**N-(4-Hydroxyphenyl)Retinamide Inhibits Invasion, Suppresses Osteoclastogenesis, and Potentiates Apoptosis through Down-regulation of IκBα Kinase and Nuclear Factor-κB–Regulated Gene Products**

Shishir Shishodia,1 Angelica M. Gutierrez,1 Reuben Lotan,2 and Bharat B. Aggarwal1

1Cytokine Research Laboratory, Department of Experimental Therapeutics and 2Thoracic/Head and Neck Medical Oncology, University of Texas M.D. Anderson Cancer Center, Houston, Texas

**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-κB (NF-κB) has also been shown to regulate proliferation, invasion, and metastasis of tumor cells, we postulated that 4-HPR modulates the activity of NF-κB. To test this postulate, we examined the effect of this retinoid on NF-κB and NF-κB–regulated gene products. We found that 4-HPR potentiated the apoptosis induced by tumor necrosis factor (TNF) and chemotherapeutic agents, suppressed RANKL-induced osteoclastogenesis, all of which are known to require NF-κB activation. We found that 4-HPR suppressed both inducible and constitutive NF-κB activation without interfering with the direct DNA binding of NF-κB. 4-HPR was found to be synergistic with Velcade, a proteasome inhibitor. Further studies showed that 4-HPR blocked the phosphorylation and degradation of IκBα through the inhibition of activation of IκBα 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-κB–dependent reporter gene expression was also suppressed by 4-HPR, as was NF-κB reporter activity induced by TNFR1, TRADD, TRAF2, NIK, and IKK but not that induced by p65 transfection. The expression of NF-κB–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-methyleurea–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 NH2-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 G1/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-κB (NF-κB; ref. 41).

NF-κB 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 IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p105, and p100. Under resting conditions, NF-κB consists of a heterotrimer of p50, p65, and IκBα 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, H2O2, and tumor necrosis factor (TNF), have been shown to activate NF-κB. The activation of NF-κB involves the phosphorylation, ubiquitination, and degradation of IκBα and phosphorylation of p65, which in turn leads to the translocation of NF-κB to the nucleus where it binds to specific response elements in the DNA. The phosphorylation of IκBα is catalyzed by IκBα kinase (IKK), which is essential for NF-κB activation by most agents. NF-κB has been shown to regulate the expression of several genes whose
products are involved in tumorigenesis (41). These include antiapoptotic genes (e.g., cIAP, survivin, TRAF, cFLIP, 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-κB and suppresses tumor cell proliferation, invasion, angiogenesis, and metastasis, which are also regulated by NF-κB, we postulated that this retinamide must mediate its effects through modulation of NF-κB activation pathway. To test this postulate, we investigated the effect of 4-HPR on NF-κB activation induced by a variety of inflammatory agents and carcinogens in various cell types, including those where NF-κB is constitutively active. We found that 4-HPR inhibited the activation of NF-κB through inhibition of IκB kinase, IκB degradation, p65 nuclear translocation, DNA binding, and NF-κB–dependent reporter gene expression. The suppression of NF-κB 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°C. Bacteria-derived human TNF, purified to homogeneity with a specific activity of 5 × 10^5 units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640, fetal bovine serum (FBS), and Lipopectamine 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 NH2-terminal domain of human NF-κB p65; anti-p50, against a peptide 15 amino acids long mapping at the nuclear localization sequence region of NF-κB p50; anti-IκBα, against amino acids 297 to 317 mapping at the COOH terminus of IκBα/MAD-3; anti-c-Rel; anti-β-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-IκBα (Ser32), phosphorylated Akt, and phosphorylated p65 (Ser536) antibody were obtained from Cell Signaling (Beverly, MA). Anti-IκBα and anti-IκBα antibodies were kindly provided by Imgenex (San Diego, CA). Velaide (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 cells U266 and RPMI 8226, and human head and neck squamous cell carcinoma (HNSSC) cell lines MDA 1986, HN5, and FADU. HNSSC cell lines were obtained from Dr. Gary Clayman (University of Texas, MD. 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 HNSSC cell lines were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glucose (1%), and vitamins (2%). All media were supplemented with 100 units/mL penicillin and 100 μg/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 μg/mL aprotinin, 0.005 μg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L Na3VO4). 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 polyionic 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 × 10^5 cells were incubated with 10 μmol/L 4-HPR for 24 hours and then treated with 1 mmol/L TNF for 16 hours at 37°C. Cells are stained with the Live and Dead reagent (5 μmol/L ethidium homodimer, 5 μmol/L calcein-AM) and then incubated at 37°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-μm-diameter pores and is coated with a reconstituted basement membrane gel (BD Biosciences). A total of 2.5 × 10^4 H1299 cells were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with 10 μmol/L 4-HPR for 12 hours and then stimulated with 1 mmol/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 μg/mL calcein-AM (Molecular Probes) in PBS for 30 minutes at 37°C and scanned for fluorescence with a Victor 3 multichannel plate 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 × 10^5 per well and allowed to adhere overnight. The medium was then replaced, and the cells were pretreated with 5 μmol/L 4-HPR for 12 hours and then treated with 5 mmol/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-κB activation. To determine NF-κB activation by TNF (which has a well-established role in inflammation, tumor proliferation, 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 × 10^5/mL) were incubated with 32P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg protein with 16 fmol DNA) from the HIV long terminal repeat, 5′-TTTTTACAAGGACCATTTCCGCTGGGGACCTTTCCCCAGGGAGCCGTGG-3′ (boldeface indicates NF-κB–binding sites), for 30 minutes at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTTTTACAACCTCATTTCCGCTCCCATTTCCAGGGAGCCGTTG-3′, was used to examine the specificity of binding of NF-κB 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 p65 or the p50 subunit of NF-κB for 30 minutes at 37°C before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm280 and radioactive bands were quantitated using ImageQuant software (Amersham Pharmacia Biotech- nology, Piscataway, NJ).

Western blot analysis. To determine the effect of 4-HPR on TNF-dependent IκBα phosphorylation, IκBα degradation, p65 translocation, and
p65 phosphorylation, cytoplasmic extracts were prepared as described previously (44) from H1299 cells (2 × 10^6/mL) that had been pretreated with 25 μmol/L 4-HPR for 24 hours and then exposed to 0.1 nmol/L TNF for various times. Cytoplasmic protein (30 μg) was resolved on 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with specific antibody against IκBα, phosphorylated IκBα, 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 × 10^6 cells in 2 mL medium), protein (30-50 μg) 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.

**Immunocolocalization of nuclear factor-κB 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 Coolscan CF color camera (Nikon, Lewisville, TX) as described previously (44).

**Nuclear factor-κB-dependent reporter gene transcription.** The effect of 4-HPR on TNF-induced NF-κB-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 × 10^3 cells per well in six-well plates. After overnight culture, the cells in each well were transfected with 2 μg DNA consisting of COX-2 promoter-luciferase reporter plasmid along with 6 μL 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’-GAGTCTCTTATTTTTTT-3’ (sense) and 5’-GCTGCTGAGGAGTTCCTGGACGTGC-3’ (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 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-κB signaling pathway, on NF-κB–regulated gene products, and on NF-κB-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-κB 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 chemotherapy drugs.

Because NF-κB 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. 1A). 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-κB 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-κB (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-κB (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-κB up-regulates the expression of several genes implicated in facilitating tumor cell survival, including cIAP1, Bfl-1, Bcl-2, TRAF1,
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-κB 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), proliferation (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-κB. Whether 4-HPR modulates NF-κB activation was investigated. We first examined the effect of 4-HPR on the activation of NF-κB induced by various agents, including TNF, pervanadate, phorbol 12-myristate 13-acetate (PMA), okadaic acid, cigarette smoke condensate, and H2O2. The DNA-binding assay (EMSA) showed that 4-HPR suppressed the NF-κB activation induced by all these agents (Fig. 4A). These results suggest that 4-HPR acted at a step in the NF-κB activation pathway that is common to all these agents.

N-(4-hydroxyphenyl) retinamide suppresses nuclear factor-κB activation in a dose- and time-dependent manner. We next determined the dose and time of exposure to 4-HPR required to suppress NF-κB activation. EMSA results showed that 4-HPR alone had no effect on NF-κB activation. However, it inhibited TNF-mediated NF-κB activation in a dose-dependent manner (Fig. 4B). The suppression of NF-κB 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-κB1) and the p65 (RelA) subunit of NF-κB, 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-κB (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-κB to the DNA. Some NF-κB inhibitors, including N-tosyl-l-phenylalanine chloromethyl ketone (TPCK; the serine protease inhibitor), herbinycin A (protein tyrosine kinase inhibitor), and caffeic acid phenethyl ester (CAPE), directly modify NF-κB to suppress its DNA binding (48–50). Whether 4-HPR mediates its effect through similar mechanisms was examined. EMSA showed that 4-HPR did not modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells (Fig. 4D). These results suggest that 4-HPR inhibits NF-κB activation by a mechanism different from that of TPCK, herbinycin A, or CAPE.

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

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

4-HPR completely suppressed TNF-induced activation of IKK. Therefore, 4-HPR appears to block the phosphorylation and degradation of IκBα in a dose-dependent manner. H1299 cells (2 × 10⁶/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. E. Velcade synergizes with 4-HPR in inhibiting TNF-induced NF-κB activation. H1299 cells (2 × 10⁶/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.

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 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 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 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 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.
We also tested the effect of 4-HPR on TNF-induced inhibition of constitutive NF-κB.

Our results up to this point show that 4-HPR inhibited NF-κB, which was supported by dominant-negative IkBα, 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 events involved in TNF-dependent reporter gene expression. Our results indicated that 4-HPR inhibited NF-κB–dependent gene transcription, which was consistent with our previous findings. We found that TNF produced an ~20-fold increase in SEAP activity over vector control (Fig. 6f), which was inhibited by dominant-negative IkBα, indicating specificity. When the cells were pretreated with 4-HPR, TNF-induced NF-κB–dependent SEAP expression was inhibited 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–dependent canonical NF-κB signaling, which activated NF-κB–dependent SEAP expression in all cells, except those transfected with the NF-κB plasmids, indicating specificity. When the cells were pretreated with 4-HPR, TNF-induced NF-κB–dependent SEAP expression was inhibited in a dose-dependent manner.
effect of 4-HPR on NF-κB 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-κB without directly interfering with the DNA binding of NF-κB. 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 μmol/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 μmol/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-κB activation but also constitutively activated NF-κB in multiple myeloma and HNSCC cells. These results agree with a previous report that showed suppression of constitutive NF-κB by 4-HPR in human prostate DU145 and PC3 cells (55). Constitutive active
NF-κB activation has been found to be critical for the survival and proliferation of various tumor cell types (41); however, the mechanism of constitutive NF-κB activation is not well understood. Some of the potential mechanisms are overexpression of IκBα without inhibition of NF-κB activity, mutations in the IκBα gene, enhanced IκBα 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-κB (41). We show in this report that 4-HPR inhibits the expression of cyclin D1 and c-myc, which are both regulated by NF-κB. Our results are consistent with recent reports where 4-HPR has been shown to block the G1-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-κB, 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-κB is known to promote angiogenesis, invasion, and 4-HPR is anti-inflammatory (39) and antiangiogenic (27). These results imply that 4-HPR exercises its anticancer properties through the inhibition of NF-κB.

NF-κB 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,

Figure 6.
A, 4-HPR inhibits TNF-induced NF-κB–dependent reporter gene expression. H1299 cells were transiently transfected with a NF-κB-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-κB–dependent reporter gene expression induced by TNFR, TRADD, TRAF, NIK, and IKKq. H1299 cells were transiently transfected with the indicated plasmids along with a NF-κB-containing plasmid linked to the SEAP gene and then left either untreated or treated with 25 μmol/L 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-κB activation and apoptosis.
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 Bel-2 family. The levels of Bel-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-κB (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 μmol/L 4-HPR is needed to suppress NF-κB activation. Although it is difficult to correlate in vitro concentrations to that in vivo, a plasma concentration of 12.9 μmol/L in the patients and 7.9 μmol/L in the tissue have been reported (51, 52). We found that when combined with 50 nmol/L Velcade, 10 μmol/L 4-HPR is sufficient to inhibit NF-κB activation. We found that 24-hour treatment was needed to suppress NF-κB 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-κB is constitutively active (see Fig. 4G and H), it suggests that the chemopreventive effects of 4-HPR could be mediated through NF-κB 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-κB–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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