had no effect on DNA binding. Addition of excess unlabeled NF- $\kappa$ B (cold oligonucleotide; 100-fold) caused complete disappearance of the band, whereas mutated oligonucleotide had no effect on the DNA binding.

***N*-(4-hydroxyphenyl) retinamide does not directly affect binding of nuclear factor- $\kappa$ B to the DNA.** Some NF- $\kappa$ B inhibitors, including *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; the serine protease inhibitor), herbimycin A (protein tyrosine kinase inhibitor), and caffeic acid phenethyl ester (CAPE), directly modify NF- $\kappa$ B to suppress its DNA binding (48–50). Whether 4-HPR mediates its effect through similar mechanism was examined. EMSA showed that 4-HPR did not modify the DNA-binding ability of NF- $\kappa$ B proteins prepared from TNF-treated cells (Fig. 4D). These results suggest that 4-HPR inhibits NF- $\kappa$ B activation by a mechanism different from that of TPCK, herbimycin A, or CAPE.

**Inhibition of nuclear factor- $\kappa$ B by *N*-(4-hydroxyphenyl) retinamide is potentiated by Velcade.** Our results until now show that 25  $\mu$ mol/L 4-HPR is required to inhibit TNF-induced NF- $\kappa$ B activation *in vitro*. This dose, however, is not physiologically achievable (51, 52). We, therefore, examined whether Velcade, a proteasome inhibitor, synergize with 4-HPR to inhibit NF- $\kappa$ B activation. We found that 0.05  $\mu$ mol/L Velcade or 10  $\mu$ mol/L 4-HPR alone had no effect on TNF-induced NF- $\kappa$ B activation (Fig. 4E). However, when 10  $\mu$ mol/L 4-HPR was combined with 0.05  $\mu$ mol/L Velcade, it completely suppressed TNF-induced NF- $\kappa$ B activation (Fig. 4E). These results suggest that physiologically achievable level of 4-HPR when combined with Velcade can abrogate NF- $\kappa$ B activation.

**Inhibition of nuclear factor- $\kappa$ B activation by *N*-(4-hydroxyphenyl) retinamide is not cell type specific.** It has been reported that NF- $\kappa$ B induction pathway in epithelial cells may differ from lymphoid cells (53). We therefore determined whether 4-HPR inhibited NF- $\kappa$ B activation in three different cell types. 4-HPR completely inhibited TNF-induced NF- $\kappa$ B activation in lung epithelial cell carcinoma (1170-I), T-cell leukemia (Jurkat), and myeloid leukemia (KBM-5) cells (Fig. 4F), indicating a lack of cell type specificity.



**Figure 4.** 4-HPR inhibits NF-κB. **A**, 4-HPR blocks NF-κB activation induced by TNF, sodium pervanadate, PMA, okadaic acid, and H<sub>2</sub>O<sub>2</sub>. H1299 cells (2 × 10<sup>6</sup>/mL) were preincubated for 24 hours at 37°C with 25 μmol/L 4-HPR and then treated with TNF (0.1 nmol/L), sodium pervanadate (250 μmol/L, 2 hours), PMA (100 ng/mL, 1 hours), okadaic acid (500 nmol/L, 4 hours), cigarette smoke condensate (10 μg/mL, 30 minutes), or H<sub>2</sub>O<sub>2</sub> (250 μmol/L, 1 hour). Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. Representative experiment of the three independent ones showing similar results. **B**, 4-HPR inhibits TNF-dependent NF-κB activation in a dose-dependent manner. H1299 cells (2 × 10<sup>6</sup>/mL) were preincubated with the indicated concentrations of 4-HPR for 24 hours at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes. Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. **C**, 4-HPR inhibits TNF-dependent NF-κB activation in a time-dependent manner. H1299 cells (2 × 10<sup>6</sup>/mL) were preincubated with 25 μmol/L 4-HPR for the indicated times at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation. Cell viability (%) is indicated. **D**, 4-HPR does not modulate the ability of NF-κB to bind to the DNA. Nuclear extracts from H1299 cells (2 × 10<sup>6</sup>/mL) treated or not treated with 0.1 nmol/L TNF for 30 minutes were treated with the indicated concentrations of 4-HPR for 2 hours at room temperature and then assayed for DNA binding by EMSA. Representative experiment of the three independent ones showing similar results. **E**, Velcade synergizes with 4-HPR in inhibiting TNF-induced NF-κB activation. H1299 cells (2 × 10<sup>6</sup>/mL) were pretreated with 10 μmol/L 4-HPR and/or 0.05 μmol/L Velcade as indicated for 24 hours and then treated with 0.1 nmol/L TNF for 30 minutes. Nuclear extracts were prepared and then assayed for DNA binding by EMSA. Representative experiment of the three independent ones showing similar results.

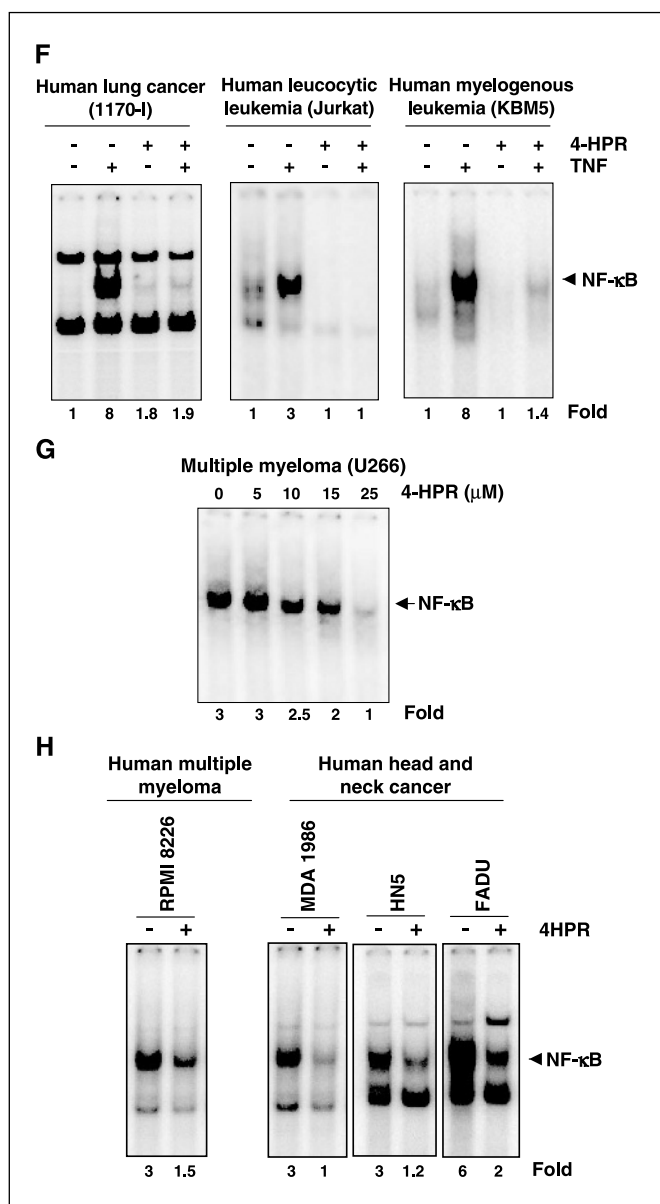
**N-(4-hydroxyphenyl) retinamide inhibits constitutive nuclear factor-κB activation.** Various tumor cells are known to express constitutively active NF-κB (41). To determine whether 4-HPR can inhibit constitutive NF-κB activation, we treated multiple myeloma (U266) cells with varying doses of 4-HPR. We found that 4-HPR inhibited the constitutive NF-κB activation in a dose-dependent manner with optimum inhibition occurring at 25 μmol/L (Fig. 4G). Whether constitutive NF-κB in other tumor cells is also affected by 4-HPR was also examined. As shown in Fig. 4H, 4-HPR inhibited constitutively active NF-κB in other multiple myeloma (RPMI 8226) and HNSCC (MDA 1986, HN5, and FADU) tumor cells.

**N-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-dependent IκBα degradation.** Because IκBα degradation is required for activation of NF-κB (41), we determined whether 4-HPR's inhibition of TNF-induced NF-κB activation was due to inhibition of IκBα degradation. We found that TNF induced IκBα degradation in control cells as early as 10 minutes,

but in 4-HPR-pretreated cells TNF had no effect on IκBα degradation (Fig. 5A, top).

**N-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-dependent IκBα phosphorylation.** We next determined whether 4-HPR affected TNF-induced IκBα phosphorylation needed for IκBα degradation. Western blot analysis using antibody that detects only the serine-phosphorylated form of IκBα indicated that TNF induced IκBα phosphorylation as early as 5 minutes, and 4-HPR completely suppressed it (Fig. 5A, middle). Thus, 4-HPR inhibited TNF-induced NF-κB activation by inhibiting phosphorylation and degradation of IκBα.

**N-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-induced IκBα kinase activation.** Because 4-HPR inhibits the phosphorylation and degradation of IκBα, we tested the effect of 4-HPR on TNF-induced IKK activation, which is required for TNF-induced phosphorylation of IκBα. As shown in Fig. 5B (top), 4-HPR completely suppressed TNF-induced activation of IKK.



**Figure 4.** Continued. *F*, suppression of inducible activation is not cell type specific. Two million KBM-5, Jurkat, or 1170-I cells were pretreated with 25 μmol/L 4-HPR for 24 hours and then treated with 0.1 nmol/L TNF for 30 minutes. The nuclear extracts were then prepared and assayed for NF-κB by EMSA as described in Materials and Methods. *G*, 4-HPR suppresses constitutive NF-κB activation in U266 cells. Two million multiple myeloma (U266) cells were exposed to varying concentrations of 4-HPR for 24 hours; then, nuclear extracts were prepared and assayed for NF-κB by EMSA as described in Materials and Methods. *H*, suppression of constitutive NF-κB is not cell type specific. Two million multiple myeloma and HNSCC cells were exposed to 25 μmol/L 4-HPR for 24 hours; then, nuclear extracts were prepared and assayed for NF-κB by EMSA as described in Materials and Methods. Representative experiment of the three independent ones showing similar results.

TNF or 4-HPR had no direct effect on the expression of IKK protein (*bottom*).

Next, we examined the effect of 4-HPR on IKK activity *in vitro*. We found that 4-HPR did not directly interfere with the IKK activity (Fig. 5C). Because treatment of cells inhibits TNF-induced IKK activity, 4-HPR must suppress the activation of IKK.

***N*-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-induced Akt activation.** TNF has been shown to activate

IKK through activation of Akt (54). Therefore, we also tested the effect of 4-HPR on TNF-induced activation of Akt. As shown in Fig. 5D, TNF induced the activation of Akt in a time-dependent manner, and 4-HPR treatment suppressed the activation.

***N*-(4-hydroxyphenyl) retinamide inhibits tumor necrosis factor-induced phosphorylation and nuclear translocation of p65.** We also tested the effect of 4-HPR on TNF-induced phosphorylation of p65, because phosphorylation is also required for transcriptional activity of p65 (41). As shown in Fig. 5E, 4-HPR suppressed p65 phosphorylation almost completely. Likewise, Western blot analysis (Fig. 5F) and immunocytochemistry (Fig. 5G) indicated that 4-HPR abolished TNF-induced nuclear translocation of p65.

***N*-(4-hydroxyphenyl) retinamide represses tumor necrosis factor-induced nuclear factor-κB-dependent reporter gene expression.** Our results up to this point show that 4-HPR inhibited the translocation of p65 inside the nucleus. To further determine that 4-HPR inhibited NF-κB-dependent gene transcription, we transiently transfected cells with the NF-κB-regulated secretory alkaline phosphatase (SEAP) reporter construct and then stimulated them with TNF. We found that TNF produced an ~20-fold increase in SEAP activity over vector control (Fig. 6A), which was inhibited by dominant-negative IκBα, indicating specificity. When the cells were pretreated with 4-HPR, TNF-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner. These results show that 4-HPR inhibits NF-κB-dependent reporter gene expression induced by TNF.

We next determined where 4-HPR acts in the sequence of TNFR1, TRADD, TRAF2, NIK, and IKK recruitment that characterizes TNF-induced NF-κB activation (41). In cells transfected with TNFR1, TRADD, TRAF2, NIK, IKKβ, and p65 plasmids, NF-κB-dependent reporter gene expression was induced; 4-HPR suppressed SEAP expression in all cells, except those transfected with p65 (Fig. 6B).

***N*-(4-hydroxyphenyl) retinamide represses tumor necrosis factor-induced cyclooxygenase-2 promoter activity.** We next determined whether 4-HPR affected COX-2 promoter activity, which is regulated by NF-κB (41). As shown in Fig. 6C, 4-HPR inhibited the TNF-induced COX-2 promoter activity in a dose-dependent manner.

## Discussion

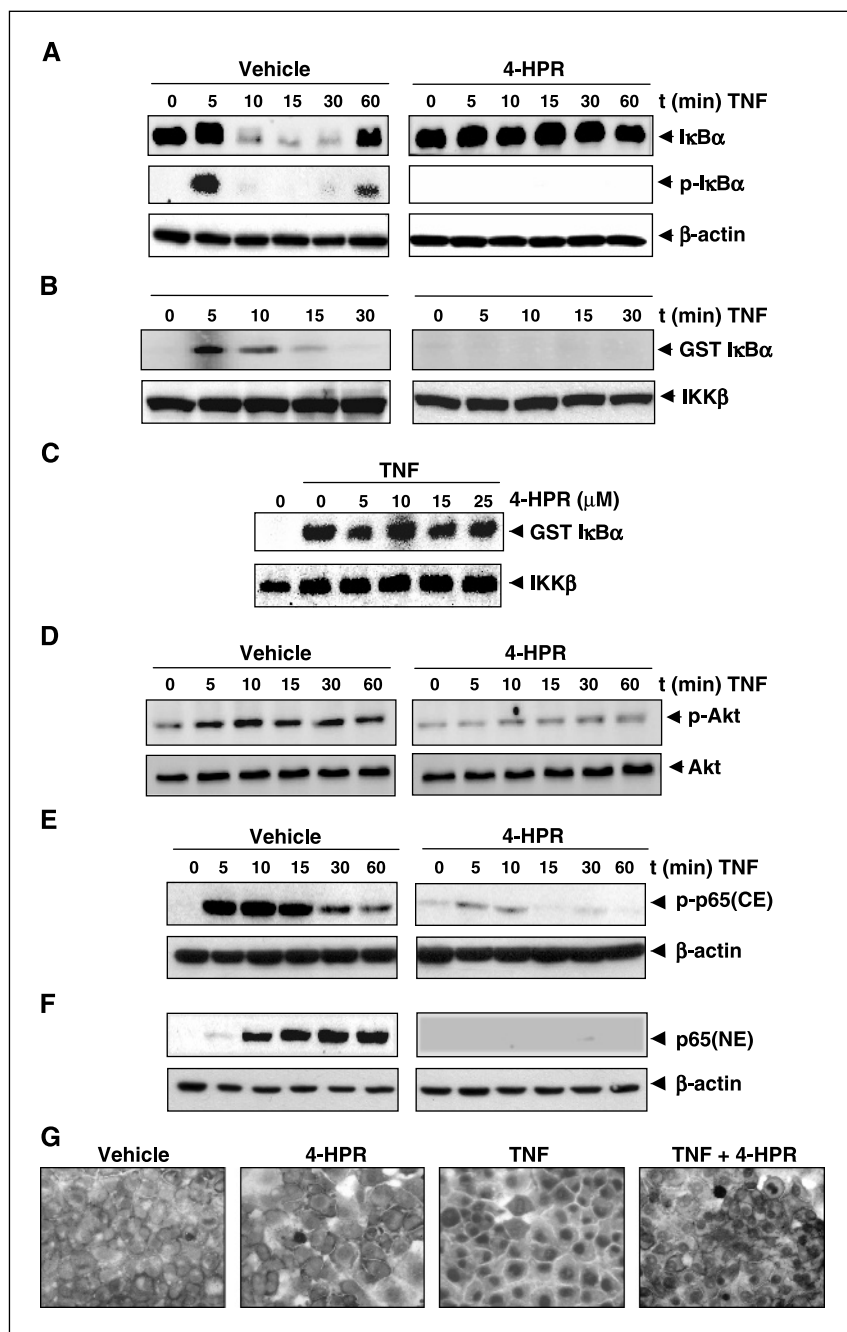
The present study was designed to investigate the effect of 4-HPR on the NF-κB activation pathway and on the NF-κB-regulated gene products that control tumor cell survival, proliferation, invasion, angiogenesis, and metastasis (see Fig. 6D). We found that 4-HPR potentiated the apoptosis induced by TNF and chemotherapeutic agents and inhibited TNF-induced invasion and RANKL-induced osteoclastogenesis. 4-HPR suppressed NF-κB activated by carcinogens, tumor promoters, and inflammatory stimuli in a variety of cell lines. This inhibition was mediated through inhibition of IKK by 4-HPR, which led to suppression of phosphorylation and degradation of IκBα. 4-HPR also inhibited the TNF-induced phosphorylation of p65, nuclear p65 translocation, and NF-κB-dependent reporter gene activity. The expression of gene products involved in antiapoptosis (IAP1, Bfl-1/A1, Bcl-2, TRAF1, and cFLIP), proliferation (cyclin D1 and c-Myc), and metastasis (MMP-9, COX-2, and VEGF) were also down-regulated by 4-HPR.

Our results indicate that 4-HPR suppresses NF-κB activated by a wide variety of agents. This is the first report to examine the

effect of 4-HPR on NF- $\kappa$ B activated by various stimuli. These results suggest that 4-HPR must act at a step common to all these agents. We found that 4-HPR blocked the activation of NF- $\kappa$ B without directly interfering with the DNA binding of NF- $\kappa$ B. Further analysis of the pathway indicated that 4-HPR targets at the level of IKK. It seems that our results differ from that of Bayon et al. where they did not find any IKK inhibition with 4-HPR. The difference in the results is actually due to the dose and time used. They used 6  $\mu$ mol/L 4-HPR for 4 hours, and this dose and time had no effect in our system either. We found that treatment of cells with 25  $\mu$ mol/L 4-HPR for 24 hours is needed to inhibit IKK activation. However, our *in vitro* kinase assay results show that 4-HPR is not a direct inhibitor of IKK. Thus, it seems that 4-HPR blocks the activation of IKK by

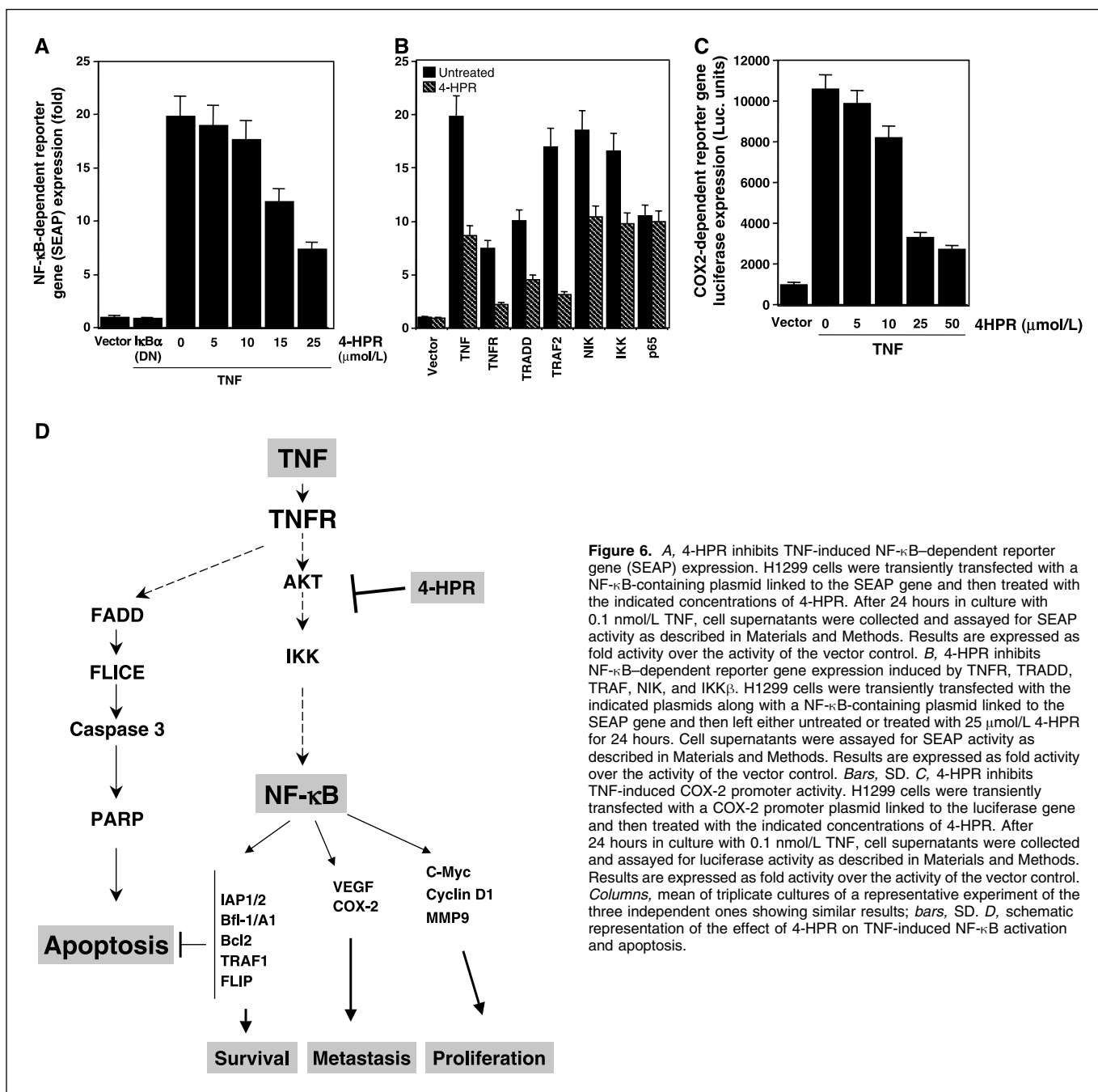
interfering with some upstream regulatory kinases. Akt, NIK, mitogen-activated protein kinase kinase kinase 1, and atypical protein kinase C are candidates because they are upstream kinases that regulate IKK (41). Indeed, our study shows that 4-HPR inhibits TNF-induced Akt activation. Suppression of TNF-induced Akt activation may lead to inhibition of IKK activation. The effect of 4-HPR on Akt activation was specific as TNF-induced extracellular signal-regulated kinase activation was unaffected (data not shown).

We found that 4-HPR inhibited not only inducible NF- $\kappa$ B activation but also constitutively activated NF- $\kappa$ B in multiple myeloma and HNSCC cells. These results agree with a previous report that showed suppression of constitutive NF- $\kappa$ B by 4-HPR in human prostate DU145 and PC3 cells (55). Constitutive active



**Figure 5.** A, 4-HPR inhibits TNF-induced phosphorylation and degradation of I $\kappa$ B $\alpha$ . H1299 cells ( $2 \times 10^6$ /mL) were incubated with 25  $\mu$ mol/L 4-HPR for 24 hours at 37°C, treated with 0.1 nmol/L TNF for the indicated times at 37°C, and then tested for I $\kappa$ B $\alpha$  (top) and phosphorylated I $\kappa$ B $\alpha$  (middle) in cytosolic fractions by Western blot analysis. Equal protein loading was evaluated by  $\beta$ -actin (bottom). B, 4-HPR inhibits TNF-induced IKK activity. H1299 cells ( $2 \times 10^6$ /mL) were treated with 25  $\mu$ mol/L 4-HPR for 24 hours and then treated with 0.1 nmol/L TNF for the indicated time intervals. Whole-cell extracts were prepared and extract (200  $\mu$ g) was immunoprecipitated with antibodies against IKK $\alpha$  and IKK $\beta$ . Thereafter, immune complex kinase assay was done as described in Materials and Methods. To examine the effect of 4-HPR on the level of expression of IKK proteins, whole-cell extract (30  $\mu$ g) was run on 10% SDS-PAGE, electrotransferred, and immunoblotted with indicated antibodies as described in Materials and Methods. C, 4-HPR has no direct effect on IKK activity. Whole-cell extracts were prepared from untreated and TNF (0.1 nmol/L)-treated H1299 cells ( $2 \times 10^6$ /mL); protein/sample whole-cell extract (200  $\mu$ g) was immunoprecipitated with antibodies against IKK $\alpha$  and IKK $\beta$ . The immune complex was treated with the indicated concentrations of 4-HPR for 30 minutes at 30°C; then, a kinase assay was done as described in Materials and Methods. Equal protein loading was evaluated by IKK $\beta$ . D, 4-HPR inhibits Akt phosphorylation. H1299 cells ( $2 \times 10^6$ /mL) were incubated with 25  $\mu$ mol/L 4-HPR for 24 hours and then treated with 0.1 nmol/L TNF for the indicated times. The whole cell extracts were analyzed by Western blotting using antibodies against the phosphorylated Akt. Equal protein loading was evaluated by Akt. Representative experiment of the three independent ones showing similar results. E, 4-HPR inhibits TNF-induced phosphorylation of p65. H1299 cells ( $2 \times 10^6$ /mL) were incubated with 25  $\mu$ mol/L 4-HPR for 24 hours and then treated with 0.1 nmol/L TNF for the indicated times. The cytoplasmic extracts were analyzed by Western blotting using antibodies against the phosphorylated form of p65. F, 4-HPR inhibits TNF-induced nuclear translocation of p65. H1299 cells ( $1 \times 10^6$ /mL) were either untreated or pretreated with 25  $\mu$ mol/L 4-HPR for 24 hours at 37°C and then treated with 0.1 nmol/L TNF for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using antibodies against p65. G, 4-HPR inhibits TNF-induced nuclear translocation of p65. H1299 cells ( $1 \times 10^6$ /mL) were first treated with 25  $\mu$ mol/L 4-HPR for 24 hours at 37°C and then exposed to 0.1 nmol/L TNF. After cytospin, immunocytochemical analysis was done as described in Materials and Methods. Representative experiment of the three independent ones showing similar results.





**Figure 6.** A, 4-HPR inhibits TNF-induced NF- $\kappa$ B-dependent reporter gene (SEAP) expression. H1299 cells were transiently transfected with a NF- $\kappa$ B-containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of 4-HPR. After 24 hours in culture with 0.1 nmol/L TNF, cell supernatants were collected and assayed for SEAP activity as described in Materials and Methods. Results are expressed as fold activity over the activity of the vector control. **B**, 4-HPR inhibits NF- $\kappa$ B-dependent reporter gene expression induced by TNFR, TRADD, TRAF, NIK, and IKK $\beta$ . H1299 cells were transiently transfected with the indicated plasmids along with a NF- $\kappa$ B-containing plasmid linked to the SEAP gene and then left either untreated or treated with 25  $\mu$ M 4-HPR for 24 hours. Cell supernatants were assayed for SEAP activity as described in Materials and Methods. Results are expressed as fold activity over the activity of the vector control. **Bars**, SD. **C**, 4-HPR inhibits TNF-induced COX-2 promoter activity. H1299 cells were transiently transfected with a COX-2 promoter plasmid linked to the luciferase gene and then treated with the indicated concentrations of 4-HPR. After 24 hours in culture with 0.1 nmol/L TNF, cell supernatants were collected and assayed for luciferase activity as described in Materials and Methods. Results are expressed as fold activity over the activity of the vector control. **Columns**, mean of triplicate cultures of a representative experiment of the three independent ones showing similar results; **bars**, SD. **D**, schematic representation of the effect of 4-HPR on TNF-induced NF- $\kappa$ B activation and apoptosis.

NF- $\kappa$ B activation has been found to be critical for the survival and proliferation of various tumor cell types (41); however, the mechanism of constitutive NF- $\kappa$ B activation is not well understood. Some of the potential mechanisms are overexpression of I $\kappa$ B $\alpha$  without inhibition of NF- $\kappa$ B activity, mutations in the *I $\kappa$ B $\alpha$*  gene, enhanced I $\kappa$ B $\alpha$  degradation, and constitutive expression of TNF and interleukin-1 (41).

The genes that are involved in the proliferation and metastasis of cancer have been shown to be regulated by NF- $\kappa$ B (41). We show in this report that 4-HPR inhibits the expression of cyclin D1 and c-myc, which are both regulated by NF- $\kappa$ B. Our results are consistent with recent reports where 4-HPR has been shown to block the G<sub>1</sub>-S transition by inhibiting the phosphorylation of Rb,

CDK4, and cyclin D protein complex (4, 38, 56). 4-HPR has also been shown to inhibit the expression of c-Myc in several tumor cell lines (33). Our results also show that the expression of COX-2, MMP-9, and VEGF, also regulated by NF- $\kappa$ B, are down-regulated by 4-HPR. Indeed, 4-HPR has been shown to down-regulate COX-2 gene expression in human colon adenocarcinoma cell lines (19). Activation of NF- $\kappa$ B is known to promote angiogenesis and invasion, and 4-HPR is anti-invasive (39) and antiangiogenic (27). These results imply that 4-HPR exercises its anticancer properties through the inhibition of NF- $\kappa$ B.

NF- $\kappa$ B is known to regulate the expression of IAP1, xIAP, Bfl-1/A1, TRAF1, Bcl-2, cFLIP, and survivin, and their overexpression in numerous tumors has been linked to survival, chemoresistance,

and radioresistance. Our results indicate that 4-HPR treatment down-regulates most of these gene products. Earlier studies have shown that 4-HPR induces apoptosis through a mitochondrial pathway regulated by proteins from the Bcl-2 family. The levels of Bcl-2 mRNA were markedly diminished by 4-HPR treatment in several tumor cell types (33, 34). Our studies also show that 4-HPR potentiated the apoptotic effects of TNF, Taxol, and doxorubicin. These effects are similar to that reported with a specific inhibitor of NF- $\kappa$ B (57). These results are also consistent with a recent report that 4-HPR potentiates the apoptotic effects of TRAIL (58), another member of the TNF superfamily.

Our results indicate that exposure of cells to 25  $\mu$ mol/L 4-HPR is needed to suppress NF- $\kappa$ B activation. Although it is difficult to correlate *in vitro* concentrations to that *in vivo*, a plasma concentration of 12.9  $\mu$ mol/L in the patients and 7.9  $\mu$ mol/L in the tissue have been reported (51, 52). We found that when combined with 50 nmol/L Velcade, 10  $\mu$ mol/L 4-HPR is sufficient to inhibit NF- $\kappa$ B activation. We found that 24-hour treatment was needed to suppress NF- $\kappa$ B activation. This suggests slow cellular accumulation of 4-HPR. Similar observations have been made by others (33–35, 55). Because 4-HPR was also effective in cells where NF- $\kappa$ B is constitutively active (see Fig. 4G and H), it suggests that the chemopreventive effects of 4-HPR could be mediated through NF- $\kappa$ B suppression.

Most retinoids are known to act through binding to the RARs and RXRs. Evidence exists for and against participation of retinoid receptors in 4-HPR-elicited cellular events. Transactivation studies have shown that 4-HPR can activate certain RAR and RXR (59), but studies in human breast carcinomas have shown that 4-HPR-mediated biological actions involve retinoid receptor-independent pathways (60). Overall, our results indicate that antiproliferative, proapoptotic, anti-invasive, antiosteoclastogenic, antiangiogenic, and antimetastatic effects assigned to 4-HPR may be mediated through suppression of NF- $\kappa$ B-regulated gene products. Considering the lack of effect on normal cells (61), 4-HPR has a great potential for prevention and treatment of cancer.

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