Nimbolide Retards Tumor Cell Migration, Invasion, and Angiogenesis by Downregulating MMP-2/9 Expression Via Inhibiting ERK1/2 and Reducing DNA-Binding Activity of NF-κB in Colon Cancer Cells

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Nimbolide, a plant-derived limonoid has been shown to exert its antiproliferative effects in various cell lines. We demonstrate that nimbolide effectively inhibited proliferation of WiDr colon cancer cells through inhibition of cyclin A leading to S phase arrest. It also caused activation of caspase-mediated apoptosis through the inhibition of ERK1/2 and activation of p38 and JNK1/2. Further nimbolide effectively retarded tumor cell migration and invasion through inhibition of metalloproteinase-2/9 (MMP-2/9) expression, both at the mRNA and protein level. It was also a strong inhibitor of VEGF expression, promoter activity, and in vitro angiogenesis. Finally, nimbolide suppressed the nuclear translocation of p65/p50 and DNA binding of NF-κB, which is an important transcription factor for controlling MMP-2/9 and VEGF gene expression. © 2011 Wiley-Liss, Inc.

Key words: nimbolide; colon cancer; MMP-2/9; ERK1/2; invasion

INTRODUCTION

Neem (Azadirachta indica) is a traditional medicinal plant, which is commonly used for treating various human ailments. Many bioactive compounds are isolated from this plant among which, nimbolide belongs to the limonoid group. Nimbolide is a tetrnortriterpenoid with α,β-unsaturated system and δ-lactonic ring (Figure 1) [1]. Although nimbolide showed non-mutagenicity, it was toxic to mice and thus investigated the possibility of using it as drug. This limonoid has been shown to exhibit various biological activities, including anti-feedant [2], anti-malarial [3], insecticidal, and anticancer [4–8] activities. This triterpenoid exhibits anti-proliferative activity in a wide variety of cancer cells, including breast cancer, lung cancer, neuroblastoma cells, osteosarcoma cells, choriocarcinoma cells, and melanoma cells [9–11]. A few studies have shown that nimbolide can affect cell cycle progression and induce apoptosis in colon cancer [8,10], oral carcinoma [12], and cervical cancer [13]. Thus, cell cycle arrest and apoptosis induction of nimbolide have been attributed for its documented anticancer effects. Recently, nimbolide has been shown to interfere with the expression of NF-κB regulated proteins like Bcl-2, Cox-2, metalloproteinase-9 (MMP-9), VEGF, etc., by inhibiting IKK [10]. Therefore, it is likely that nimbolide will interfere with cell migration, invasion, angiogenesis, and the present study was done to check whether nimbolide actually inhibit the above tumor-associated processes. The study shows for the first time that nimbolide inhibits cell migration, invasion, and angiogenesis by downregulating MMP-2/9 expression via inhibiting ERK1/2 and reducing translocation of NF-κB and its DNA-binding activity in colon cancer cells.

Additional Supporting Information may be found in the online version of this article.

Abbreviations: MMP, metalloproteinase; HRP, horse-radish peroxidase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MTT, 3-(4-5 dimethylthiozol-2-yl) 2-5diphenyl-tetrazolium bromide; PI, propidium iodide; PBS, phosphate-buffered saline; RIPA, radio immunoprecipitation assay; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; PMA, phorbol-12-myristyl-13-acetate; MMLV-RT, Moloney Murine Leukemia Virus Reverse Transcriptase; APMA, p-aminophenyl-mercuric acetate; EMSA, electrophoretic mobility shift assay; TBE, Tris-borate EDTA; HUVEC, human umbilical ven endothelial cell; SAPK, stress-activated protein kinase; JNK, c-Jun amino-terminal kinase; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; PKC, protein kinase C.

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MATERIALS AND METHODS

Reagents and Antibodies

Nimbolide was extracted from neem leaves as mentioned elsewhere [14]. Antibodies to poly (ADP-ribose) polymerase, procaspase-9, procaspase-3, cleaved caspase-9, cleaved caspase-3, cyclin A, cyclin D1, phospho-ERK1/2, phospho-JNK 1/2, phospho-P38 (Cell Signaling Technology, Inc., Danvers, MA), NF-κB, IκB (Santa Cruz Biotechnology Inc., Santa Cruz, CA), histone, and β-actin (Sigma–Aldrich, St. Louis, MO) and horseradish peroxidase (HRP)-conjugated secondary antibodies anti-rabbit IgG, anti-mouse IgG (Cell Signaling Technology, Inc.), and anti-goat IgG (Sigma–Aldrich) were used. All other reagents were purchased from Sigma–Aldrich.

Cell Culture and Maintenance

WiDr, colon adenocarcinoma cells were sent as a gift by Professor William R. Wilson, Auckland Cancer Society Research Centre (University of Auckland, New Zealand), and HCT-116, colon adenocarcinoma cells were obtained from Rajiv Gandhi Center for Biotechnology (Thiruvananthapuram). The cells were grown in monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM; Sigma–Aldrich) containing 10% fetal bovine serum (FBS; Sigma–Aldrich) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO2 at 37°C. For all experiments DMEM containing 2.5% FBS was used.

Cell Viability Assay

Cell growth assays were carried out as described elsewhere with slight modifications [15]. Cells grown in 96-well microtiter plates (10,000 cells/well) were incubated for 24/48 h with or without different concentrations of nimbolide (0.156–2.5 μM). Then the medium was removed and fresh medium was added along with 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; 5 mg/ml) to each well. The plates were incubated for another 2½ h and the formazan crystals formed were solubilized with acidic isopropanol. The color developed was quantitated (Measuring wave length: 570 nm, Reference wave length: 630 nm) with a 96-well plate reader (BIO-TEK Instruments, Winoski, VT). The cell viability was expressed as percentage over the control.

Annexin/Propidium Iodide Staining

For annexin/propidium iodide (PI) staining, cells (10,000 cells/well) were seeded in culture slides and treated with or without nimbolide (1.25 μM) for 16 h. Then they were washed with phosphate-buffered saline (PBS) and incubated with annexin-fluorescein isothiocyanate and PI as per the protocol described in the annexin V apoptosis detection kit (Santa Cruz Biotechnology Inc.). After 10–20 min, the cells were washed with PBS and the greenish apoptotic cells were viewed using an Olympus fluorescent microscope (IX 51) and photographed.

Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential was measured by using a Mitochondrial Membrane Sensor Kit as described by the manufacturer (Clontech Laboratories Inc., Mountain View, CA). After 12 h treatment with 1.25 μM of nimbolide, the cells were washed with serum-free medium and mitosensor reagent was added to the cells. Cells were then incubated at 37°C in a humidified, 5% CO2 incubator for 15–20 min. Cells were washed with incubation buffer and examined with Olympus fluorescent microscope and documented.

Caspase-3/7 Assay

Activation of caspases was determined using Caspase-Glo 3/7 Assay kit (Promega, Madison, WI). The assay makes use of a luminogenic caspase-3/7 substrate with tetrapeptide sequence DEVD (D—Asp Aspartic acid; E—Glu Glutamic acid; V—Val Valine), that can be cleaved by caspases 3/7 produced in the apoptotic cells. The cells were seeded onto 96-well plate and treated with different concentrations of nimbolide for 16 h. After treatment, 100 μl of Caspase-Glo 3/7 reagent was added to each well and the contents were incubated for 2 h at room temperature. Luminescence was measured in Sirius single tube luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Relative luminescence units were calculated after normalizing for the protein content.

Western Blot Analysis

The cells treated with or without nimbolide were washed with PBS and lysed in ice-cold Radio Immuno Precipitation Assay (RIPA) buffer. Whole
cell extracts (100 µg protein) were resolved on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membrane, probed with anti-poly (ADP-ribose) polymerase, anti-procaspase-9, anti-procaspase-3, anti-cleaved caspase-9, anti-cleaved caspase-3, anti-cyclin A, anti-cyclin D1, anti-phospho-ERK1/2, anti-phospho-JNK1/2, anti-phospho-P38, anti-NF-κB, anti-IκB, anti-histone, and anti-β-actin. HRP-conjugated secondary antibodies (anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG) were used and the bands were visualized as per the protocols given in the West Pico Chemiluminescence Detection Kit (Pierce Biotechnology Inc., Rockford, IL).

Flow Cytometric Analysis

For flow cytometric analysis of cell cycle, 1.5 × 10^6 cells were treated with and without 1.25 µM of nimbolide for 8 h. Then cells were harvested and fixed in 70% ice-cold ethanol. Cells were washed and resuspended in 0.2–0.5 mg/ml RNase A for 1 h at 37°C. DNA was stained using PI (final concentration 10 µg/ml) and kept in dark until analysis. Samples were analyzed using FACSAria flow cytometer (BD Biosciences, San Jose, CA). From each sample 10^5 cells were analyzed. The cell cycle distribution was estimated according to standard procedures. The percentage of cells in different cell cycle phases (G0/G1, S, or G2/M) were calculated using FACS Diva DNA analysis software.

Semi-Quantitative RT-PCR and Real-Time RT-PCR Analysis of MMP-2, MMP-9, and VEGF-A

The cells were grown in a 60 mm diameter dish, and incubated with nimbolide (0.625–2.5 µM) with and without phorbol-12-myristyl-13-acetate (PMA). After treatment, total RNA was isolated using Pure Link RNA Mini Kit (Invitrogen Corporation, Carlsbad, CA) following manufacture’s protocol. The cDNA was synthesized with 4 µg of total RNA through Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and random primers (Promega Corporation). PCR was done with specific primers (MMP-2 sense 5’-CAGGCTTCTCTGTTTTCACAC-3’, antisense 5’-AAGCACCAGGGTGGTTTCTCCTC-3’ (400 bp), MMP-9 sense 5’-TGAGTACGTGACCTATGACAT-3’, antisense 5’-GCCGACCCACCACCTCCTCCTC-3’ (173 bp), VEGF-A sense 5’-ATCTGCATGGTGATGTTGGA-3’ (400 bp), 18s RNA, non-regulated housekeeping gene was used as an internal control to normalize input cDNA. The real-time PCR was performed with 1 µg of cDNA and 2 pM primers per reaction in 7900 HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA) using MESA green qPCR Mastermix for SYBR Assay (Eurogentec, Seraing, Belgium).

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Gelatin Zymography

Cells were seeded into the 60 mm plate and treated with 100 ng/ml PMA and different concentrations of nimbolide (0.625–2.5 µM) for 24 h. Subsequently, the conditioned medium was collected, concentrated, and gelatin zymography were performed with slight modifications to examine the activities of MMP-2 and MMP-9 [16]. Briefly, samples were mixed with loading buffer and electrophoresed on 8% SDS–PAGE containing 0.1% gelatin. After incubating at 37°C for 24 h in zymography reaction buffer the gel was stained with Coomassie blue R-250 for 1 h and destained. Non-staining bands representing the levels of the active form of MMP-2 and MMP-9 were quantified by densitometric measurement.

MMP-2/9 Activity Assay

Gelatinolytic activity was analyzed by using Immozyme Gelatinase MMP-2/9 Activity Assay Kit (Merck KGaA, Darmstadt, Germany). Culture supernatant (subjected to concentration), collected after 24 h nimbolide treatment (0.625–2.5 µM) with and without PMA, was diluted in activation buffer and incubated with substrate working solution for 6 h at 37°C. MMP-2/9 is exclusively released in their inactive proforms and, therefore, detection of MMP-2/9 activity required pre-incubation with the synthetic MMP-2/9 activator p-aminophenyl-mercuric acetate (APMA). Fluorescence was measured at excitation wavelength of 320 nm and emission wavelength of 405 nm in microplate reader (Tecan, Mannedorf, Switzerland) and analyzed using Magellan™ software.

MMP-2/9 and VEGF Promoter Activity Assay

MMP-2 and MMP-9 luciferase reporter plasmids were obtained as kind gifts from C.D. Kim (Pusan National University, Busan, Korea), and Douglas D. Boyd (MD Anderson Cancer Center, Houston, USA) originally constructed by Hiroshi Sato (Cancer Research Medicine, Kanazawa University, Kanazawa, Japan [17]), respectively. VEGF luciferase promoter was given by Dr. G. Pages (Institute of Developmental Biology and Cancer, Nice, France). The cells were grown to 70% confluency and transfected with pRL-Null (Promega Corporation), MMP-9 and MMP-2 luciferase reporter plasmids using Metafectene (Biontex Laboratories GmbH, Planegg, Germany). After transfection, the cells were treated with and without 1.25 µM nimbolide/PMA and luciferase reporter assays were performed by Dual Luciferase Reporter Assay System (Promega Corporation) following the manufacturer’s protocol and luciferase activity was measured by a luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Fire-fly luciferase activity was normalized for transfection.
efficiency using Renilla Luciferase activity (pRL-null) and protein content.

Electrophoretic Mobility Shift Assay

The DNA-binding activities of NF-κB in nuclear extracts were assessed by electrophoretic mobility shift assay (EMSA) [18] with slight modifications using the LightShift kit from Pierce. Nuclear proteins were extracted from cells using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology Inc.). Complementary oligonucleotide probes containing the NF-κB-binding motifs (NF-κB—5′ probe, 5′-TTGTTACAAGGGACTTTCCGGGGAC-3′, NF-κB—3′ probe, 5′-CCACGCCCTCCCTGGAAAGTC-3′, CCCCCAGCCAAAGTCCCTTGTAACA-3′), were end labeled with biotin using Biotin 3′-end labeling kit (Pierce Biotechnology Inc.) according to the manufacturer’s protocol. Nuclear protein (25 μg) was incubated with 20 fmol of biotin-labeled oligonucleotide for 20 min at room temperature in 1X binding buffer consisting of 50 ng of poly (dl.dC), 2.5% glycerol, 0.05% Nonidet P-40, 50 mM KCl, 5 mM MgCl₂, and 10 mM EDTA. Products of binding reactions were resolved by electrophoresis on a 6% polyacrylamide gel in 0.625 M Tris-borate buffer for 15 min at room temperature, the membrane was electroblotted and UV crosslinked to a Biodyne B Pre-Cut Modified Nylon Membrane, 0.45 μm (Pierce). After incubation in blocking buffer for 15 min at room temperature, the membrane was incubated with streptavidin–HRP conjugate for 30 min at room temperature. Detection of protein–oligonucleotide complex was performed using a LightShift chemiluminescent EMSA kit (Pierce Biotechnology Inc.). The membrane was incubated with chemiluminescent substrate for 5 min, and exposed on X-ray film and developed. Band intensity of protein–oligonucleotide complex was performed with ImageQuant software (Bio-Rad Laboratories, Hercules, CA).

Scratch Wound Healing Assay

Cells were seeded at 5 × 10⁵ cells/well in 24-well plates and then pre-incubated for 24 h in serum-free DMEM (Sigma–Aldrich) before creating a wound across the cell monolayer with a plastic tip. Cells grown in culture medium with 2.5% FBS was added to the lower chamber. Migration was induced by 100 ng/ml of PMA, a known cell migrating agent. Cell migration was assayed by counting the number of cells that had migrated from the upper side to the lower side of the membrane after 24 h of incubation. Migrated cells were fixed with cold methanol and stained with 0.5% crystal violet solution. In case of assessing cell invasion, BD BioCoat Matrigel Invasion Chambers were used (BD Biosciences, Bedford, MA).

In Vitro Tube Formation Assay

Human umbilical vein endothelial cells (HUVECs) when plated on Matrigel, align themselves to form tube like structures. For this assay 500 μg of Matrigel (BD Biosciences) was coated on the wells of 96-well plate and incubated for 30 min at 37°C to solidify the gel. 1.5 × 10⁴ HUVECs were then plated on the Matrigel and cultured in DMEM containing 0.625 μM of nimbolide for 24 h. The enclosed network of complete tubes were observed and photographed under light microscope.

Statistical Analysis

All statistical calculations were carried out with the GraphPad Prism software program (version 5 for Windows). Values are expressed as the mean ± standard deviation (SD). The differences among the mean values from at least three independent experiments were analyzed with one-way ANOVA followed by Tukey’s post hoc t-test analysis. The significance of the difference from the respective control for each experimental test conditions was assayed by Student’s t-test for each paired experiment. Differences between means were considered statistically significant at P < 0.05.

RESULTS

Nimbolide Inhibits Proliferation and Causes S Phase Arrest Through Repression of Cyclin A/Cyclin D1

To quantitate the effects of nimbolide on cell growth, cell viability was assessed by reduction of MTT at 24/48 h with various concentrations of nimbolide. Colon cancer cells WiDr and HCT-116 exhibited a concentration/time-dependent growth inhibition with nimbolide treatment (Figure 2A, Supplementary Figure 1A).

We checked whether nimbolide-induced cell growth inhibition was due to perturbation of cell cycle, and cell phase distribution analysis showed an accumulation of cells in S phase upon treatment with nimbolide (1.25 μM; Figure 2B). We checked whether nimbolide-induced cell growth

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inhibition was due to modulation of cell cycle regulatory proteins and have found that cyclin A and cyclin D1 levels were decreased in a concentration-dependent manner when treated with nimbolide (Figure 2C,D). Cyclin A is required for the cells to proceed through S phase and abrogation of its level by nimbolide correlates with the stalling of cells in S phase.
Overall, these results indicate that nimbolide inhibits tumor cell proliferation and exerts its growth inhibitory actions through alterations of cyclin levels.

Nimbolide Induces Apoptosis Through Activation of Caspases, p38, JNK1/2, and Inhibition of ERK1/2

There are several reports suggesting apoptosis induction as a mode of cell death by nimbolide in a variety of tumor cells. Nimbolide treatment induces changes that are characteristic of apoptosis, like, appearance of phosphatidyl serine on the outer cell membrane (Figure 2E) and reduction in mitochondrial membrane potential (Figure 2F). Activation of caspases was initially determined by the expression levels of procaspase-3/9, upon treatment with nimbolide (1.25 μM) in a time-dependent manner (Figure 3A,B). This was further confirmed by assessing concomitant levels of cleaved fragments of caspases 3 and 9 (Figure 3C). It is evident that caspase-9 was activated nearly 20-fold compared to the untreated control while the activation of caspase-3 was nearly twofold (Figure 3E,F). A bioluminescent-based caspase-3/7 assay showed similar fold activation for the caspases (Figure 3D). Cleaved PARP corresponds to the actions of the executioner caspases and was increased to threefold upon treatment with nimbolide (Figure 3G,H). These results suggest that nimbolide-treated human colon cancer cells undergo cell death through activation of caspases leading apoptosis.

The lysates from the cells treated with nimbolide were immunoblotted for the activated forms of ERK1/2 with an antibody specific for phosphorylation on residues Thr202 and Tyr204 of ERK1/2. Levels of phosphorylated ERK were downregulated in a time-dependent manner in nimbolide-treated cells by about 50% (Figure 4A,B). These results demonstrate that inhibition of ERK is necessary for nimbolide to exert its antiproliferative effect. At the same time, phosphorylation of p38 and JNK1/2 increased by more than 3- and 1.5-fold, respectively (Figure 4A,C,D).

Prevention of In Vitro Cell Migration by Nimbolide

Inhibitory effect of nimbolide on colon cancer cell migration was evaluated by scratch wound migration assay. All experiments for anti-migratory/invasive effect of nimbolide were carried out in presence of PMA. A concentration of 0.625 μM nimbolide for 24 h effectively reduced the PMA-induced migration of cells in to the wounded area, which was determined as movement of cells (μm) into the wound (Figure 5A, Supplementary Figure 1B,C). PMA-induced migration was inhibited by >90% after treatment with nimbolide for 24 h (Figure 5C).

The Effect of Nimbolide on Transwell Migration/Invasion

Inhibitory effect of 0.625 μM nimbolide on colon cancer cell migration was evaluated by a modified Boyden’s chamber. The influence of nimbolide on migration through a membrane of 8 μm pore size was examined in WiDr and HCT-116 cells (Figure 5B, Supplementary Figure 1D). As shown in Figure 5D and Supplementary Figure 1D,E, treatment with nimbolide reduced the PMA-induced transwell migration of WiDr and HCT-116 cells by 3- and 2-fold, respectively.

We then evaluated for the anti-invasive property of nimbolide, if any. As expected, PMA-treated cells showed increased invasiveness when compared to cells treated with control medium alone. Nimbolide successfully diminished the number of cells invaded through the basement membrane matrix-coated culture inserts (Figure 5B). As shown in Figure 5D, treatment with nimbolide reduced the PMA-induced transwell invasion of WiDr cells by over sevenfold. These results clues regarding involvement of MMPs in nimbolide-mediated inhibition of invasion and prompted us to evaluate the prominent MMPs involved in cell migration/invasion.

Nimbolide Inhibits PMA-Induced MMP-2/9 Expression, Secretion, and Activity in Tumor Cells and in Culture Supernatants

Cellular movements are enhanced by the degradation of extracellular components by activating a set of genes. In a recent report, nimbolide was shown to inhibit MMP-9 via NF-κB; MMPs are one of the important gene targets which have NF-κB-binding sites in the promoter regions [10,19]. In this study, we evaluated whether nimbolide could regulate the expression of MMP-2/9 that are found to be activated in various malignant tumors.

Nimbolide Downregulates MMP-2/9 mRNA Levels

For this experiment, we used PMA as an agent that would induce MMPs expression in cells. Semi-quantitative RT-PCR analysis showed that PMA-stimulated expression of MMP-2/9 in WiDr cells was inhibited by different concentrations of nimbolide (Figure 6A). This finding was subsequently validated by real-time RT-PCR. The decrease in the expression of MMP-2/9 in nimbolide-treated cells was nearly fivefold (Figure 6B).

Nimbolide Hinders MMP-2/9 Promoter Activity

The effects of nimbolide on PMA-induced MMP-2 promoter activity was investigated by luciferase reporter gene assay using the amplified 1,584 bp fragment that was cloned into pGL3 Basic vector. This promoter portion bears multiple-binding sites for transcription factors including NF-κB, CREB,
Figure 3. Procaspase-3/9 expression in nimbolide-treated WiDr cells. (A) Cells were treated with 1.25 μM of nimbolide for indicated time points. The cell lysates were evaluated for levels of Procaspase-3/9 expression by Western blotting as described in Materials and Methods Section. (B) The protein expression levels were expressed as ratio to the expression of β-actin. The above experiment was repeated at least three times. *P < 0.05, **P < 0.01. Activation of caspase-3/9 by nimbolide in WiDr cells. (C) Cells were treated with 1.25 μM of nimbolide for indicated time points. The cell lysates were evaluated for levels of active fragments of caspase-3/9 by Western blotting as described in Materials and Methods Section. (E,F) The protein expression levels for cleaved caspase-9 and cleaved caspase-3 were expressed as ratio to the expression of β-actin. The above experiment was repeated at least three times. ***P < 0.001. Nimbolide activates caspase-3/7 in WiDr cells. (D) Cells were treated with indicated concentrations of nimbolide for 16 h. After treatment, cells were lysed and caspase activities were determined as described under Materials and Methods Section. The experiment was repeated another time with similar result and the caspase activity was expressed as fold activation over the untreated control. The mean fold activation was significantly higher in the nimbolide-treated sample as analyzed by Student’s t-test. Bars indicate SD. *P < 0.05, **P < 0.01. Cleavage of PARP by nimbolide in WiDr cells. (G) Cells were treated with nimbolide for indicated time points. The cell lysates were evaluated for levels of cleaved PARP by Western blotting as described in Materials and Methods Section. (H) The protein expression levels were expressed as ratio to the expression of β-actin. The above experiment was repeated at least three times. ***P < 0.001.
and AP-1 [20]. MMP-9 promoter activity was investigated by luciferase reporter gene assay using a proximal 0.67 kb fragment of the human MMP-9 promoter. This promoter portion also bears multiple-binding sites for transcription factors including NF-κB, Sp-1, and AP-1 sufficient for an optimal induction of MMP-9 promoter activity by cytokines [21]. PMA caused a twofold increase in the luciferase activity of both MMP-2 and MMP-9 reporter plasmids, which was subsequently reduced to the basal levels on incubation with nimbolide (Figure 6C). Even though the MMP-2/9 expression was controlled by nimbolide, the extent to which it influenced the transcription differed, that is, MMP-9 transcription was relatively more inhibited than MMP-2.

Inhibition of MMP-2/9 Enzymatic Activity by Nimbolide

After we tested for nimbolide-mediated inhibition of MMP-2/9 at transcription levels, we examined whether the reduction in the intracellular MMP-2/9 mRNA content leads to a reduction in MMP-2/9 activity in the culture supernatant. To this end, conditioned media were tested by an MMP-2/9-specific activity assay. Similar to the results obtained by RT-PCR, the PMA-induced MMP-2/9 activity was reduced on co-incubation with 1.25 μM nimbolide. PMA treatment induced more than fourfold increase in the MMP-2/9 activity and treatment with nimbolide diminished the activity to that in unstimulated tumor cells (Figure 6D). This clearly shows that nimbolide decreases the expression and activity of MMP-2/9.

Inhibition of Gelatinolytic Activity of Nimbolide

We evaluated effects of nimbolide on extracellular MMP-2/9 functional activity in WiDr cells treated with PMA, by gelatin-zymography. WiDr cells showed secretion of the two distinct 72 and 92 kDa type IV collagenases, MMP-2, and MMP-9, respectively, whose activities were stimulated by PMA. In contrast to MMP-9 levels, a high basal content of MMP-2 was evident, possibly due to larger spectrum of MMPs activating MMP-2 than MMP-9. Treatment of WiDr cells with PMA for 24 h caused a strong increase in latent MMP-2/9 levels characterized by a lytic band at 72 and 92 kDa, respectively (Figure 6E). Incubation with increasing concentrations of nimbolide caused a dose-dependent decrease in PMA-induced MMP-2/
Figure 5. Evaluation of cell migration by scratch wound healing assay. (A) Cells were seeded in 24-well plates and then pre-incubated for 24 h in serum-free DMEM before creating a wound across the cell monolayer with a plastic tip. Cells were allowed to migrate with and without PMA/nimbolide. Cell migration into the wound surface was then monitored by microscopy after 24 h and reported as the width of the remaining wounded area relative to the initial wound area. (C) This assay was independently repeated three times and the values are plotted as graph. The mean number of cells was significantly lower in the nimbolide-treated sample as analyzed by Student’s t-test. Bars indicate SD. $^{***}P < 0.001$. Inhibition of transwell migration/invasion of WiDr cells by nimbolide. (B) Migration of WiDr cells was determined with modified Boyden’s chamber method. Response of cells were assayed using 24-well migration chamber with an upper well having a membrane of 8 μm pore size and 12 mm of diameter. In case of assessing cell invasion, BD BioCoat Matrigel Invasion Chambers were used. Cells on the upper side of the membrane were carefully removed by using a wet cotton swab. The experiment was done as described in the Materials and Methods Section. (D) The experiment was repeated three times with similar results and the number of cells migrated/invasde was counted and plotted in the graph. The mean number of cells was significantly lower in the nimbolide-treated sample as analyzed by Student’s t-test. Bars indicate SD. $^{***}P < 0.001$. 

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Figure 6. Semi-quantitative RT-PCR analysis of MMP-2/9 inhibition by nimibolide. (A) The cells were grown in 60 mm dish and incubated with different concentrations of nimibolide. After treatment, total RNA was isolated using Pure Link RNA Mini Kit following manufacturer’s protocol. RNA was reverse transcribed into cDNA and then amplified by using specific primers by protocols described in the Materials and Methods Section. The amplified PCR products were electrophoresed on 2% agarose gel and documented. Real-time RT-PCR analysis of MMP-2/9 inhibition by nimibolide. (B) The real-time PCR was performed by 1 μg of cDNA and 2 pmol primers per reaction in 7900 HT Fast Real-Time PCR system using MESA green qPCR Mastermix for SYBR Assay. Fold increase of the target mRNAs were normalized with that of β-actin and is plotted as a graph. Each value is presented as the mean ± SD of determinations from two independent experiments. The mean fold change was significantly lower from the corresponding PMA-treated group as analyzed by Student’s t-test. ***P < 0.001. Nimibolide inhibits MMP-2/9 reporter activity. (C) The cells grown at 70% confluence was transfected with pRL-Null, MMP-2, and MMP-9 luciferase reporter plasmids using Metafectene. After transfection, the cells were treated with and without nimibolide and luciferase reporter assays were performed by Dual Luciferase Reporter Assay System following the manufacturer’s instruction. The relative luminescence units were normalized for the pRL-Null vector and protein content and expressed as fold activation. Each value is presented as the mean ± SD of determinations from three independent experiments. The mean fold change was significantly lower from the corresponding PMA-treated group as analyzed by Student’s t-test. ***P < 0.001. Inhibition of MMP-2/9 activity by nimibolide. (D) Gelatinolytic activity was analyzed by using Innozyme Gelatinase MMP-2/9 Activity Assay Kit following the manufacturer’s protocol. Each value is presented as the mean ± SD of duplicate determinations from two independent experiments. The mean fold change was significantly lower from the corresponding PMA-treated group as analyzed by Student’s t-test. ***P < 0.001. Inhibition of gelatinolytic activity by nimibolide. (E) Cells were treated with 100 ng/ml PMA and different concentrations of nimibolide (0.625–2.5 μM) for 24 h. Subsequently, the conditioned medium was collected, concentrated using amicon ultra centrifuge tubes (10 kDa cutoff) and samples were electrophoresed on 8% SDS–PAGE containing 0.1% gelatin, stained with Coomassie blue R-250. Non-staining bands representing the levels of the active form of MMP-2 and MMP-9 were quantified by densitometric measurement. (F) Fold change of the bands corresponding two distinct 72- and 92-kDa type IV collagenases, MMP-2 and MMP-9, respectively were normalized with that the untreated control and plotted as a graph. Each value is presented as the mean ± SD of determinations from three independent experiments. The mean fold change was significantly lower from the corresponding PMA-treated group as analyzed by Student’s t-test. ***P < 0.001.
9 activity with a maximum inhibition at 2.5 μM nimbolide (Figure 6F).

In summary, we have shown nimbolide indeed brings down the mRNA levels of MMP-2/9 and it does so by transcriptional regulation. Decreased gelatinolytic activity in the culture supernatant was also seen upon treatment with nimbolide.

Nimbolide Inhibits VEGF-A Expression, Promoter Activity in Tumor Cells and In Vitro Tube Formation of HUVECs

Angiogenesis plays an important part in the process of metastasis and is seen along with tumor progression. Since VEGF-A plays a key role in prompting angiogenesis, we assayed mRNA levels of VEGF-A in nimbolide-treated cells by semi-quantitative RT-PCR. The results show that PMA stimulated expression of VEGF-A in WiDr cells was inhibited by varying concentrations of nimbolide (Figure 7A). We also confirmed this by real-time RT-PCR which showed that nimbolide treatment decreased PMA-induced VEGF mRNA by more than 10-fold (Figure 7B).

The effects of nimbolide on PMA-induced VEGF promoter activity (Luciferase reporter plasmid containing gene spanning -1,176/+54 region of VEGF promoter, constructed in pGL2 basic vector) was used to investigate and the results show that nimbolide treatment brought down the transcriptional regulation to the basal level (Figure 7C).

In vitro angiogenic assays were carried out to test the specific effect of nimbolide treatment on tube formation assay using HUVECs on Matrigel. The concentration and time of nimbolide treatment were carried out under conditions that did not produce appreciable cytotoxic effect on cells. Nimbolide successfully inhibited tube formation of endothelial cells (Figure 7D).

In summary, we have shown nimbolide indeed brings down the mRNA levels of VEGF-A and it does so by transcriptional inhibition in tumor cells.

Figure 7. Semi-quantitative RT-PCR analysis of VEGF inhibition by nimbolide. (A) The cells were grown in a 60 mm dish and incubated with different concentrations of nimbolide. After treatment, total RNA was isolated using Pure Link RNA Mini Kit following manufacturer’s protocol. Specific amount of RNA was reverse transcribed into cDNA and then amplified by using specific primers by protocols described in the Materials and Methods Section. The amplified PCR products were electrophoresed on 2% agarose gel and documented. Real-time RT-PCR analysis of VEGF-A inhibition by nimbolide. (B) The real-time PCR was performed in 7900 HT Fast Real-Time PCR system using MESA green qPCR Mastermix for SYBR Assay. Fold change of the VEGF-A was normalized with that of β-actin and was plotted as a graph. Each value is presented as the mean ± SD of determinations from three independent experiments. The mean fold change was significantly lower from the corresponding PMA-treated group as analyzed by Student’s t-test.

***p < 0.001. Inhibition of VEGF reporter activity by nimbolide. (C) The cells grown at 70% confluence were transfected with pRL-Null, VEGF-Luciferase reporter plasmids using Metafectene. After transfection, the cells were treated with and without nimbolide/ PMA and luciferase reporter assays were performed by Dual Luciferase Reporter Assay System following the manufacturer’s instructions. The relative luminescence units were normalized for the pRL-Null vector/protein content. Each value is presented as the mean ± SD of determinations from three independent experiments. The mean fold change was significantly lower from the corresponding PMA-treated group as analyzed by Student’s t-test. **p < 0.001. In vitro tube formation assay. (D) 1.5 × 10^5 HUVECs were plated on the Matrigel and cultured in DMEM with and without nimbolide for 24 h. The enclosed network of complete tubes/incomplete structures were observed and photographed under light microscope.

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subsequently leading to stunted in vitro formation of HUVECs.

Nimbolide Blocks PMA-Induced ERK1/2 Activation and NF-κB–DNA Binding

ERK1/2 is known to control the expression of several transcription factors like AP-1, NF-κB, which in turn has been implicated in the induction of several MMPs expression [17,21–23].

Nimbolide Prevents PMA-Induced Phosphorylation of ERK1/2

Treatment of WiDr cells with PMA augmented phosphorylation of ERK1/2 which was reversed by different concentrations of nimbolide (Figure 8A,C). JNK1/2 also gets activated in presence of PMA, but nimbolide fails to inhibit the PMA-induced JNK1/2 activation. In fact, there is an overall activation of JNK1/2 after nimbolide treatment.

Nimbolide Inhibits PMA-Induced NF-κB–DNA Binding

PMA induced nuclear p65/p50 levels in WiDr cells were reversed by treatment with 1.25 μM nimbolide. Even though nuclear p65/p50 levels were inhibited by nimbolide, the extent to which it inhibited these proteins differed and p50 was relatively more inhibited than p65 (Figure 8B,D). However, there was no change in the cytoplasmic IκB levels when treated with nimbolide (Figure 8E).

To test whether nimbolide interferes with the DNA-binding of NF-κB we performed EMSA. Stimulation of WiDr cells with PMA (100 ng/ml) caused enhancement in the binding of proteins to NF-κB oligonucleotide whereas treatment with nimbolide caused inhibition of such binding (Figure 8F). This result suggests that NF-κB pathway is important in nimbolide-induced MMP downregulation. In summary, the results suggest that nimbolide inhibits PMA-induced ERK phosphorylation, nuclear p50/p65 levels, and reduced binding of NF-κB to DNA.

DISCUSSION

The growth inhibitory and apoptotic effects of nimbolide, one of the major neem limonoids, on various cancer cells is being increasingly documented in a number of recent studies [4,6,8–13,24,25]. In many in vitro and in vivo studies, nimbolide has shown cell cycle regulatory effects [8,11,13,25]. Flow cytometric analysis of U937 cells showed that nimbolide treatment resulted in cell cycle disruption by decreasing the number of cells in G0/G1 phase [25]. In HT-29 cells too, nimbolide treatment caused a 6.5-fold increase in the number of cells in the G2/M phase compared with the control cells [8]. Further analysis revealed that nimbolide-mediated G2/M arrest was accompanied by the upregulation of cyclin D2 and downregulation of cyclin A and cyclin E. Our study suggests that nimbolide inhibits cell proliferation by interfering with cell cycle kinetics, by inducing an S phase arrest primarily caused through the repression of cyclin A/cyclin D1. Downregulation of different cyclins, either individually or in combinations, by nimbolide have been linked with inhibition of cell proliferation or cell cycle arrest as shown in earlier studies [8,10,12,13]. However, when cell damage is irreparable, the arrested cells initiate apoptotic cell death [26]. Likewise, when exposed to higher doses of nimbolide and for longer treatment periods, cells often displayed cell death features and a remarkable increase in the number of cells in the sub-G1 fraction, with a reciprocal decrease of cells in all phases [8,10,11,13,25]. In our study cells treated with nimbolide exhibited apoptotic features like increased appearance of annexin V positive cells, decrease in mitochondrial membrane potential, and evidence of biochemical effectors of apoptosis such as activation of caspase-9, 3/7 were seen in a time-dependent manner. PARP cleavage, a hallmark of caspase-dependent apoptosis, was also documented in nimbolide-treated cells in a time-dependent manner.

We also investigated whether nimbolide regulates MAPK pathways for its antiproliferative and apoptosis inducing abilities. Since MAPKs are the key components of intracellular signaling for cell fate determination, we examined the phosphorylation status of ERK1/2, p38, and JNK1/2 in nimbolide-treated WiDr cells. ERK1/2 is an important candidate protein which assists cell division and proliferation. An activated ERK dimer can regulate targets in the cytosol and also translocate to the nucleus where it phosphorylates a variety of transcription factors regulating gene expression [27]. In this study, nimbolide inhibited phosphorylation of ERK1/2 in a time-dependent manner. On the other hand, stress-activated protein kinases (SAPK)/c-Jun amino-terminal kinases (JNK), which are members of the MAPK family are activated by a variety of environmental stresses, inflammatory cytokines, and growth factors [28]. Our results show that nimbolide-activated p38 and JNK1/2 in a time-dependent manner eventhough their activation levels differed. There is one report showing involvement of MAPKs in nimbolide-induced activation of ERK1/2 and p38, which is in contrast to our finding [9]. It could be due to the inherent biological differences in the cell lines used.

Although cell proliferation is an essential prerequisite for the development of a malignant tumor, angiogenesis and metastasis are major causes of cancer morbidity and mortality. Matrix digestion serves as a prelude for tumor cell to become invasive and MMPs play a pivotal role in extracellular matrix (ECM) digestion and even
epithelial–mesenchymal transition (EMT) [29]. Initiation of metastasis involves invasion, which has many phenotypic similarities to EMT, including a loss of cell–cell adhesion and an increase in cell mobility [30]. Hence, one of the objectives of our study was to explore the possible effects of nimbolide on tumor cell migration and invasion. In vivo, cells free themselves to migrate by breaking the ECM connections by different MMPs and such events occur during many physiological and pathological conditions [31,32]. PMA, a tumor promoter known to activate various signaling cascades

Figure 8. Modulation of PMA-induced pERK1/2, pJNK1/2 expression by nimbolide. (A) Cells were treated with PMA followed by different concentrations of nimbolide for 4 h. The cell lysates were evaluated for levels of pERK1/2, pJNK1/2 expression by Western blotting as described in Materials and Methods Section. (C) Fold change was normalized with that of β-actin and was plotted as a graph. Each value is presented as the mean ± SD of determinations from three independent experiments. The mean fold change was significantly lower from the corresponding PMA-treated group as analyzed by Student’s t-test. **P < 0.001. (E) Modulation of PMA-induced NF-κB subunit expression by nimbolide. Cells were treated with PMA/nimbolide for 4 h. The nuclear lysates were evaluated for p65/p50 and cytoplasmic levels of IκB by Western blotting as described in Materials and Methods Section. (D) p65/p50 levels were expressed as ratio to the expression of histone. **P < 0.001. (F) Nimbolide induces diminished binding of PMA-induced NF-κB. WiDr cells were incubated with nimbolide for 4 h with or without PMA. Nuclear extracts were prepared and assayed for NF-κB–DNA binding using EMSA.
including protein kinase C (PKC), was used as an agent to induce migration and invasion of cells. It is known to activate transcription factors through various intermediate signaling molecules [33–35]. There are several studies that have used PMA as an agent to activate tumor promoting signaling events and in our study too we used it as an agent to induce migration and invasion [36,37]. Nimbo- lide was able to inhibit the promigratory effect of PMA which is evident from the decreased mRNA levels of MMP-2/9 which is shown by semi-quantitative and real-time PCR. It is affirmed by two recent reports showing that the nimbolide suppresses the TNF-α-DMBA-induced MMP activity of cancer cells [10,38]. Tumor cell migration and invasion requires efficient degradation of the ECM by MMPs and their activation is persistently observed during tumor progression. MMPs have been extensively studied in colorectal cancers and are required for invasion during metastasis. Among this battery of endopeptidases, MMP-2/9 are prevalent based on their appearance at malignant sites. MMP-2, the most abundant among all MMPs, is secreted as a zymogen that requires activation which is an important event in the regulation of ECM degradation by MMPs [40]. Ours is the first study that looked at the mRNA levels, MMP promoter activity, biochemical and functional activities of MMP-2/9 in response to nimbolide treatment in tumor cells. The ability of nimbolide to suppress the PMA-induced expression of MMP-2/9 suggests that it has a role in preventing metastasis and tumor invasion.

Efficient metastasis is always promoted by VEGF, released during ECM processing or through endogenous production by the tumor cells, all leading to bioavailability of VEGF, triggering a network of signaling pathways that promote angiogenesis. Repression of VEGF and its receptors reduce tumor growth in multiple tumor models demonstrating the vital role of VEGF in tumor progression [41]. Our study showed that nimbolide inhibits PMA-induced VEGF-A mRNA expression and promoter activity which corroborates with a recent report [10]. Moreover, the extent of inhibition of VEGF expression by nimbolide, prompted us to carry out in vitro tube formation assay by HUVECs. There was a near complete inhibition of Matrigel-induced in vitro tube formation of HUVECs and is the first report showing inhibition of in vitro angiogenesis by nimbolide.

Dysregulation of MAPK pathway is much observed in various malignant tumors [42]. Importantly MAP kinase signaling plays significant role in MMPs regulation, particularly MMP-2/9 in many experimental systems. Nimbolide successfully abrogated PMA-induced phosphorylation of ERK1/2 in a concentration-dependent manner,
which suggests the involvement of MAPK signaling in tumor cell migration.

MAPKs regulate transcription factor NF-κB, which act independently or coordinately to regulate numerous genes involved in the regulation of MMPs expression [43,44]. It was previously reported that the inhibition of ERK and JNK results in the suppression of MMP-9 promoter activity as well as the NF-κB and AP-1 activities [16]. It is known that NF-κB has critical role in transcription of genes involved in apoptosis, inflammation, and tumor progression [43]. It has been recently shown that nimbolide inhibited the activation of NF-κB and the suppression of NF-κB expression as reported that the inhibition of ERK and JNK results in down-regulation of NF-κB-dependent tumor promoting genes such as MMP-2/9, VEGF-A, cyclin D1/2. 

In conclusion, this study demonstrates that nimbolide decreased the DNA binding of NF-κB. Since NF-κB is an important transcription factor for controlling various genes in cell survival, our results indicate that the anti-tumor activity of nimbolide could be through the downregulation of NF-κB-dependent tumor promoting genes such as MMP-2/9, VEGF-A, cyclin D1/2.

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REFERENCES


