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Nimbolide, a Limonoid Triterpene, Inhibits Growth of Human Colorectal Cancer Xenografts by Suppressing the Proinflammatory Microenvironment

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Abstract

Purpose—Extensive research over the past decade has revealed that the proinflammatory microenvironment plays a critical role in the development of colorectal cancer (CRC). Whether nimbolide, a limonoid triterpene, can inhibit the growth of CRC was investigated in the present study.

Experimental Design—The effect of nimbolide on proliferation of CRC cell lines was examined by MTT assay, apoptosis by caspase activation and poly-ADP ribose polymerase cleavage, nuclear factor-kappa B (NF-κB) activation by DNA-binding assay, and protein expression by Western blotting. The effect of nimbolide on the tumor growth *in vivo* was examined in CRC xenografts in a nude mouse model.

Results—Nimbolide inhibited proliferation, induced apoptosis, and suppressed NF-κB activation and NF-κB-regulated tumorigenic proteins in CRC cells. The suppression of NF-κB activation by nimbolide was caused by sequential inhibition of IκB kinase (IKK) activation, IκBα phosphorylation, and p65 nuclear translocation. Furthermore, the effect of nimbolide on IKK activity was found to be direct. *In vivo*, nimbolide (at 5 and 20 mg/kg body weight), injected intraperitoneally after tumor inoculation, significantly decreased the volume of CRC xenografts. The limonoid-treated xenografts exhibited significant down-regulation in the expression of proteins involved in tumor cell survival (Bcl-2, Bcl-xL, c-IAP-1, survivin, Mcl-1), proliferation (c-Myc, cyclin D1), invasion (MMP-9, ICAM-1), metastasis (CXCR4), and angiogenesis (VEGF). The limonoid was found to be bioavailable in the blood plasma and tumor tissues of treated mice.

Conclusions—Our studies provide evidence that nimbolide can suppress the growth of human CRC through modulation of the proinflammatory microenvironment.

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Keywords

Colorectal cancer; Inflammation; Limonoid; Nimbolide; NF- κ B

INTRODUCTION

More than 1 million new cases of colorectal cancer (CRC) are diagnosed worldwide each year (1). Despite major advances in detection, surgery, and chemotherapy, CRC is the second-leading cause of cancer deaths in the United States, with about 143,460 new cases and 51,690 deaths being claimed in 2012 (2). Studies have demonstrated that inflammatory response modulated by the proinflammatory transcription factor nuclear factor-kappaB (NF- κ B) plays a crucial role in the development of CRC (3).

The association between NF- κ B activation and the promotion of CRC has been shown by numerous lines of evidence. First, constitutive NF- κ B can often be seen in various CRC cell lines, xenograft animal models, and human CRC tissues (4–6). Second, CRC cells with activated NF- κ B are resistant to chemotherapeutics (7). Third, NF- κ B activation can support CRC growth by modulating expression of proteins involved in cell proliferation (8), cell death (9), cell invasion, metastasis, and angiogenesis (10–13). These statements suggest that agents with the potential for blocking NF- κ B activation have therapeutic efficacy against CRC.

When the cell is in a resting condition, NF- κ B stays in an inactive state in the cytoplasm as a heterotrimer consisting of the subunits p65 and p50 and the inhibitory subunit I κ B α . In most cancers, however, including CRC, NF- κ B is found in an active state. In CRC, the classic mechanism of NF- κ B activation is the activation of I κ B kinase (IKK) complex (14). Activated IKK phosphorylates I κ B α , targeting it for ubiquitination and subsequent proteolytic degradation. The p65/p50 subunit is then liberated and migrates to the nucleus, where it binds to a specific DNA sequence and activates the transcription of more than 500 genes (15, 16).

Over the past quarter century, agents derived from natural sources have gained considerable attention from researchers and clinicians because of their safety, efficacy, and immediate availability. At least 70% of all drugs approved by the Food and Drug Administration in the past 30 years originated from natural sources (17). However, natural agents have not been popularly accepted, mainly due to their poorly defined molecular mechanisms. One such little-investigated agent is nimbolide, which was first isolated from the leaves and flowers of neem (*Azadirachta indica*) (18, 19). Nimbolide, a tetranortriterpenoid limonoid, is one of the important contributors to the cytotoxicity of neem extracts (20). This limonoid has been shown to possess numerous biological activities such as antifeedant (21), antimalarial (22), antimicrobial (23), and anticancer (19, 20, 24–30) activities. The limonoid exhibits antiproliferative activity in a wide variety of tumor cells, including neuroblastoma, osteosarcoma, choriocarcinoma (31), cervical cancer (32), leukemia, and melanoma (29) cells.

How nimbolide mediates all of these effects is not completely understood. However, the limonoid has been shown to modulate numerous cell signaling molecules such as proliferating cell nuclear antigen (PCNA), p21, cyclin D1, glutathione S-transferase pi (GST-P), p53, Fas, Bcl-2, Bax, Bid, Apaf-1, cytochrome C, survivin, and caspases (33). The anticancer activities of nimbolide have been attributed to its α , β -unsaturated ketone structural element (34). Amide derivatives modified on the lactone ring were also found to enhance the cytotoxic activity of nimbolide (28). However, most of the anticancer activities assigned to nimbolide have been based on *in vitro* studies. Only a limited number of animal studies revealing the anticancer activities of nimbolide have been conducted.

Because of the critical role of NF- κ B in tumor cell survival, proliferation, invasion, and angiogenesis, we hypothesized that nimbolide may modulate this cell signaling pathway in CRC. Our results, to be discussed, indicate that the limonoid inhibits the NF- κ B activation pathway in CRC cells through direct interaction with IKK, leading to suppression of I κ B α phosphorylation, inhibition of p65 nuclear translocation, down-regulation of NF- κ B-regulated gene products, inhibition of cell proliferation, and induction of apoptosis. Furthermore, the limonoid inhibited the growth of CRC xenografts in nude mice; this result was associated with inhibition of NF- κ B activation and down-regulation of NF- κ B-regulated gene products.

MATERIALS AND METHODS

Materials

Nimbolide (Fig. 1A) was isolated from *Azadirachta indica* leaves, as reported (35). The purity of the triterpene was determined by high-performance liquid chromatography (HPLC). Penicillin, streptomycin, Dulbecco's modified Eagle's medium, DMEM/F12 medium, RPMI 1640 medium, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). Antibodies against p65, Mcl-1, cyclin D1, matrix metalloproteinase-9 (MMP-9), poly (ADP-ribose) polymerase (PARP), inhibitor of apoptosis protein-1 (IAP-1), Bcl-2, Bcl-xL, intercellular adhesion molecule-1 (ICAM-1), c-Myc, STAT3, pSTAT3, and caspase-3, and -9 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific anti-I κ B α (Ser^{32/36}) and anti-p65 (Ser⁵³⁶) were purchased from Cell Signaling (Danvers, MA). Vascular endothelial growth factor (VEGF) antibody was purchased from NeoMarkers (Fremont, CA). Anti-I κ B α , IKK- α , and IKK- β antibodies were obtained from Imgenex (San Diego, CA). The antibodies against survivin and CXCR4 were purchased from R & D Systems (Minneapolis, MN) and Abcam (Cambridge, MA), respectively. Anti-DR5 was purchased from ProSci, Inc. The reagents for immunohistochemical (IHC) analyses were obtained from DakoCytomation (Carpinteria, CA). The antibody against β -actin and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell lines

Human colon cancer cell lines HCT-116, HT-29, and Caco-2 were obtained from the American Type Culture Collection (Manassas, VA). The HCT-116 cells were cultured in DMEM medium, whereas the HT-29 and Caco-2 cells were cultured in RPMI 1640 medium.

The luciferase-transfected HCT-116 cells were cultured in DMEM/F12 medium. All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Measurement of cell proliferation by the MTT method

The effect of nimbolide on the proliferation of human colon cancer cells was determined by measuring mitochondrial dehydrogenase activity, using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) as the substrate (24).

Clonogenic assay

The clonogenic assay determines the ability of cells in a given population to form colonies. HCT-116 cells were seeded in 6-well plates and treated with various concentrations of nimbolide. After 12 hours, the nimbolide was washed off; the cells were allowed to form colonies for 10 days and were then stained with 0.3% crystal violet solution (25).

Live/dead assay

To measure cell viability, we used the live/dead assay, which assesses intracellular esterase activity and plasma membrane integrity (24).

Immunocytochemical and confocal microscopic analyses

To localize p65 in control and nimbolide-treated HCT-116 cells, an immunocytochemical analysis was performed. The control and nimbolide-treated cells were plated on a poly-L-lysine-coated glass slide with use of a Cytospin 4 centrifuge (Thermo Shandon, Pittsburgh, PA) and air-dried. The cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and blocked with 5% normal goat serum for 1 hour. After a brief washing, the cells were incubated overnight with rabbit polyclonal p65 antibody (dilution, 1:100); they were then incubated with goat anti-rabbit immunoglobulin G Alexa 488 (1:100) for 1 hour, counterstained for nuclei with diamidino-2-phenylindole (DAPI-100 ng/mL) for 5 minutes, and mounted with mounting medium. The cells were analyzed with use of a laser scanning Leica TCS SP2 confocal microscope equipped with diode and Ar/ArKr lasers adjusted to 405 nm and 488 nm excitations, respectively.

IKK assay

To examine the effect of nimbolide on IKK activity, an immune complex kinase assay was performed with use of GST-IκBα as the substrate (36).

Quantitative real time RT-PCR analysis

To examine the effects of nimbolide on mRNA expression, we performed quantitative real time PCR as described previously (37). The total RNA isolated using TRI reagent from control and treated cells were reverse transcribed using Revert Aid reverse transcriptase following the manufacturer's protocol. The reagents for reverse transcription were obtained from Fermentas (Pittsburgh PA). The PCR reaction was performed using SYBR^(R) Green Supermix and CFXTM Real Time system from BIORAD (Hercules, CA). The sequences of primers used to amplify the fragments of gene of interest were as follows: Bcl-2, 5'-

GTGTGGAGAGCGTCAACC-3' (forward) and 5'-CTTCAGAGACAGCCAGGAG-3' (reverse); c-IAP-1, 5'-GCCTGATGCTGGATAACTGG-3' (forward) and 5'-GGCGACAGAAAAGTCAATGG-3' (reverse); Survivin, 5'-CTGTGGGCCCCCTTAGCAAT-3' (forward) and 5'-TAAGCCCGGGAATCAAAACA-3' (reverse); c-Myc, 5'-CGTCTCCACACATCAGCACAA-3' (forward) and 5'-TCTTGGCAGCAGGATAGTCCTT-3' (reverse); ICAM-1, 5'-CGACTGGACGAGAGGGATTG-3' (forward) and 5'-TTATGACTGCGGCTGCTACC-3' (reverse); MMP-9, 5'-TGGGCTACGTGACCTATGACAT-3' (forward) and 5'-GCCAGCCCACCTCCACTCCTC-3' (reverse); and GAPDH, 5'-GAGTCAACGGATTTGGTCGT-3' (forward) and 5'-TTGATTTTGGAGGGATCTCG-3' (reverse). The data were analyzed by comparative CT method and the fold change was calculated by 2^{-CT} method (38).

Animals

The athymic nu/nu mice (4 weeks old) were obtained from the Department of Experimental Radiation Oncology at The University of Texas MD Anderson Cancer Center (Houston, TX). The mice were housed four per cage in standard plexiglass cages in a room maintained at constant temperature and humidity. Mice were housed under 12-hour light-and-darkness cycles and were fed a regular chow diet with water *ad libitum*. Before the experiment was initiated, mice were checked to ensure that they were lesion-free and pathogen-free. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at MD Anderson Cancer Center.

Xenograft implantation of the tumor

The HCT-116 cells stably transfected with luciferase (39) were harvested from subconfluent cultures after a brief exposure to 0.25% trypsin and 0.2% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum. The cells were washed once in serum-free medium and resuspended in phosphate-buffered saline. Cell suspensions consisting of single cells with >90% viability were used for the injections. Mice were anesthetized with ketamine-xylazine solution, and 2×10^6 cells in 50 μ L PBS was injected subcutaneously into the leg of each mouse with use of a 27-gauge needle.

Experimental protocol

Seven days after tumor implantation, the mice were randomly assigned to the following treatment groups (4 mice/group): (i) untreated control (50 μ L DMSO, intraperitoneally [i.p.], daily for 10 days) and (ii) nimbolide (5 mg/kg body weight, i.p., daily for 10 days) and nimbolide (20 mg/kg body weight, i.p., daily for 10 days). Tumor size was measured every other day by Vernier calipers with use of the formula $1/2 \times L \times W^2$, where L represents tumor length and W represents tumor width (40). Tumor volumes were also measured on days 0 and 10 by using the IVIS 200 bioluminescence imaging system and Living Image software (Caliper Life Sciences, Hopkinton, MA). In brief, animals were injected intraperitoneally with D-luciferin potassium salt at a dose of 150 mg/kg of body weight and then anesthetized with a 2.5% isoflurane/air mixture. After incubation for 10 minutes in an acrylic chamber, mice were kept in a vertical position and the total number of photons emerging from the

active luciferase within the animals was detected. The signal intensity was quantified as the sum of all detected photons within the region of interest per second per steradian.

Two hours before the mice were euthanized, they were given nimbolide, and blood was collected from each mouse by cardiac puncture; serum and plasma were separated. The tumor was excised and divided into two parts: the first was formalin-fixed and paraffin-embedded for IHC analysis, and the second was snap-frozen in liquid nitrogen and stored at -80°C for analysis of proteins by Western blotting.

Immunolocalization of cyclin D1, MMP-9, CXCR4, VEGF, and Ki67 in tumor tissues

We used an IHC method to localize the expression of cyclin D1, MMP-9, CXCR4, VEGF, and Ki67 in tumor tissues, as described before (41).

Western blot analysis

The protein extract from colon cancer cells and tumor tissues was prepared, and Western blot analysis was carried out as described earlier (41).

Preparation of nuclear extracts and electrophoretic mobility shift assay

The nuclear extract from colon cancer cells and tumor tissues was prepared as described before (41). The nuclear extracts were then analyzed for NF- κ B activation by electrophoretic mobility shift assay.

Analysis of nimbolide uptake by high-performance liquid chromatography

Nimbolide was extracted from tumor tissues and blood plasma as described earlier (42). In brief, the tissues (100 mg) were homogenized in 400 μL of ethanol for 30 seconds. This was followed by the sequential addition of 500 μL of water and hexane, with homogenization for 15 seconds after each addition. The samples were then centrifuged at 5,000 rpm for 5 minutes at 4°C , frozen at -80°C , and then lyophilized. The pellets were reconstituted with mobile phase that consisted of a mixture of acetonitrile and water at a ratio of 60:40. The samples were run at a flow rate of 0.5 mL/minute with the detector operating at a wavelength of 230 nm. Tissues from vehicle-fed mice were set as controls that reflected the baseline.

The blood samples obtained from each mouse were centrifuged for 8 minutes at 2,000 rpm to obtain the blood plasma. The blood plasma was treated with acetone (2:1) for deproteination and centrifuged again for 5 minutes at 3,000 rpm. The supernatant fraction was frozen at -80°C , lyophilized, reconstituted in the mobile phase, and analyzed by HPLC.

Statistical methods

Different parameters were monitored in normal and treated groups. The data were first analyzed by one-way ANOVA. The two groups were later compared by an unpaired Student's *t* test, which revealed significant differences between the means. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The aim of this study was to determine whether nimbolide has anticancer activities against CRC with use of both *in vitro* and *in vivo* models and if so, to determine how nimbolide manifests these effects. For the *in vitro* studies, we used human colon cancer cell lines (HCT-116, HT-29, and Caco-2) that possess different sensitivities. For the *in vivo* studies, we used a xenograft nude mouse model.

Nimbolide inhibits proliferation and induces apoptosis in CRC cells

First, we used the MTT assay to determine whether nimbolide can inhibit the growth of CRC cells. The limonoid suppressed the proliferation of HCT-116, HT-29, and Caco-2 cell lines in a dose- and time-dependent manner. At concentrations as low as 2 μ M, nimbolide inhibited the proliferation of cancer cells after 3 days of treatment (Fig. 1B). At the highest concentration, significant inhibition of cell growth was observed after 1 day. These results indicate that nimbolide exhibits potent antiproliferative effects against CRC cells.

To confirm the results observed by the MTT assay, we treated the CRC cells with various concentrations of nimbolide for 24 hours and then performed a live/dead assay. As indicated in Fig. 1C, cytotoxicity was induced in 52.5%, 38.4%, and 43.1% of HCT-116, HT-29, and Caco-2 cells, respectively at 10 μ M nimbolide. These results suggest that HCT-116 cells are relatively more sensitive to the limonoid.

Next, we examined whether nimbolide can induce apoptosis in colon cancer cells. HCT-116 and HT-29 cells were treated with different concentrations of nimbolide for 12 hours and then caspase activation and PARP cleavage were examined by Western blot analysis. The limonoid induced caspase activation and PARP cleavage at the highest concentration (Fig. 1D).

Nimbolide suppresses the expression of tumorigenic proteins

Because nimbolide inhibited the survival of CRC cells, we investigated whether this triterpene can inhibit the expression of gene products involved in tumor cell survival. Nimbolide inhibited the expression of antiapoptotic proteins such as Bcl-2, Bcl-xL, c-IAP-1, and survivin, all of which are known to play a major role in cell survival (Fig. 2A, *upper*).

We next examined whether nimbolide can inhibit the expression of cell proliferative proteins. The triterpene inhibited the expression of cyclin D1 and c-Myc, both of which are known to contribute to cell proliferation (Fig. 2A, *middle*). The limonoid also inhibited the expression of proteins involved in tumor cell invasion, metastasis, and angiogenesis (MMP-9, ICAM-1, CXCR4, VEGF) in a concentration-dependent manner (Fig. 2A, *lower*). The effect of nimbolide on the expression of gene products was greatest at a concentration of 10 μ M.

Whether nimbolide inhibits expression of tumorigenic proteins at the transcriptional level was investigated. We found that nimbolide suppressed the mRNA expressions of Bcl-2, c-IAP-1, survivin, c-Myc, ICAM-1 and MMP-9 in HCT-116 cells (Fig. 2B). A more prominent effect was observed at the mRNA levels of Bcl-2, c-IAP-1, and survivin.

We also examined whether nimbolide can suppress the ability of colon cancer cells to form colonies. Cells were treated with 2–10 μ M nimbolide for 12 hours, washed, and then allowed to form colonies for 10 days in fresh medium. At the highest concentration of nimbolide, the total number of colonies was reduced from 907 to 152 (Fig. 2C, *left* and *right*).

Nimbolide inhibits constitutive NF- κ B activation in CRC cells

Because Bcl-2, Bcl-xL, c-IAP-1, survivin, cyclin D1, c-Myc, MMP-9, ICAM-1, CXCR4, and VEGF are all regulated by NF- κ B, we investigated whether nimbolide could suppress the expression of these gene products by inhibiting NF- κ B activation. We found that HCT-116 cells exhibited constitutive NF- κ B and that treatment with nimbolide inhibited NF- κ B activation in a time-dependent manner. We further found that 10 μ M nimbolide almost completely suppressed constitutive NF- κ B after 12 hours (Fig. 3A, *left*).

Whether nimbolide inhibits STAT3 activation, another proinflammatory transcription factor was investigated. Nimbolide neither inhibited STAT3 phosphorylation nor expression of STAT3 (Fig 3A, *right*), indicating that the inhibition of NF- κ B activation by this limonoid may be specific.

We investigated whether inhibition of NF- κ B activation by nimbolide was due to suppression of I κ B α phosphorylation. HCT-116 cells were treated with 10 μ M nimbolide for 15 minutes to 4 hours, and the cytoplasmic fraction was isolated and analyzed for I κ B α phosphorylation by Western blotting. Results indicated that I κ B α phosphorylation was significantly suppressed after 2 hours of nimbolide treatment (Fig. 3B, *left*).

Because IKK is required for I κ B α phosphorylation and since nimbolide inhibits I κ B α phosphorylation, we investigated the effect of nimbolide on IKK activation. The results indicated that HCT-116 cells expressed constitutive IKK and that treatment of these cells with nimbolide suppressed IKK activity in a time-dependent manner. We found that IKK activity was completely suppressed after 30 minutes of nimbolide treatment (Fig. 3B, *right*).

We also sought to determine whether nimbolide inhibits IKK activation by direct binding. For this, we incubated immunoprecipitated IKK complex with the limonoid at various concentrations and performed an IKK assay. We found that nimbolide directly inhibits IKK activity (Fig. 3C, *left*).

Because p65 shuttles between the nucleus and cytoplasm, we investigated whether nimbolide affects the nuclear retention of p65 in HCT-116 cells. Immunocytochemical analysis revealed the constitutive presence of p65 in HCT-116 cells. When the cells were treated with nimbolide, p65 was redistributed from the nucleus to the cytoplasm (Fig. 3C, *right*).

To confirm the results observed by immunocytochemical analysis, we treated the cells with 10 μ M nimbolide for 15 minutes to 4 hours, extracted the nuclear fraction, and analyzed it for p65 content. Results indicated that the level of p65 was decreased by the limonoid treatment in a time-dependent manner (Fig. 3D). Because phosphorylation of p65 at Ser⁵³⁶

is required for p65's transcriptional activity, we sought to determine the effect of nimbolide on p65 phosphorylation. As shown in Fig. 3D, treating the cells with the triterpene suppressed p65 phosphorylation in a time-dependent manner.

Nimbolide inhibits tumor growth in xenograft nude mice model

We determined whether nimbolide can inhibit tumor growth in a xenograft-implanted nude mice model. Luciferase-transfected HCT-116 cells were xenograft-implanted into the leg of mice. One week later, mice were randomized into three groups on the basis of IVIS imaging (Fig. 4A). Nimbolide was given at two different doses (5 and 20 mg/kg body weight, i.p.) for 10 days. As determined by noninvasive bioluminescence imaging on day 10, nimbolide significantly suppressed tumor growth in nude mice at a dose as low as 5 mg/kg. More specifically, administration of nimbolide at 20 mg/kg of body weight reduced tumor growth by almost 90% by day 10 (Fig. 4B, *left* and *right*). When we measured the tumor volume with Vernier calipers every other day, we found that tumor growth had increased rapidly in the vehicle-treated group (Fig. 4C). A comparison of tumor volumes between days 0 and 10 showed a 4.2-fold increase in tumor volume in the vehicle-treated group. However, in the nimbolide treatment groups, an insignificant increase in tumor volume was observed. Consistent with observations based on IVIS imaging, the tumor growth had been significantly suppressed by the nimbolide treatment. Furthermore, the limonoid did not affect the body weight of mice (Fig. 4D).

Nimbolide down-regulates expression of proteins involved in tumor cell development

We investigated the expression of tumor-associated gene products in control and nimbolide-treated mice. As revealed in Fig. 5, treatment with nimbolide down-regulated the expression of gene products in tumor tissues. First, nimbolide down-regulated the expression of cell survival proteins including Bcl-2, Bcl-xL, c-IAP-1, survivin, and Mcl-1 (Fig. 5A). Because nimbolide down-regulated the expression of cell survival proteins, whether this limonoid can modulate PARP cleavage and caspase 3 activity in tumor tissues was also examined. The PARP cleavage was detectable (Fig. 5A), under the conditions no significant activation of caspase-3 (data not shown) was noted in tumors from mice treated with nimbolide. Second, nimbolide down-regulated the expression of c-Myc and cyclin D1, both known to promote tumor growth (Fig. 5B, *top*). And third, nimbolide down-regulated the expression of proteins involved in invasion, metastasis, and angiogenesis (MMP-9, CXCR4, ICAM-1, and VEGF) (Fig. 5B, *bottom*).

Up to this point, our results indicated that nimbolide can down-regulate the expression of proteins associated with tumor development. Because these gene products are known to be regulated by NF- κ B, we investigated the effects of nimbolide on NF- κ B activation in tumor tissues. As revealed in Fig. 5C, nimbolide inhibited constitutive NF- κ B in tumor tissues.

We also investigated whether nimbolide inhibits STAT3 activation in tumor tissues. Consistent with *in vitro* data, the phosphorylation and total content of STAT3 was unaffected by nimbolide treatment (Fig. 5D, *left*). However, nimbolide was found to induce DR5 in tumor tissues (Fig. 5D, *right*).

To confirm the results observed by Western blot analysis, we performed IHC analyses of the selected proteins. The results indicated that the tumor tissues expressed cyclin D1, MMP-9, CXCR4, and VEGF. However, the expression of all of these proteins was almost completely suppressed by the nimbolide treatment at a concentration as low as 5 mg/kg of body weight (Fig. 6A).

We also sought to determine the effects of nimbolide treatment on the expression of Ki67, another marker for tumor proliferation. The results indicated that the tumor tissues expressed Ki67, and the expression of the protein was decreased by the nimbolide treatment (Fig. 6B).

Nimbolide is bioavailable in plasma and CRC tumors

We examined the availability of nimbolide in the blood plasma and tumors of the treated mice. As indicated by HPLC analysis, nimbolide was bioavailable in the plasma obtained from treated mice. More specifically, nimbolide levels of 222 ng/mL and 409 ng/mL of plasma were detected in the mice treated with nimbolide at 5 mg/kg and 20 mg/kg of body weight, respectively (Fig. S1B). Similarly, nimbolide levels of 345 ng/g and 868 ng/g of tumor tissue were obtained from the mice treated with nimbolide at 5 mg/kg and 20 mg/kg of body weight, respectively (Fig. S1C). Overall, these results reflect the bioavailability of nimbolide in blood plasma and tumor tissues.

DISCUSSION

With the development of new therapeutics, the incidence of CRC has decreased during the past two decades. Targeted therapies against VEGF (bevacizumab) or against EGFR (cetuximab) are now commonly used as treatments for CRC (43–45). However, patients develop resistance to such treatments; thus, new strategies are required to replace or complement current therapies. In the present study, we investigated whether nimbolide, a limonoid triterpene, can exert anticancer activity against CRC via modulation of proinflammatory pathways in animal model.

We found that nimbolide inhibited proliferation and induced apoptosis in CRC cells. The overexpression of proteins associated with cell survival and cell proliferation (46) has been shown to contribute to tumor development. Down-regulation of the expression of proteins involved with cell survival and proliferation may contribute to the decreased growth of colon cancer cells. The observed antiproliferative and apoptosis-inducing properties of nimbolide are in agreement with those observed by others in leukemia (24, 29), colon cancer (25, 47), neuroblastoma (20), and choriocarcinoma (31) cells. In a previous study, we demonstrated that nimbolide at lower concentrations can sensitize colon cancer cells to TRAIL by modulating apoptotic pathways (25). In the present study, we provide evidence that higher concentrations of nimbolide alone can activate caspase-3 and caspase-9. The ability of nimbolide to inhibit the expression of proteins involved in tumor invasion, metastasis, and angiogenesis (MMP-9, ICAM-1, CXCR4, VEGF) further supports the role of nimbolide against CRC. We also found that nimbolide inhibits the expression of tumorigenic proteins both at mRNA and protein levels. However, nimbolide's affect on ICAM-1 and MMP-9 expression at mRNA level was less prominent. It is likely that a subtle change at mRNA is sufficient to modulate the translation of ICAM-1 and MMP-9. We found

that HCT-116 colon cancer cells exhibited constitutive NF- κ B and that nimbolide suppressed this activation. Although nimbolide has been shown to inhibit inducible and constitutive NF- κ B activation in leukemia and multiple myeloma cells (24), this is the first report demonstrating the inhibition of constitutive NF- κ B in colon cancer cells. Constitutive NF- κ B has been found to be crucial for the survival and proliferation of various tumor cell types by regulating the expression of proteins involved in tumor development (48). Therefore, it is likely that nimbolide exerts its inhibitory effects on tumor survival and growth by inactivating NF- κ B.

One of the possible mechanisms for the constitutive activation of NF- κ B in tumor cells is through IKK activation (49). In our observations, the limonoid inhibited constitutive IKK activation, which may have accounted for nimbolide's inhibition of constitutive NF- κ B activation. An analysis of the kinetics of inhibition of IKK activation, I κ B α phosphorylation, NF- κ B activation, and NF- κ B-regulated gene products on nimbolide treatment revealed that IKK inhibition occurred much earlier (after 30 minutes) than did inhibition in I κ B α phosphorylation (2 hours), NF- κ B inactivation (6 hours), and suppression in NF- κ B-regulated gene products (12 hours). These data indicate that inhibition in IKK activity is an upstream event that leads to the sequential suppression of I κ B α phosphorylation and inhibition of NF- κ B activation. IKK has also been reported to phosphorylate the p65 subunit of NF- κ B at Ser⁵³⁶. Inhibition of p65 phosphorylation by nimbolide further suggests that its inhibitory effect on NF- κ B activation is mediated through IKK. We found that nimbolide inhibited IKK activity by direct binding. In agreement with these observations, a previous study from this laboratory indicated that nimbolide inhibits IKK activity by modifying Cys¹⁷⁹ in the active site of IKK.

We found that the limonoid, at relatively lower concentrations (5 and 20 mg/kg of body weight), inhibited the survival and growth of CRC in a xenograft-implanted nude mouse model. In agreement with these observations, nimbolide (10–100mg/kg) was shown to exhibit chemopreventive activity against 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced oral carcinogenesis (50). In another hamster model of oral oncogenesis, the limonoid was shown to exhibit antiproliferative and apoptosis-inducing activities (33). Our study, however, is the first to demonstrate the potential of the limonoid in inhibiting the growth of CRC in a xenograft nude mouse model.

We found that nimbolide can mediate antitumor activity *in vivo* by modulating the expression of numerous tumorigenesis-related proteins. First, nimbolide down-regulated the expression of Bcl-2, Bcl-xL, c-IAP-1, survivin, and Mcl-1, all known to promote tumor survival. Second, nimbolide down-regulated the expression of c-Myc and cyclin D1, which are known to be overexpressed in CRC and to promote tumor growth (51). Third, nimbolide down-regulated the expression of proteins involved in tumor invasion, metastasis, and angiogenesis such as MMP-9, ICAM-1, CXCR4, and VEGF. Fourth, constitutively active NF- κ B, known to regulate the expression of all of these proteins, was also inhibited by the limonoid treatment. That nimbolide was unable to suppress STAT3 activation suggests that the inhibitory effect of nimbolide on NF- κ B activation may be specific. Finally, expression of Ki67, a marker of proliferation, was also decreased by the limonoid treatment. The inhibition of CXCR4 expression by nimbolide may have clinical relevance since increased

expression of CXCR4 has been associated with increased risk of recurrence and poor survival in CRC (52). In this study, for the first time, we reported the bioavailability of nimbolide. Consistent with these observations, tumor volume was significantly decreased in nimbolide-treated mice, further supporting the antitumor activity of nimbolide *in vivo*. The observations that nimbolide induces DR5 in tumor tissues suggest that induction of DR5 could be another mechanism for its anticancer activities. In a previous study, we have demonstrated that nimbolide can generate ROS that was indispensable for induction of DR5 and DR4 in cancer cells (25). Thus it is more likely that nimbolide exert anticancer activity through generation of ROS.

Overall, the results from our cell-based and *in vivo* studies support the apoptosis-inducing, antiproliferative, antiinvasive, antimetastatic, and antiangiogenic activities of nimbolide. The underlying mechanism by which this limonoid exhibits anti-CRC activities seems to be through inhibition of NF- κ B activation. The efficacy of nimbolide in inhibiting the growth of the CRC xenograft at relatively lower concentrations strengthens its therapeutic value. Thus, this study provides strong evidence for evaluating the safety and efficacy of nimbolide by clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance

Despite major advances in detection, surgery and chemotherapy, colorectal cancer (CRC) has been the second leading cause of cancer deaths in the United States. Although targeted therapies are now commonly used for CRC, many cases develop resistance to such treatments. Thus, safe and effective agents are required to replace or compliment current therapies. Here, we show that nimbolide, a limonoid triterpene, can inhibit the growth of CRC. Using both *in vitro* and *in vivo* CRC models, we show for the first time that nimbolide suppressed the proinflammatory microenvironment as indicated by the suppression of nuclear factor (NF)- κ B activation and modulation of expression of NF- κ B regulated gene products linked to survival, proliferation, invasion, and angiogenesis. These observations provide new insights into the molecular mechanism of action of nimbolide and opens up possibility for evaluating its safety and efficacy in humans.

FIGURE 1. Nimbolide inhibits growth and induces apoptosis in colorectal cancer cells
 A, Molecular structure of nimbolide. B, Cells were treated with indicated concentrations of nimbolide, and cell growth was analyzed on days 0, 1, 3, and 5 by MTT assay. C, Cytotoxicity was measured by the live/dead assay. D, Nimbolide induces caspase activation and PARP cleavage in CRC cells. Whole-cell extracts from treated cells were analyzed by Western blotting by using indicated antibodies. *-indicates the significance of difference compared with control; $P < 0.05$. NL, nimbolide.

FIGURE 2. Nimbolide inhibits the expression of tumorigenic proteins in CRC cells

A, Whole-cell extracts from treated cells were analyzed by Western blotting by using indicated antibodies. B, Nimbolide suppresses mRNA levels of tumorigenic proteins. HCT-116 cells were treated with indicated concentrations of nimbolide for 12 h, total RNA was extracted and examined for mRNA levels by qRT-PCR after normalization to GAPDH data. C, Nimbolide reduces colony formation by colon cancer cells. HCT-116 cells were treated with indicated concentrations of nimbolide for 12 hours. Cells were then washed, allowed to form colonies for 10 days, stained with crystal violet, and then counted. NL, nimbolide.

FIGURE 3. Nimbolide down-regulates constitutive NF- κ B activation in colon cancer cells through inhibition of IKK activity

A (*left*), HCT-116 cells were treated with 10 μ M nimbolide for the times indicated. Nuclear extracts were prepared and assayed for NF- κ B activation by using EMSA. A (*right*), Nimbolide neither inhibits STAT3 phosphorylation nor total STAT3 in HCT 116 cells. The whole cell extract from normal and treated cells were analyzed by Western blotting for pSTAT3 and STAT3. B (*left*), Nimbolide inhibits phosphorylation of I κ B α in HCT-116 cells. Cells were treated with 10 μ M nimbolide for the times indicated. Cytoplasmic extracts were analyzed by Western blotting by using a phosphospecific I κ B α antibody (Ser^{32/36}). B (*right*), Nimbolide inhibits IKK activation in HCT-116 cells. Cells were incubated with 10 μ M nimbolide for the times indicated. Whole-cell extracts were immunoprecipitated with an

antibody against IKK- β and analyzed with use of an immune complex kinase assay. The effect of nimbolide on IKK protein expression was determined by Western blot analysis by using IKK- α and IKK- β antibodies. C (*left*), Nimbolide directly inhibits IKK activation in HCT-116 cells. Whole-cell extracts were immunoprecipitated with an IKK- β antibody. The immunoprecipitated complex was incubated with the indicated concentrations of nimbolide, and an immunocomplex kinase assay was performed. C (*right*), D, Nimbolide induces redistribution of p65 and inhibits p65 phosphorylation in HCT-116 cells. C (*right*), Cells were incubated with 10 μ M nimbolide for 4 hours and analyzed for p65 localization by immunocytochemical analysis with use of a confocal microscope. D, Cells were incubated with 10 μ M nimbolide for the times indicated, and the nuclear extract was analyzed for p65 and p-p65. PARP was used as a loading control. NL, nimbolide.

FIGURE 4. Nimbolide inhibits the growth of colorectal tumors in nude mice

A, Outline of the experimental protocol. Group I was given DMSO (50 μ L, i.p. daily) for 10 days. Groups II and III were given nimbolide at 5 mg/kg and 20 mg/kg of body weight (i.p. daily), respectively, for 10 days. B, Bioluminescence imaging of live anesthetized mice implanted with CRC tumors (*left*); measurements of tumor volume on the 10th day with use of bioluminescence imaging (*right*, $n = 4$). C, Measurements of tumor volume with use of Vernier calipers and the formula $1/2 \times L \times W^2$, where L and W represent the length and width of tumors, respectively, on various days. Data represent mean $\pm$ SD of tumor volume of 4 mice. D, Effect of nimbolide on the body weight of mice over the treatment time.

FIGURE 5. Nimbolide inhibits NF- κ B activation and down-regulates NF- κ B-regulated gene products

A–B, Protein extracts prepared from colorectal tumor tissues of mice were analyzed by Western blotting by using antibodies against (A) antiapoptotic, (B, *top*) proliferative, (B, *bottom*) invasive, and angiogenic proteins. β -actin was used as an internal control. C, Nimbolide inhibits NF- κ B activation in colorectal tumor tissues. Nuclear extracts prepared from tumor tissues were assayed for NF- κ B activation by EMSA. D, Nimbolide induces DR5 but does not affect STAT3 phosphorylation or total STAT3 in tumor tissues. The whole cell extract from tumor tissues were analyzed by Western blotting for pSTAT3, STAT3 and DR5. NL, nimbolide.

FIGURE 6. Nimbolide inhibits the expression of cyclin D1, MMP-9, CXCR4, VEGF, and Ki67 in xenograft tumors from mice

The sections were stained and analyzed as described in the Materials and Methods section. Values across each photomicrograph represent percent mean $\pm$ SD of positive cells.