Nimbolide inhibits invasion and migration, and down-regulates uPAR chemokine gene expression, in two breast cancer cell lines

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Abstract

Objectives: Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women, worldwide. Urokinase type plasminogen activator (uPA) is a serine protease that is involved in cancer progression, especially invasion and metastasis of breast cancer. Nimbolide is a potent cytotoxic limnoid isolated from *Azadirachta indica*. Our previous studies have shown that nimbolide elicits pleiotropic effects on breast cancer cells; however, its roles in invasion and migration have not previously been fully elucidated.

Materials and methods: Protein expression of pEGFR, VEGFR, NFκB, IKKα, IKKβ, MMP-2, MMP-9 and TIMP-2 were analysed by western blotting. We also analysed expressions of *uPA*, *uPAR* genes and chemokines by real-time PCR. Breast cancer cell invasion was assessed by transwell invasion assay and cell migration analysed by scratch wound healing assay.

Results: Our results showed that reduced protein expression of pEGFR, VEGFR, NFκB, IKKα, β, MMP-2, MMP-9 and TIMP-2 was higher in nimbolide-treated breast cancer cells. mRNA expression of *uPA*, *uPAR*, chemokines and their receptors were also significantly reduced in response to nimbolide treatment. Nimbolide inhibited breast cancer cell migration and invasion as shown in transwell invasion and wound healing assays.

Conclusion: These results clearly proved inhibitory effects of nimbolide on tumour cell invasion and

migration by down-regulating proteins critically involved in regulation of cell invasion and metastasis, suggesting a possible therapeutic role of nimbolide for breast cancer.

Introduction

Epidermal growth factor receptor (EGFR; HER1) is a proto-oncogene that encodes a 170 kDa transmembrane protein (1). In common with insulin-like growth factor (IGF), epidermal growth factor (EGF) also plays a vital role in progression of breast cancer by its interaction with the EGF receptor (EGFR). EGF and EGFR binding results in dimerization and auto-phosphorylation of the receptor and subsequent recruitment of downstream molecules (MAPK and PI3K/Akt) that mediate cell proliferation, invasion and migration (2). EGFR and its ligands are frequently co-expressed in a large subset of primary breast carcinomas (3). EGFR triggers kinase pathways with the final outcome being nuclear stimulation of the dimeric complex NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) that activates expression of genes, which play a key role in development and progression of cancers, such as proliferation, migration and apoptosis (4). This transcription factor is localized in the cytosol and is blocked by the inhibitor of κB (IκB). Activation of NFκB may result from different signalling pathways triggered by a variety of cytokines, growth factors and tyrosine kinases. Moreover, activation of other signalling pathways, such as Ras/MAPK and PI3K/Akt, is also involved in activation of NFκB. NFκB induces expression of cell adhesion molecules (ICAM-1, E-selectin) and proteins involved in invasion (MMPs). Numbers of angiogenic factors, including vascular endothelial growth factor (VEGF), are also promoted by NFκB.

Metastasis is the process by which a tumour cell leaves the primary site, travels to a distant location

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(often *via* the circulatory system), and establishes a secondary tumour. To metastasize, cancer cells must invade through their basement membrane and surrounding extracellular matrix (ECM). Matrix metalloproteinases (MMPs) compose a family of structurally related, zinc-dependent endopeptidases that are capable of degrading protein components of the ECM (5,6). Members of this family of proteolytic enzymes are present in both normal and pathological tissues in which matrix remodelling is involved, including embryonic development, wound healing, arthritis, and angiogenesis as well as in tumour invasion and metastasis (7,8); MMP activity is regulated by specific inhibitors, tissue inhibitors of MMP (TIMPs) (9). MMP-2 and MMP-9 have important roles in basement membrane turnover (10). Proteolytic effect of MMPs facilitates migration of endothelial cells through altered extracellular matrix towards a source of angiogenic stimulus. Hence, MMPs are an integral component for the angiogenic process. It has been reported that there is a link between EGFR function and MMP expression that may contribute to the invasive phenotype.

Activation of the chemokine and urokinase plasminogen activator (uPA)/uPA receptor (uPAR) system is also a central mediator of tumour cell migration and invasion. Chemokines are chemotactic cytokines that cause site-directed migration of leukocytes induced by inflammatory cytokines, growth factors and pathogenic stimuli. Chemokine signalling results in transcription of genes involved in cell invasion, motility and survival (11,12). uPA is a serine protease that engages in cancer progression, especially invasion and metastasis (13). It is a multifunctional protein with potential activity in both proteolysis and signal transduction (14). As a protease, its best known reaction is catalysis of conversion of the zymogen plasminogen, into active plasmin. It can activate precursor forms of certain MMPs (15). Formation of active MMPs allows further degradation of the ECM, especially interstitial and type IV collagens.

Nimbolide was first derived from leaves and flowers of neem (*Azadirachta indica*). Neem is a traditional medicinal plant, commonly used to treat a variety of human ailments (16). Many bioactive compounds are isolated from this plant among which, nimbolide belongs to the limonoid group; it is the major component of leaves of *A. indica*. Nimbolide is a tetranortriterpenoid consisting of a classic limonoid skeleton with an α , β -unsaturated ketone system and δ -lactone ring (17). It has been shown to exhibit numerous types of biological activity, including, being anti-malarial (18), anti-feedent (19) and anti-malignant (20–23). Nimbolide significantly suppresses viability of HeLa cells in a dose-dependent manner by inducing cell cycle arrest in

the G0/G1 phase, accompanied by p21 accumulation and down-regulation of cell cycle regulatory proteins cyclin B, cyclin D1 and proliferating cell nuclear antigen (PCNA) (24). It has been shown to interfere with expression of cell survival proteins (Bcl-2, Bcl-x_L, IAP-1 and IAP-2), proliferation (cyclin D1), invasion (MMP-9) and angiogenesis (VEGF), by inhibiting IKK/NF κ B (22).

Nimbolide has been found to down-regulate NF κ B-regulated proteins such as Bcl-2, Cox-2, MMP-9, VEGF, and more, by inhibiting IKK in the WiDr colon cancer cell line (25). In a previous study of ours, nimbolide repressed cell proliferation by inhibiting the IGF1/IGF1R-P13K/Akt pathway and induced apoptosis by activation of both extrinsic and intrinsic apoptotic pathways, in prostate cancer cells (26). Recent findings of Karkare *et al.* (27) demonstrated that nimbolide suppressed glioblastoma viability and restrained tumour growth by inhibiting CDK4/6 activity, leading to Rb hypophosphorylation and cell cycle arrest, and by inhibiting growth factor pathways hyperactivated in glioblastoma, including those of PI3K-Akt, MAP kinase and JAK-STAT. The role of nimbolide in invasion and metastasis of breast cancer cells has not, up to now, been studied. Thus, the present investigation aimed to reveal molecular mechanisms underlying inhibition of invasion and metastasis in human breast cancer cell lines MCF-7 (oestrogen receptor (ER) positive) and MDA-MB-231 (ER negative). In this report, we focused on proteins which are known to play important roles in control of angiogenesis, tumour cell invasion and metastasis.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and β -actin antibody were purchased from Sigma Chemical Pvt Ltd, St. Louis, MO, USA. Nimbolide (98% purity) was purchased from Asthagiri Research Foundation, Chennai, India. Polyvinylidenedifluoride (PVDF) membrane was from Millipore, Billerica, MA, USA. Trypsin-EDTA, foetal bovine serum (FBS), antibiotics, antimycotics and phosphate-buffered saline (PBS) were from Gibco, Burlington, ON, Canada. Primary antibodies against EGFR, VEGFR, NF κ B, IKK α and IKK β , MMP-2, MMP-9 and TIMP-2 were from Cell Signaling, Beverly, MA, USA and Santa Cruz Biotechnology, Santa Cruz, CA, USA. Secondary antibodies, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG were obtained from Genei, Bangalore, India. The enhanced chemiluminescence (ECL) kit was procured from Thermo Scientific,

Table 1. Primer Sequence

S.No.	Primers	Forward	Reverse	Tm	bp
1	uPA	tgtgagactcactggcttgg	cacagcattttgggtgac	58	146
2	uPAR	agctatcgactggcttgaa	tgtctgatgagccacaggaa	58	142
3	CXCR1	gttccctggggtttaactc	cctcagggtgaagctgagac	60	145
4	CXCR4	ctccaagctgtcacactcca	gagtcgatgctgatccaat	60	120
5	CCR2	gctcttggctgtaggattgc	tttcttttccacgaccatc	58	119
6	CXCL8	gtctgctagcaggctccac	gcttcacatgtcctcaca	58	123
7	CXCL12	ctccacatcctccagttct	cctgcttggtgcacagtta	58	131
8	CCL2	ccccagtcacctgctgttat	cagatcctctggccacaat	58	136
9	CXCR2	cgctcgtcactgatgtca	gagtgagaccacttgcaca	58	135
10	GAPDH	cgaccactttgtcaagctca	cccctcttcaagggtctac	58	238

Waltham, MA, USA. Primers used in the present study are shown in Table 1. All chemicals used were extra pure and of analytical grade.

Cell lines and culture

ER-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cell lines were obtained from the NCCS, Pune. Cells were grown in T75 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). They were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. On reaching confluence, cells were trypsinized and passaged.

Western blot analysis

Cells were seeded 1×10^6 per dish, in 100 mm dishes and treated with DMSO (vehicle), 2 µM or 4 µM nimbolide for MCF-7 cells, 4 µM or 6 µM nimbolide for MDA-MB-231 cells. After 24 h treatment, cells were lysed in RIPA buffer containing 1x protease and phosphatase inhibitor cocktail, and protein concentrations were determined by Lowry's method (28). Cell lysates (50 µg) were electrophoresed in 12% SDS polyacrylamide gel, then transferred to PVDF membranes, which were incubated with primary antibodies against pEGFR, VEGFR, NFκB, IKKα and IKKβ, MMP-2, MMP-9 and TIMP-2 and β-actin, in Tris-buffered saline. After washing, membranes were incubated with HRP-conjugated anti-mouse IgG (1:5000) or goat anti-rabbit IgG (1:5000). Protein bands were detected using ECL Kit chemiluminescence system and quantified with Chemi Doc XRS Imaging System (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR

mRNA expression of *uPA* and *uPAR* were examined using real-time PCR, total RNA being isolated using Tri

Reagent (Sigma, St. Louis, MO, USA) (29). Total RNA (2 µg) from each sample was reverse transcribed using commercial Superscript III first strand cDNA synthesis kit (Invitrogen, Eugene, OR, USA) according to the manufacturer's protocol; list of primers and internal control sequence are given in Table 1. Real-time PCR was carried out in an MX3000p PCR system (Stratagene, La Jolla, CA, USA). The reaction was performed using MESA Green PCR master mix (containing all necessary PCR components along with SYBR green dye) (Eurogentec, San Diego, CA, USA). Specificity of amplification product was determined by melting curve analysis for each primer pair. Data were analysed by the comparative C_T method and fold change was calculated by the $2^{-\Delta\Delta C_T}$ method as described previously (30).

Reverse transcription-polymerase chain reaction

mRNA expression of chemokines CCL2, CXCL12, CXCL8 and their receptors CCR2, CXCR1, CXCR2 and CXCR4 respectively were analysed by conventional PCR. Primers used in the present study are shown in Table 1. Conventional PCR was performed with Fast PCR Master Mix (KAPA, Wilmington, MA, USA) in an Eppendorf master cycler. Amplification sequence protocol was 35 cycles of 94 °C for 5 min, 58 °C for 30 s, extension 72 °C for 30 s and final extension 72 °C for 10 min. Amplification products were analysed electrophoretically on 2.0% agarose gel containing 0.1 µg/ml ethidium bromide. PCR experiments were performed in triplicate. Data were quantified by Quantity One software (Bio-Rad, USA).

Wound healing assay

Effects of nimbolide on MCF-7 and MDA-MB-231 cell migratory activity was examined by wound healing assay. Cells were seeded in six-well plates and were cultured in 5% CO₂ incubator for 24 h, until

completely confluent. The cell monolayer was scratched with a 200 μ l pipette tip to create a wound, and cells were washed twice in PBS (to remove any floating) which was then replaced with serum-free RPMI medium. Cells were subjected to the indicated treatment for 24 h, and cells migrating from the leading edge were photographed at 0 and 24 h using phase-contrast microscopy.

Matrigel invasion assay

Transwell inserts 6.5 mm diameter, with 8 μ m pore membranes coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA), were used to assess invasive potentials of the breast cancer cells. Cells were detached, washed twice in PBS, and resuspended in serum-free DMEM with nimbolide or without nimbolide (control). 5×10^5 cells in 0.2 ml were placed in the

upper chamber of a transwell insert and the lower chamber was filled with 400 μ l DMEM 10% foetal bovine serum, as chemo-attractant. After 24-h incubation, cells in the upper chamber that had not migrated were gently scraped away and adherent cells present on the lower surface were stained with Diff-Quick stain; they were then photographed using fluorescence microscopy (NIKON Eclipse 80i, Ku, Tokyo, Japan).

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVA followed by Student–Newman–Keul’s (SNK) tests for comparison between treatment and control values, using Statistical Package for Student, version 7.5 (SPSS) software. $P < 0.05$ was considered to be statistically significant.

Fig. 1. Inhibition of pEGFR, VEGFR, IKK α , IKK β and NF κ B protein expression in MCF-7 and MDA-MB-231 cells. Cells were cultured and treated with or without nimbolide (2, 4 μ M for MCF-7 and 4, 6 μ M for MDA-MB-231 cells) in serum-free DMEM medium for 24 h. Total cell lysate was subjected to western blot analysis. Proteins (50 μ g) from control and nimbolide-treated cell lysates were separated in SDS-PAGE and protein levels of pEGFR, VEGFR, IKK α , IKK β and NF κ B were detected. β -actin was used as internal control. Immunoreactive bands were detected with enhanced chemiluminescence reagent (chemi documentation) using Chemi Doc XRS Imaging System, Bio-Rad (USA). Each bar represents mean $\pm$ SEM of three independent observations. ‘*’ represents statistical significance between control versus nimbolide treatment groups at $P < 0.05$ level, using Student’s–Newman–Keul’s test.

Results

Nimbolide inhibited expression of pEGFR, VEGFR, IKK α , IKK β and NF κ B proteins

Western blot analysis indicated that nimbolide significantly reduced expression of pEGFR and VEGFR proteins at 2, 4 μ M in MCF-7, and 4, 6 μ M in MDA-MB-231 cells compared to control (Fig. 1). IKK α and IKK β enzyme complex is part of the upstream NF κ B signal transduction cascade. NF κ B is an important transcription factor involved in cell survival. Nimbolide (2, 4 μ M in MCF-7 cells and 4, 6 μ M in MDA-MB-231 cells) significantly reduced expression of IKK α , IKK β and NF κ B proteins in both the cell lines (Fig. 1).

Effect of nimbolide on expression of uPA and uPAR mRNA

Strong clinical and experimental evidence has accumulated that cell surface interaction of uPA with uPAR facilitates extravasation and intravasation of cancer cells by regulating local proteolysis and attachment of cells to components of the extracellular matrix (31). mRNA expression of uPA and uPAR was assessed by real-time PCR in both the MCF-7 and MDA-MB-231 cell lines. Nimbolide significantly reduced mRNA expression of uPA and uPAR in both cell lines (Fig. 2).

Effect of nimbolide on expression of chemokine mRNAs

Chemokines compose a family of small cytokines (signalling proteins) secreted by cells. The major role of chemokines is to act as chemoattractants to guide cell migration. CCL2 and CCR2 mRNA expressions were assessed by semi-quantitative PCR (Fig. 3a). CCL2, an inflammatory chemokine, binds to its receptor CCR2 and mediates inflammation. Nimbolide treatment (2, 4 μ M in MCF-7 cells and 4, 6 μ M in MDA-MB-231 cells) significantly reduced CCL2 mRNA expression compared to control cells. mRNA expression of CCR2 decreased in nimbolide-treated MCF-7 cells, but not in MDA-MB-231 cells. Figure 3b shows mRNA expressions of CXCL12 and CXCR4. CXCL12 is mainly involved in cancer cell metastasis; it binds to CXCR4 and is involved in site-specific migration. mRNA expression of CXCL12 and CXCR4 significantly decreased in nimbolide-treated breast cancer cells compared to control. Figure 3c shows mRNA expressions of CXCL8, CXCR1 and CXCR2. CXCL8 plays a major role in angiogenesis as it binds to CXCR1 and CXCR2. It has been found that nimbolide treatment significantly

Fig. 2. Effect of nimbolide on mRNA expression of uPA and uPAR. Cells were treated with or without nimbolide (2, 4 μ M for MCF-7 and 4, 6 μ M for MDA-B-231 cells) in serum-free DMEM for 24 h; total RNA was immediately extracted and converted to cDNA. mRNA expression of genes were analysed by real-time PCR using SYBR green dye and protein expression by western blotting. Target gene expression normalized to GAPDH mRNA expression and the results expressed as fold change from control (Fig. 2). Protein levels were quantified using densitometry analysis and are expressed in relative intensity arbitrary unit (Fig. 2). β -actin was used as an internal control. ‘*’ represents statistical significance between control versus nimbolide treatment groups at $P < 0.05$ level using Student’s–Newman–Keul’s test.

reduced CXCL8 and CXCR2 mRNA expressions compared to control. CXCR1 expression was reduced in nimbolide-treated MDA MB-231 cells, but there was no change in CXCR1 expression in MCF-7s.

Effects of nimbolide on expression of MMP2, MMP-9 and TIMP-2 proteins

Matrix metalloproteinases are zinc-dependent endopeptidases. They are capable of degrading a wide range of extracellular matrix proteins, but also can process a number of bioactive molecules. MMPs also play a major role in various types of cell behaviour, such as proliferation, migration and angiogenesis. Nimbolide significantly reduced expression of MMP-2, MMP-9 proteins

Fig. 3. Effect of nimbolide on mRNA expression of chemokines. (a) mRNA expression of CCL2 and CCR2. (b) CXCL12, CXCR1 and CXCR2. (c) CXCL8, CXCR1 and CXCR2 in the breast cancer cells. Cells were treated with or without nimbolide (2, 4 μM for MCF-7 and 4, 6 μM for MDA-MB-231 cells) in serum-free DMEM for 24 h. RT-PCR was performed for CXCL8, CXCR1 and CXCR2 mRNA expression. Each bar represents mean ± SEM of three independent observations. '*' represents statistical significance between control versus nimbolide and other treatment groups at $P < 0.05$ level using Student's–Newman–Keul's test.

Fig. 4. Effects of nimbolide on MMP2, MMP-9 and TIMP-2 protein expression. Each bar represents mean $\pm$ SEM of three independent observations. ‘*’ represents statistical significance between control versus nimbolide and other treatment groups at $P < 0.05$ level using Student’s–Newman–Keul’s test.

and increased TIMP-2 expression at 2, 4 μ M concentrations in MCF-7 cells and 4, 6 μ M in MDA-MB-231 cells (Fig. 4).

Nimbolide inhibited migration of MCF-7 and MDA-MB-231 cells

Inhibitory effect of nimbolide on breast cancer cell migration was evaluated by scratch wound healing assay (Fig. 5a,b). At time point 0 h, scraped cells were photographed. After nimbolide (2, 4 μ M for MCF-7 cells and 4, 6 μ M for MDA-MB-231 cells) treatment for 24 h, migration of cells into wounded areas was effectively reduced. This result suggests that nimbolide inhibited migration of the breast cancer cells.

Nimbolide inhibited invasion of MCF-7 and MDA-MB-231 cells

Cell invasion was determined by transwell invasion assay. Nimbolide (2, 4 μ M for MCF-7 cells and 4, 6 μ M for MDA-MB-231 cells) significantly inhibited invasion of both types of cell (Fig. 6). Quantitative data derived from three independent experiments supported the

information that nimbolide effectively prevented invasion characteristics of MCF-7 and MDA-MB-231 cells.

Discussion

Abnormal population growth and metastasis of cancer cells, are regarded as the most important biological characteristics of malignancies. Presence of metastasis is the main cause of morbidity and mortality in millions of patients with cancer. In present study, we demonstrated that nimbolide inhibited breast cancer cell invasion and migration. In an earlier study by our team, we observed that breast cancer cell viability decreased gradually with increased concentration of nimbolide, with IC₅₀ values of 4.0 μ M and 2.7 μ M, over 24 and 48 h, respectively in MCF-7 and 6.0 μ M and 3.2 μ M for 24 and 48 h, respectively in MDA-MB-231 cells (32). EGF is a growth factor that stimulates cell population growth, proliferation and differentiation, by binding to its receptor. EGF and EGFR are frequently co-expressed in a large subset of primary breast carcinomas (3). Signal transduction through the EGFR axis has been implicated in mediating multiple processes involved in tumour progression and metastasis, including invasion, angiogenesis, proliferation

Fig. 5. Nimbolide inhibited migration of (a) MCF-7 and (b) MDA-MB-231 cells. Cells were scratched with a pipette tip and washed twice in PBS and photographed (0 h), then they were treated with nimbolide. Control cells were treated with SFM for 24 h. Migration was photographed under phase-contrast microscopy ($200\times$). After 24 h, numbers of cells of the control group were shown to have migrated. However, lower migration of cells after nimbolide treatment indicates the anti-migratory activity of nimbolide.

and inhibition of apoptosis (33). Nimbolide treatment significantly reduced protein expression of EGFR in both the cell lines. Thus, nimbolide interfered with operation of the EGF system, thereby inhibiting cell proliferation and metastasis of our breast cancer cells.

EGFR mediates its function through *via* kinase signalling pathways such as that of Ras/Raf/Erk. Thus, inhibiting activation of growth factor receptors, especially EGFR, is a promising strategy for treatment of breast cancer (34). EGFR triggers a kinase pathway resulting in a final outcome concerned with nuclear stimulation of the dimeric complex of NF κ B that activates transcription of tumour cell growth linked genes (4). Involved is its family of pleiotropic transcription factors that regulates transcription of a many genes that play key roles in embryonic development, lymphoid differentiation, apoptosis, and immune and inflammatory responses (35). This transcription factor is localized in the cytosol and is blocked by I κ B. The major target of

Akt is the NF κ B pathway. Activation of NF κ B typically occurs through site-specific phosphorylation and subsequent degradation of I κ B by IKK α ; IKK β enzyme complex is part of the upstream NF κ B signal transduction cascade. This allows translocation of NF κ B into the nucleus to bind to NF κ B-specific DNA-binding sites and regulate gene transcription. In the present study, nimbolide significantly reduced expression of IKK α , IKK β and NF κ B proteins in both the breast cancer cell lines we investigated. Gupta *et al.* (22) found that nimbolide inhibited constitutive and inducible NF κ B activation in myeloid and leukemic cells. Our earlier studies have shown that inhibition of cell expansion by nimbolide was associated with induction of apoptosis in the same breast cancer cell lines (32). Efficient metastasis is always promoted by VEGF, released during ECM processing or through endogenous production by tumour cells. This all leads to bioavailability of VEGF, triggering a network of signalling pathways that promote

Fig. 6. Nimbolide inhibited invasion of MCF-7 and MDA-MB-231 cells. Cells were treated with nimbolide in serum-free medium for 24 h in Matrigel invasion chambers. Cells invading to the bottom of membranes were stained and photographed (200 × magnification) using fluorescence microscopy (NIKON Eclipse 80i). Each membrane was extracted and quantified at 560 nm. Each bar represents the mean ± SEM of three independent observations. '*' represents statistical significance between control versus nimbolide and other treatment groups at $P < 0.05$ level using Student's–Newman–Keul's test.

angiogenesis. Repression of VEGF and its receptors reduce tumour growth in multiple tumour models demonstrating its vital role in tumour progression (36). In the current study, a significant reduction in expression of VEGFR protein in nimbolide-treated our breast cancer cell lines, was observed.

Metastasis is the process by which a tumour cell leaves the primary tumour, travels to a distant site *via* the circulatory system, and establishes a secondary tumour. To metastasize, cancer cells must invade through the basement membrane and extracellular matrix. Proteolysis of the ECM is an important step in metastasis and is associated with up-regulated production and activity of several ECM-degrading proteases. Matrix metalloproteinases are zinc-dependent proteases that play major roles in proteolytic degradation of ECM components and aid in tumour invasion and metastasis. MMPs, secreted as latent zymogens, are activated by plasmin and their activity is regulated by a family of tissue inhibitors of metalloproteinase (37). TIMPs have been shown to perform numerous biological functions in blocking tumour metastasis, principally by their action of inhibiting matrix metalloproteinases at different steps of the metastatic process (38). The initial step of tumour

cell invasion is characterized by breakdown of the basement membrane, a process known to be dependent on type IV collagen-degrading enzymes, mainly MMP-2 and MMP-9 (8). Expression of MMP-2 & 9 proteins was significantly reduced and TIMP-2 expression increased in the nimbolide-treated breast cancer cells. Previous studies have also reported that protein expression and activity of MMP-2 & 9 was significantly reduced in myeloid leukaemia cell lines (22) and colon cancer cells (25).

High level of expression of one or more proteases often correlates with migration of cancer cells through the digested extracellular matrix, and contributes to tumour cell invasion and metastasis of virtually all malignancies, including breast cancer (37). One such key protease is uPA, involved in multiple physiological and pathological processes including cell migration, angiogenesis, wound healing and metastasis (39,40). uPA binds to its receptor, which facilitates conversion of plasminogen to plasmin. Plasmin, either directly or indirectly through metalloproteinases, can degrade components of the extracellular matrix contributing to invasion and metastases. In breast cancer, high levels of uPA and/or its inhibitor (PAI-1) significantly correlate

Fig. 7. Schematic representation of molecular mechanisms for anti-invasive and anti-migrative activity of nimbolide. Nimbolide down-regulated protein expression of pEGFR, VEGFR, NFκB, MMP-2, MMP-9, and mRNA expression of chemokines. Protein expression of TIMP-2 increased on nimbolide treatment. Red down arrow indicates that these molecules were up-down regulated by nimbolide, whereas green up arrow indicates increased expression.

with tumour aggressiveness and poor outcome (41). Here, mRNA expression of uPA and uPAR were significantly reduced in nimbolide-treated breast cancer cell lines. Results are consistent with previous reports revealing that ENLE treatment at 50 and 100 µg/ml to PC-3 and LNCaP prostate cancer cell lines significantly inhibited mRNA expression of uPA and its receptor uPAR (42).

Chemokines, a family of chemoattractant cytokines, have been shown to play different important roles in the various stages of tumour development and metastasis, and may provide a gateway to targeted anti-cancer therapies (43). They are produced by tumour cells (and also by stromal cells) of both primary and the distant metastatic microenvironment. Wide and differential distribution of chemokines and their receptors accounts for pleiotropic actions of chemokines in breast cancer, including modulation of growth, angiogenesis, invasion and metastasis. Up-regulation of chemokine molecules in tumour biology compared to normal cells hints that chemokines might be thought of “magic bullets,” as targeting them can potentially specifically select cancer cells and their metastases, leaving non-malignant cells unharmed. CCL2 is a pro-inflammatory chemokine and

associating with its receptor CCR2 infiltrates inflammatory leukocytes. CCL2 recruits inflammatory leukocytes (such as tumour-associated macrophages) in the tumour microenvironment and facilitates cancer cell progression (44). Results of the present study reveal significant reduction in mRNA expression of CCL2 in both our nimbolide-treated cell lines. mRNA expression of CCR2 decreased in nimbolide-treated MCF-7 cells, but not in MDA-MB-231 cells.

With mRNA expression of CXCL8 (a novel angiogenic factor), CXCR2 has been shown to be significantly reduced in nimbolide-treated cells. CXCL8, a pro-angiogenic chemokine, simulates both endothelial cell proliferation and migration through CXCR2 & 1 (45). CXCL-8 enhances *in vitro* growth, motility and invasion as well as MMP-9 and VEGF production (46). Elevated levels of VEGF expression in breast cancer are accompanied by high expression levels of CXCL8 (47). In the present study, low mRNA expression of CXCL8 and CXCR2 also demonstrated that nimbolide abrogated angiogenesis of the breast cancers by targeting multiple angiogenic factors. CXCR1 mRNA expression was reduced in nimbolide-treated MDA-MB-231 cells, but no change was observed in the MCF-7 cells. Muller

et al. (48) proposed that the chemokine CXCR4/CXCL12 axis may provide directional cues for site-specific metastasis of breast cancer. CXCL12 is a CXC type chemokine that activates downstream signalling through binding G protein-coupled receptor. CXCL12 promotes tumour invasion by inducing MMP-9 which degrades extracellular matrix components (49). In this study, nimbolide treatment has caused significant reduction in mRNA expression of CXCR4 and CXCL12, which again proves the potent anti-invasive role of nimbolide. CXCR4 is the key component of metastatic implantation into bone marrow and represents an important therapeutic target for metastatic bone disease in breast cancer metastasis. NF κ B has previously been shown to increase expression of CXCR4 in MCF-7 and MDA-MB 231 breast cancer cells (50). Here, NF κ B system reduced after nimbolide treatment, in both the cell lines. These results may provide the reason for reduced expression of chemokines.

Initiation of metastasis involves invasion, which has many phenotypic similarities to epithelial–mesenchymal transition (EMT), including loss of cell–cell adhesion and increase in cell mobility. Hence, one of the objectives of this study was to explore possible effects of nimbolide on tumour cell migration and invasion. *In vivo*, cells free themselves to migrate by breaking ECM connections by the action of different MMPs; such events occur during many physiological and pathological conditions (51). Invasion occurrence was performed by *in vitro* Matrigel invasion chamber assay to evaluate anti-invasive potential of nimbolide. Nimbolide significantly repressed invasive potential of the breast cancer cells. Similarly, nimbolide inhibited cell migration, determined by wound healing assay. Upon nimbolide treatment, both MCF-7 and MDA-MB-231 cells resulted in reduced expression of MMP-2 and MMP-9 with significantly increased level of TIMP-2 expression. Reduction in expression of MMP-2 and MMP-9 would have been probably been due to reduction in chemokines, pEGFR expression and its down-stream signalling molecules by nimbolide treatment. Thus nimbolide reduced EGF signalling and inhibited proliferation. Accordingly, migration and invasive potential of MCF-7 and MDA-MB-231 cells was considerably suppressed by nimbolide treatment.

Nimbolide has been found to modulate expression and/or activity of transcription factors involved in critical pathways of carcinogenesis, including carcinogen activation and detoxification, growth arrest and apoptosis, and proinflammatory-mediated signalling pathway (52). Findings of the current study are in agreement with previous reports in which similar trends have been observed. Our results concerning mRNA and protein

levels showed that nimbolide down-regulated expression of uPA, uPAR, chemokines, pEGFR, VEGFR, NF κ B, IKK α , IKK β , MMP-2, MMP-9 and up-regulated expression of TIMP-2, suggesting that nimbolide inhibits angiogenesis, invasion and metastasis of breast cancer cells. Altogether, data of the present study highlight (Fig. 7) that nimbolide significantly reduced migration and invasive potential of the breast cancer cells tested. Thus, they suggest that this phytochemical can be considered a promising candidate for molecular targeted therapy of challenging breast cancers. Further studies *in vivo* will be a still more definitive to prove its potency.

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Conflict of interest

None declared.

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