

## Down-Regulation of uPA and uPAR by 3,3'-Diindolylmethane Contributes to the Inhibition of Cell Growth and Migration of Breast Cancer Cells

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### ABSTRACT

3,3'-Diindolylmethane (DIM) is a known anti-tumor agent against breast and other cancers; however, its exact mechanism of action remains unclear. The urokinase plasminogen activator (uPA) and its receptor (uPAR) system are involved in the degradation of basement membrane and extracellular matrix, leading to tumor cell invasion and metastasis. Since uPA-uPAR system is highly activated in aggressive breast cancer, we hypothesized that the biological activity of B-DIM could be mediated via inactivation of uPA-uPAR system. We found that B-DIM treatment as well as silencing of uPA-uPAR led to the inhibition of cell growth and motility of MDA-MB-231 cells, which was in part due to inhibition of VEGF and MMP-9. Moreover, silencing of uPA-uPAR led to decreased sensitivity of these cells to B-DIM indicating an important role of uPA-uPAR in B-DIM-mediated inhibition of cell growth and migration. We also found similar effects of B-DIM on MCF-7, cells expressing low levels of uPA-uPAR, which was due to direct down-regulation of MMP-9 and VEGF, independent of uPA-uPAR system. Interestingly, over-expression of uPA-uPAR in MCF-7 cells attenuated the inhibitory effects of B-DIM. Our results, therefore, suggest that B-DIM down-regulates uPA-uPAR in aggressive breast cancers but in the absence of uPA-uPAR, B-DIM can directly inhibit VEGF and MMP-9 leading to the inhibition of cell growth and migration of breast cancer cells. *J. Cell. Biochem.* 108: 916–925, 2009. © 2009 Wiley-Liss, Inc.

**KEY WORDS:** 3, 3'-DIINDOLYLMETHANE; BREAST CANCER; uPA; uPAR; MDA-MB-231; MCF-7; MIGRATION; VEGF; MMP-9

Breast cancer is the leading cause of cancer deaths in women in the United States. The American Cancer Society estimates 192,370 new cases of breast cancer in the year 2009, and it is the second leading cause of death in women in the United States [Jemal et al., 2009]. Although significant progress has been made in reducing breast cancer-related deaths, it is still unsatisfactory; suggesting that further development of novel preventive and/or therapeutic approaches is urgently needed. One such approach could be the use of naturally occurring dietary substances that are classically known to be non-toxic to normal cells but effective in the killing of cancer cells. Epidemiological studies have shown beneficial effect of high dietary intake of fruits and vegetables against carcinogenesis [Heber and Bowerman, 2001]. Among many such agents, indole compounds present in a variety of cruciferous vegetables are showing great promise against several cancers [Steinmetz and Potter, 1991; Higdon et al., 2007; Bradlow, 2008]. In particular, our earlier work has shown a therapeutic effect of

Indole-3-Carbinol (I3C) using severe combined immunodeficient (SCID)-human mouse model of experimental bone metastasis [Rahman et al., 2006a]. I3C is chemically unstable in the acidic environment of the stomach and is rapidly converted into its dimeric form, 3,3'-diindolylmethane (DIM). DIM itself has been shown to exert anti-carcinogenic effects in experimental animals [Hong et al., 2002b]; however, the molecular mechanisms by which DIM exerts its biological effects remain under active investigation.

Previous studies from our laboratory have shown beneficial effects of DIM against breast cancer [Rahman and Sarkar, 2005; Rahman et al., 2006b; Wang et al., 2008], which is consistent with published results from other investigators [Chang et al., 2005; Hsu et al., 2008]. In an attempt to explain the mechanism of action of DIM, we have shown earlier that DIM induces apoptotic cell death in breast cancer cells by inactivating Akt and NF- $\kappa$ B signaling [Rahman and Sarkar, 2005] and by the down-regulation of survivin [Rahman et al., 2006b]. In addition to breast cancer, the anti-cancer

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properties of DIM have been demonstrated in several other cancer models as well [Nachshon-Kedmi et al., 2004a,b; Bhuiyan et al., 2006; Kim et al., 2007; Kong et al., 2007; Li et al., 2007; Ali et al., 2008; Hsu et al., 2008]. In prostate cancer cells, we showed that B-DIM (a formulated DIM) inhibits angiogenesis and invasion [Kong et al., 2007]. These results also suggested, for the first time, a potential role of uPA in B-DIM-induced killing of prostate cancer cells. In a recent mechanistic study [Ahmad et al., 2009], we showed that down-regulation of uPA or its receptor, uPAR by the use of specific siRNA leads to a significant inhibition of prostate cancer cell growth and migration, suggesting that uPA system may be a potential therapeutic target for the treatment of human malignancies.

It is well known that the uPA, or urokinase-type plasminogen activator, is a member of the urokinase plasminogen activator system, a serine protease family comprising of uPA, plasminogen activator inhibitors (PAI's), tissue-type plasminogen activator (tPA) and the uPA receptor (uPAR). The urokinase plasminogen system is believed to play an important role in cell migration, angiogenesis, cancer cell invasion and metastasis [Sheng, 2001; Rao, 2003]. In recent years the involvement of uPA family members in the progression of human cancers [Dass et al., 2008] has gained significant attention; however, mechanistic studies to ascertain the role of uPA-uPAR in mediating the biological effects of chemopreventive agents, especially in breast cancer, are lacking, which prompted us to conduct the current study.

Association of uPA with its receptor, uPAR, provides an inducible, transient, and localized cell surface proteolytic activity machinery [Wang, 2001; Pillay et al., 2007], which is recognized as a major factor causatively involved in tumor cell invasion and metastasis, and thus represents a very potent therapeutic target. We, therefore, hypothesized that uPA may play an important role in mediating the biological activity of DIM in breast cancer cells. Our current study was designed to use a highly aggressive breast cancer line, MDA-MB-231 and a lesser aggressive breast cancer cell line, MCF-7, and investigated the role of uPA as well as its receptor, uPAR, in the growth, proliferation and migration of these cells by DIM treatment. For our studies, we used a specially formulated DIM (marketed by BioResponse, Boulder, CO) and referred to as B-DIM in this study. This preparation of DIM has a highly improved bioavailability [Anderton et al., 2004] and is also currently available for human use. The results reported here clearly suggest the potential mechanistic role of B-DIM in the inhibition of cell growth and tumor progression, which was mediated by inhibiting uPA-uPAR system-dependent as well as -independent manner involving inactivation of VEGF and MMP-9 in breast cancer. Based on our results, we believe that B-DIM could be useful for the prevention of breast tumor progression and/or its treatment in the near future.

## MATERIALS AND METHODS

### CELL LINES AND REAGENTS

Breast cancer cell lines, MDA-MB-231 as well as MCF-7 were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml

streptomycin. All cells were cultured in a 5% CO<sub>2</sub>-humidified atmosphere at 37°C. B-DIM, a formulated DIM with higher bioavailability, was kindly provided by Dr. Michael Zeligs (Bio-Response) and was dissolved in DMSO to make 25 mM stock solutions and stored at -20°C in multiple aliquots. Antibodies against uPA and uPAR were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the monoclonal antibody against β-actin was purchased from Sigma-Aldrich (St. Louis, MO). The VEGF quantikine ELISA kits and MMP-9 fluorescent assay kits were purchased from R&D Systems.

### PLASMIDS, siRNAs, AND TRANSFECTION CONDITIONS

For over-expression studies, uPA and uPAR-cDNA containing vectors were purchased from Origene Technologies (Rockville, MD). MCF-7 cells were transiently transfected with uPA or uPAR cDNA or the vector control, using Lipofectamine 2000 following the transfection protocol described earlier [Ahmad et al., 2008]. For silencing studies, uPA/uPAR small interfering RNAs (siRNAs) were obtained from Santa Cruz, and were transfected into MDA-MB-231 cells using DharmaFECT3 siRNA transfection reagent (Dharmacon, Inc., Lafayette, CO). Cells were allowed to propagate for 18–24 h post-transfection before treatment with varying concentrations of B-DIM as described under individual experiments.

### CELL GROWTH INHIBITION STUDIES BY 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY

MDA-MB-231 cells were seeded at a density of  $2 \times 10^3$  cells per well while MCF-7 cells were seeded at a density of  $5 \times 10^3$  cells per well in 96-well microtiter culture plates. After overnight incubation, cells were transfected with cDNA or siRNA for uPA/uPAR, as mentioned for individual experiments. Twenty-four hours post-transfection, liquid medium was removed and replaced with a fresh medium containing DMSO (vehicle control) or different concentrations of B-DIM diluted from a 25 mM stock. After 72 h of incubation, 25 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in phosphate-buffered saline, PBS) was added to each well and incubated further for 2 h at 37°C. Upon termination, the supernatant was aspirated and the MTT formazan, formed by metabolically viable cells, was dissolved in isopropanol (100 µl) by mixing for 30 min on a gyratory shaker. The absorbance was measured at 595 nm on Ultra Multifunctional Microplate Reader (TECAN, Durham, NC). Each treatment had eight replicate wells and the amount of DMSO in reaction mixture never exceeded 0.1%. Moreover, each experiment was repeated at least three times.

### CELL VIABILITY STUDIES BY TRYPAN BLUE ASSAY

MDA-MB-231 cells were seeded in six-well culture plates and treated with B-DIM as described above. Upon completion of incubation, culture medium (with floating dead cells) was collected and pooled with the adherent cells removed from the plate by trypsinization. The cells were briefly spun and re-suspended in the normal culture medium. Cell viability was assessed by adding 50 µl of Trypan Blue solution (0.4% in PBS) to 50 µl of the cell suspension. After 2 min, the number of living cells, which did not retain the dye

was counted using a hemocytometer, and was compared to the total number of cells (living + dead) to calculate the viability percentage.

### SOFT AGAR COLONIZATION ASSAYS

MDA-MB-231 and MCF-7 cells were first transfected with appropriate siRNA/cDNA in six-well plates, allowed to grow for 24 h and then collected by trypsinization. Cells ( $3 \times 10^4$ ) were then plated in 0.5 ml of culture medium containing 0.3% (w/v) top agar layered over a basal layer of 0.7% (w/v) agar (with culture medium and the supplements) in 24-well plates. At the time of seeding, the culture was supplemented with different concentrations of B-DIM or the DMSO. After appropriate culture time (18 days for MDA-MB-231 and 30 days for MCF-7 cells), colonies (>50 cells) were counted. Experiments were carried out in quadruplicate, and results presented are representative of two independent observations.

### WESTERN BLOT ASSAY

Cell lysates were obtained from cells using cold RIPA buffer as described previously [Ahmad et al., 2008]. Protein concentration was measured by BCA Protein Assay (Pierce, Rockford, IL). After the resolution through 12% polyacrylamide gels under denaturing conditions, proteins were transferred to nitrocellulose membranes, and appropriate primary antibodies were added. This was followed by incubation with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the chemiluminescence detection system (Pierce). For re-probing the blots, membranes were incubated for 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.7) and 100 mM  $\beta$ -mercaptoethanol, washed and incubated with desired primary followed by secondary antibodies and the signals were detected as described above.

### ELISA ASSAY FOR VEGF AND MMP-9

To investigate the effect of uPA-uPAR on the production of VEGF and MMP-9, over-expression or silencing of uPA-uPAR was performed in six-well plates. After 24 h of seeding, cells were either transfected with uPA, uPAR or vector control cDNA or with non-specific (control) or uPA-uPAR siRNAs. Cells were then treated with DMSO control or B-DIM in serum-free media and incubated for 48 h. The culture media were collected, centrifuged to remove cellular debris, and stored at  $-70^\circ\text{C}$  until used for the assay. The cells in the plate were trypsinized, and the total number of cells was determined by cell counting. For assessing the levels of VEGF and MMP-9 secreted in the conditioned medium, commercially available VEGF and MMP-9 ELISA kits (R&D Systems) were used following instructions supplied by the manufacturer and the results were normalized to the cell number.

### CELL MIGRATION ASSAY

Cell migration was assessed using 24-well inserts (BD Biosciences) with 8- $\mu\text{m}$  pores according to the manufacturer's instructions. Briefly, transiently transfected cells ( $5 \times 10^4$ ) with serum-free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium. After 24 h of incubation, the cells in the upper chamber were removed, and the

cells that had migrated through the membrane were stained with 4  $\mu\text{g/ml}$  calcein AM in Hanks buffered saline at 37°C for 1 h. The fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled cells were also photographed under a fluorescent microscope.

### DATA ANALYSIS

The experimental results presented in the figures are representative of three or more independent observations. The data are presented as the mean values  $\pm$  SE. Comparisons between groups were evaluated by Student's *t* test. Values of  $P < 0.05$  were considered to be statistically significant.

## RESULTS

### EFFECT OF B-DIM TREATMENT ON PROLIFERATION AND VIABILITY OF AGGRESSIVE BREAST CANCER CELLS

We initially assessed the dose- as well as time-dependent effect of B-DIM treatment on MDA-MB-231 cells (aggressive breast cancer cell line). As seen in Figure 1A, cell proliferation was reduced in a dose-dependent manner as assessed by MTT assay. Ten micromolars B-DIM treatment resulted in  $\sim 12\%$  inhibition of cell growth after 72 h whereas 25  $\mu\text{M}$  B-DIM inhibited the growth by about 26% over the same period. We also tested the viability of MDA-MB-231 cells treated with B-DIM, using trypan blue dye exclusion method. As observed in Figure 1B, treatment with B-DIM resulted in a dose-dependent inhibition of cell viability of MDA-MB-231 cells. B-DIM, at 10  $\mu\text{M}$  final concentration inhibited cell viability by 7% after 72 h of exposure. At 25  $\mu\text{M}$  B-DIM concentration, the inhibition of cell viability was 15%. The combined results, from MTT and trypan blue exclusion assays, suggest that B-DIM treatment results in a significant inhibition of cell growth and viability of highly aggressive human breast cancer cell line.

In order to further elucidate the mechanism of growth inhibitory effect of B-DIM against aggressive breast cancer cells, we assessed the level of expression of uPA and uPAR by western blot analysis using MDA-MB-231 cells after exposure to B-DIM for 72 h. Our rationale was based on the information that the expression and activation of uPA and its receptor, uPAR, is known to play an important role in the survival and progression of human tumors [Dass et al., 2008]. Moreover, our earlier studies with prostate cancer cells provided evidence in support of the involvement of uPA in the progression of prostate cancers [Kong et al., 2007; Ahmad et al., 2009]. Our results are shown by the representative blots in Figure 1C documenting that B-DIM treatment results in a dose dependent down-regulation of uPA as well its receptor, uPAR. These results led us to conduct further mechanistic studies as presented below.

### EFFECT OF B-DIM TREATMENT AND SILENCING OF uPA AND uPAR IN HIGHLY METASTATIC MDA-MB-231 CELLS

MDA-MB-231 cell line has higher endogenous levels of uPA/uPAR expression and, therefore, we hypothesized that uPA and/or uPAR

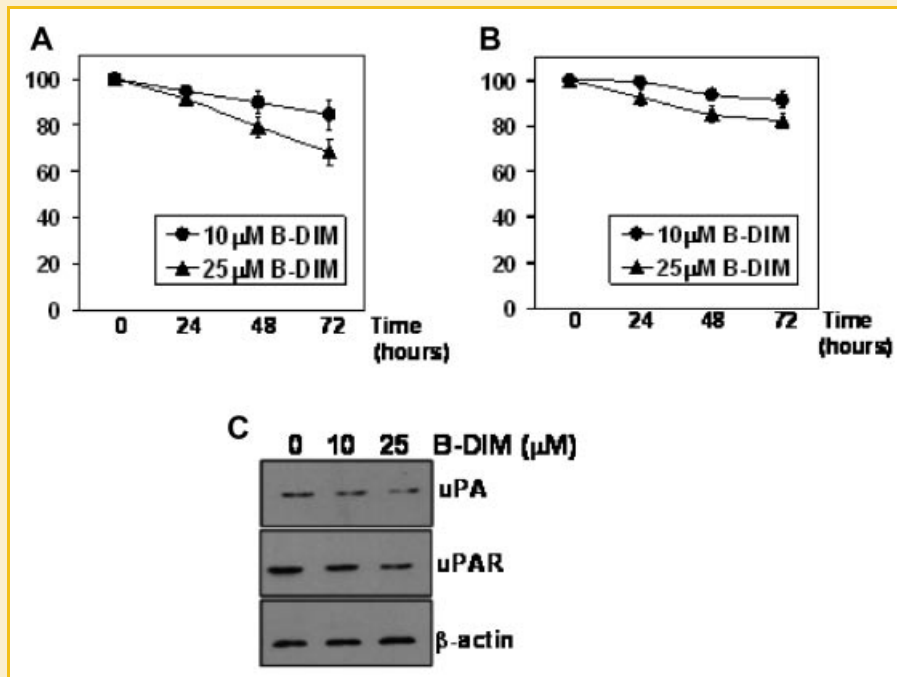


Fig. 1. Evaluation of (A) cell proliferation and (B) cell viability in B-DIM-treated MDA-MB-231 cells by MTT and trypan blue staining, respectively. Cells were either vehicle-treated (DMSO-control) or treated with 10 and 25  $\mu\text{M}$  B-DIM for 72 h and then analyzed. The amount of DMSO never exceeded 0.1% during the treatment. The number of cells counted/OD value in DMSO (control)-treatment was considered 100% and the number of cells in B-DIM-treated cells was calculated relative to this control. Each data point represents mean  $\pm$  SE of eight replicates from, at least, three independent experiments. C: Effect of B-DIM treatment on expression of uPA and uPAR in MDA-MB-231 cells.  $\beta$ -Actin protein was used as protein loading control.

might contribute to the aggressiveness of these cells. We used siRNA approach for silencing the expression of endogenous uPA and uPAR and evaluated the effect of such silencing on the proliferation of MDA-MB-231 cells. The study was performed simultaneously with B-DIM treatment. As a control, we used non-specific siRNA (NS) which was found to have no effect on the growth of MDA-MB-231 cells. In the presence of non-specific siRNA (control), exposure to B-DIM caused dose-dependent inhibition of cell growth (Fig. 2A). Silencing of uPA-uPAR, by the use of specific siRNAs, also caused significant inhibition of cell growth (0  $\mu\text{M}$  B-DIM-NS versus 0  $\mu\text{M}$  B-DIM-uPA/uPAR-si). The effect of silencing of uPA as well as uPAR on the growth of MDA-MB-231 cells was found to be highly significant ( $P < 0.01$ ) compared to non-specific control. These results suggest that uPA as well its receptor, uPAR, play a significant role in the proliferation of highly metastatic MDA-MB-231 breast cancer cells.

Interestingly, we observed that when cells were treated with B-DIM in conjunction with silencing of uPA/uPAR, the effect of B-DIM was significantly reduced (10/25  $\mu\text{M}$  B-DIM treatment in NS versus uPA/uPAR-si) suggesting that the inhibition of MDA-MB-231 cell growth by B-DIM occurs via down-regulation of uPA/uPAR and that the cell growth inhibitory effects of B-DIM could be attenuated in breast cancer cells by silencing the expression of uPA/uPAR.

Subsequently, we investigated the effect of B-DIM treatment and uPA/uPAR silencing for the ability of MDA-MB-231 cells to form viable colonies in soft agar (anchorage-independent growth assay).

B-DIM treatment resulted in 18.3% less colonies in soft agar compared to DMSO-treated control cells in the presence of non-specific siRNA (Fig. 2B). However, when uPA siRNA was used, treatment with 25  $\mu\text{M}$  B-DIM could result in only 3.8% less colonies while the same treatment in the presence of uPAR siRNA resulted in only 5.0% less colonies, compared to respective controls. Silencing of uPA inhibited anchorage-independent growth of MDA-MB-231 cells by more than 16% while silencing of uPAR inhibited it by more than 15% ( $P < 0.001$  for both, compared to NS control). These results correlate with those reported in Figure 2A and, taken together, these results demonstrate that a significant fraction of inhibitory effect of B-DIM on the growth and anchorage-independent colony formation of MDA-MB-231 breast cancer cells is due to down-regulation of uPA system.

#### EFFECT OF B-DIM TREATMENT AND uPA/uPAR TRANSFECTION IN BREAST CANCER CELLS WITH LOW INVASIVE POTENTIAL AND LOW ENDOGENOUS EXPRESSION OF uPA/uPAR

Next we tested the efficacy of B-DIM against cells that have low or no endogenous levels of uPA-uPAR. Breast cancer cell line, MCF-7, has considerably low invasive potential compared to MDA-MB-231 cells. Also, MCF-7 cells have virtually no detectable endogenous levels of uPA and very low levels of uPAR as compared to MDA-MB-231 cells, which further supports our hypothesis that uPA and uPAR might play a role in the aggressiveness of breast cancer cell lines. We found that B-DIM was effective in inhibiting cell proliferation

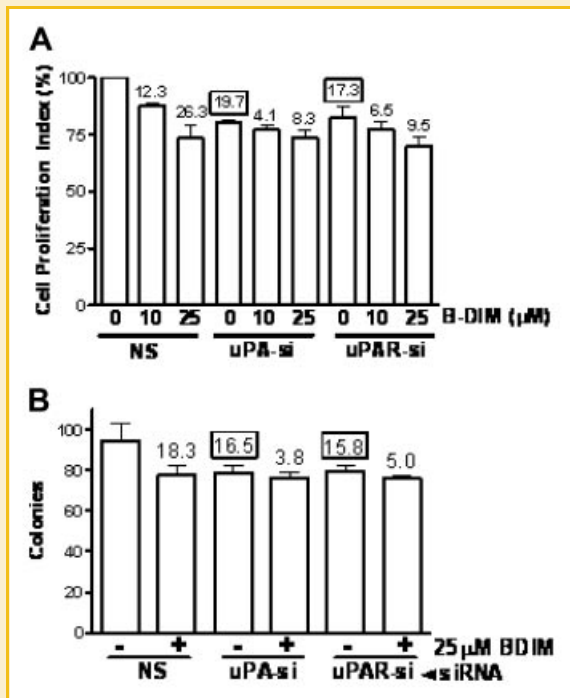


Fig. 2. Evaluation of (A) cell proliferation and (B) anchorage-independent growth in B-DIM-treated MDA-MB-231 cells by MTT and soft agar colonization assay, respectively. Cells were either vehicle-treated (DMSO-control) or treated with B-DIM, as indicated, for 72 h and then analyzed. Silencing of uPA/uPAR was achieved by the use of specific siRNAs. Non-specific siRNA (NS) was used as silencing control. The amount of DMSO never exceeded 0.1% during the treatment. The number of cells counted/OD value in DMSO (control)-treatment was considered 100% and the number of cells in B-DIM-treated cells was calculated relative to this control. The values (in squares) over some bars represent % decrease relative to the NS-0 μM B-DIM, a measure for the base-line effect of uPA/uPAR silencing while the other values (without squares) mentioned over some bars represent % decrease corresponding to the respective 0 μM B-DIM treatment, a measure for the effect of B-DIM treatment.

of MCF-7 cells, suggesting the pleiotropic effects of B-DIM mediated by alterations in multiple signaling pathways (Fig. 3A). In order to test whether uPA and uPAR play a role in proliferation and aggressiveness of cancer cells, we transfected MCF-7 cells with vectors containing uPA and uPAR cDNA constructs. Figure 3B shows that the transfections resulted in higher expression of respective proteins. Transfection of uPA cDNA resulted in 19.0% increase (Fig. 3C) whereas uPAR transfection resulted in 14.0% increase in cell proliferation, compared to vector-transfected controls ( $P < 0.001$  in both cases). We also treated the vector (control)-transfected cells as well as uPA/uPAR-transfected cells with B-DIM to test the effect of transfections on the sensitivity of these cells to B-DIM treatment. We found that uPA as well as uPAR transfection significantly attenuated the inhibitory effects of B-DIM treatment. For example, uPA transfection decreased the inhibitory effect of 25 μM B-DIM from 46.7% to 14.0% while uPAR decreased the inhibitory effect to 18.7%. Transfections with uPA/uPAR significantly altered the sensitivity of MCF-7 cells to B-DIM treatment and over-expression of uPA/uPAR was found to significantly overcome the inhibitory effects of B-DIM on the cell growth.

We subsequently tested the effect of uPA/uPAR transfection on B-DIM treatment by soft agar assay. As seen in Figure 3D, 25 μM B-DIM treatment resulted in a significant loss in colony formation after a month of growth under anchorage-independent conditions of MCF-7 cells. Transfections with both uPA and uPAR resulted in increased anchorage-independent growth (as depicted by bars without B-DIM treatment) ( $P < 0.01$ ). These increases represent the basal-level induction of anchorage-independent colonies by uPA/uPAR transfections. Treatment with 25 μM B-DIM revealed that transfections with uPA/uPAR significantly altered the sensitivity of MCF-7 cells to B-DIM treatment. These results were consistent with our results from the MTT assay (Fig. 3C).

#### EFFECT OF B-DIM TREATMENT AND uPA/uPAR EXPRESSION ON VEGF AND MMP-9 SECRETION

VEGF and MMP-9 are reliable markers of tumor aggressiveness and metastasis and uPA/uPAR system is known to be involved in the activation of MMP-9, which causes the degradation of extracellular matrix, resulting in increased invasion [Dass et al., 2008]. We, therefore, tested the relevance of uPA-uPAR system in breast cancer by studying the effect of uPA/uPAR silencing on the production of VEGF and MMP-9 in MDA-MB-231 cells by ELISA. Conditioned media was collected from test samples and subjected to ELISA for the quantitation of VEGF and MMP-9. B-DIM treatment resulted in a dose-dependent down-regulation in the secretion of VEGF (Fig. 4A) as well as MMP-9 (Fig. 4B) by MDA-MB-231 cells. Silencing of uPA as well as uPAR significantly reduced the B-DIM-induced down-regulation of VEGF as well as MMP-9. B-DIM was still able to down-regulate VEGF and MMP-9 but the degree of down-regulation was only a fraction of that observed in cells transfected with non-specific siRNAs.

We further extended this study to MCF-7 cells and the data shown in Figure 4C,D demonstrated that B-DIM treatment caused dose-dependent down-regulation of VEGF and MMP-9 in MCF-7 cells. It is important to note that the differences in the basal levels of secreted VEGF and MMP-9 in MDA-MB-231 and MCF-7 cells are consistent with their metastatic potential. In support of the putative role of uPA and uPAR in metastasis, we observed that transfection with uPA and uPAR resulted in an increase in the secretion of VEGF and MMP-9 activity. uPA transfection resulted in 13.3% increase in VEGF and 41.5% increase in MMP-9 production while transfection with uPAR resulted in 11.3% increase in VEGF and 30.2% increase in MMP-9 production (Fig. 4C,D, see the values in squares over the bars). All the values were found to be significant, compared to vector-transfected control cells ( $P < 0.05$ ).

#### EFFECT OF uPA/uPAR EXPRESSION ON THE MIGRATION OF BREAST CANCER CELLS

While VEGF and MMP-9 assays, as described above, are a reliable indication of the process of metastasis, migration assay is a more direct way to visualize and quantitate the motility of cancer cells in vitro. Therefore, we performed migration assay to study the effect of uPA and uPAR on the migration of breast cancer cells. As shown in Figure 5A, silencing of uPA and uPAR in the highly aggressive cell line, MDA-MB-231, resulted in a significant decrease in the number

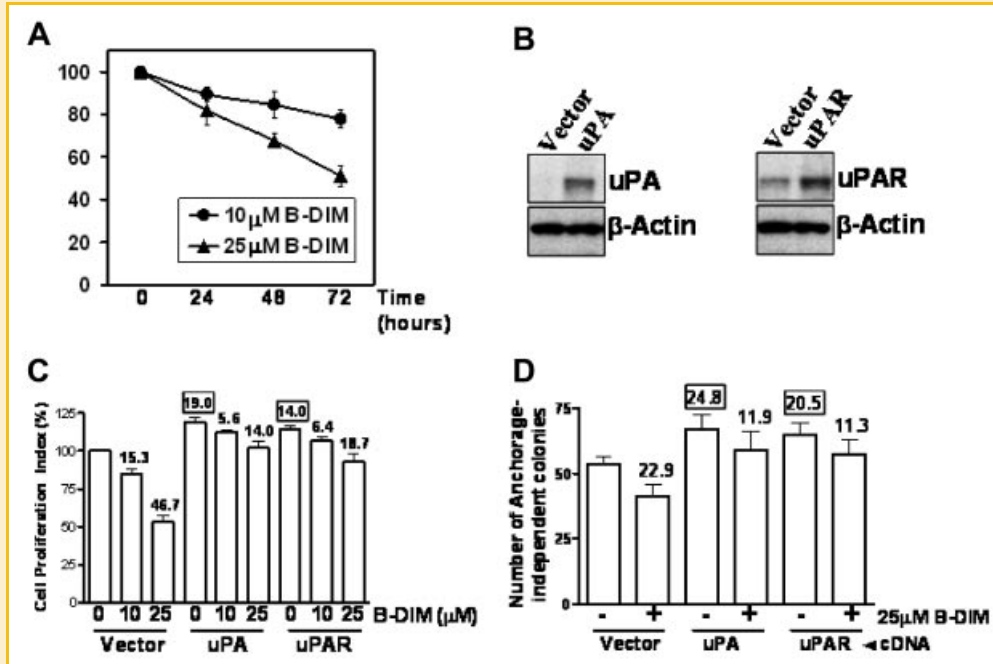


Fig. 3. A: Dose- and time-dependent effect of B-DIM treatment on the proliferation of MCF-7 cells. B: Western Blot analysis confirming the increased expression of uPA and uPAR after transfection with specific cDNAs.  $\beta$ -Actin protein was used as protein loading control. Evaluation of (C) cell proliferation and (D) anchorage-independent growth in B-DIM-treated MCF-7 cells by MTT and soft agar colonization assay, respectively. Cells were transiently transfected with vector or uPA/uPAR-containing plasmid and were either vehicle-treated (DMSO-control) or treated with B-DIM, as indicated, for 72 h. The number of cells counted/OD value in DMSO (control)-treatment was considered 100% and the number of cells in B-DIM-treated cells was calculated relative to this control as the percentage surviving cells.

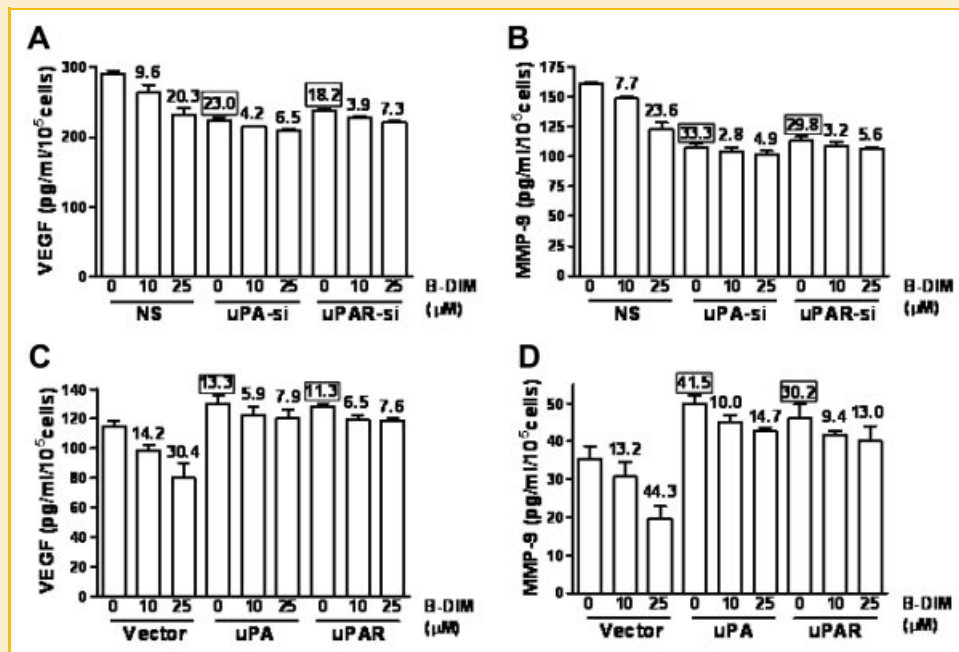


Fig. 4. Effect of B-DIM on the secretion of (A) VEGF and (B) MMP-9 in MDA-MB-231 and (C) VEGF and (D) MMP-9 in MCF-7 cells treated with indicated doses of B-DIM and/or silenced/transfected for uPA/uPAR. The culture media was collected and VEGF/MMP-9 was assayed using ELISA kits. Results were normalized to the cell number and expressed as pg/ml/10<sup>5</sup> cells. The values (in squares) over some bars represent % decrease relative to the NS/vector-0  $\mu$ M B-DIM, a measure for the base-line effect of uPA/uPAR silencing/over-expression while the other values (without squares) mentioned over some bars represent % decrease corresponding to the respective 0  $\mu$ M B-DIM treatment, a measure for the effect of B-DIM treatment.

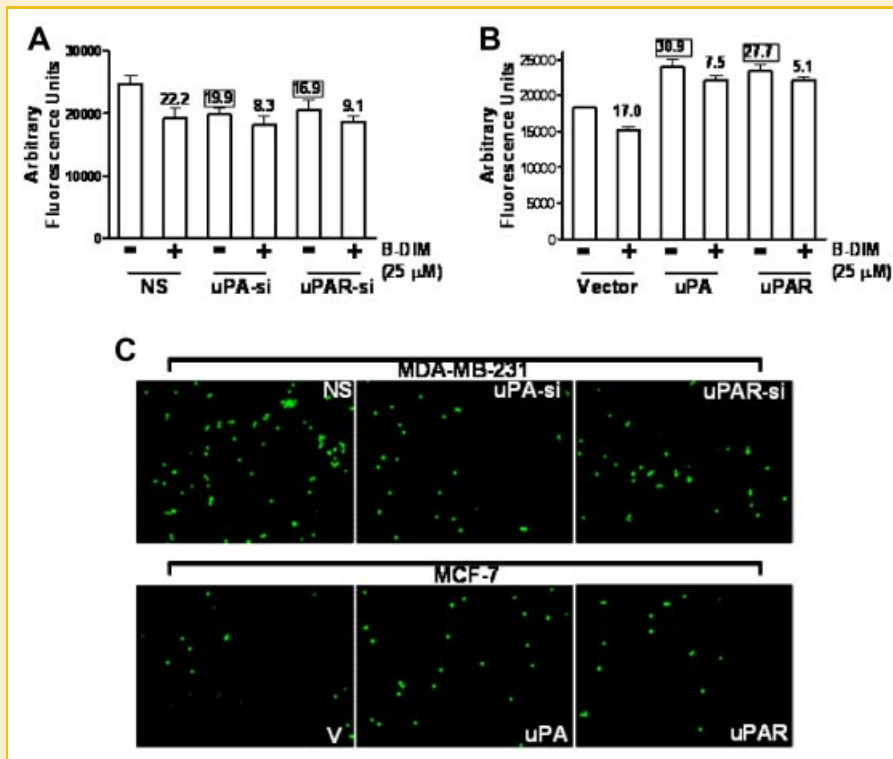


Fig. 5. Effect of B-DIM treatment and silencing of uPA/uPAR on migration of (A) MDA-MB-231 cells; and effect of B-DIM treatment and over-expression of uPA/uPAR on migration of (B) MCF-7 cells. Cells were seeded into the upper chamber of the system and transfected with cDNA/siRNA for 24 h followed by B-DIM treatment in serum-free media. Bottom wells were filled with complete media and the cells that migrated through the membrane were stained with 4  $\mu$ g/ml Calcein AM. The fluorescently labeled cells were detached from the membrane by trypsinization and the fluorescence of migrated cells was read at excitation/emission wavelengths of 530/590 nm. The values (in squares) over some bars represent % change relative to the NS/vector-0  $\mu$ M B-DIM, a measure for the base-line effect of uPA/uPAR over-expression/silencing while the other values (without squares) mentioned over some bars represent % decrease corresponding to the respective 0  $\mu$ M B-DIM treatment, a measure for the effect of 25  $\mu$ M B-DIM. C: Representative pictures showing the effect of uPA/uPAR silencing on migration of MDA-MB-231 cells and the effect of over-expression of uPA/uPAR on migration of MCF-7 cells. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

of cells migrating across the membrane. On the other hand, transfection with uPA and uPAR was found to enhance the migration of less aggressive MCF-7 cells significantly (Fig. 5B). In MCF-7 cells, uPA transfection caused 30.9% increase in the number of migratory cells while uPAR transfection caused an increase of 27.7% (Fig. 5B). Treatment with 25  $\mu$ M B-DIM resulted in a significant loss of migration in these cells but the transfection with uPA or uPAR was found to significantly decrease the inhibitory effect of B-DIM treatment. B-DIM treatment (25  $\mu$ M) of MDA-MB-231 cells resulted in 22.2% less migration, uPA-silencing resulted in 19.9% less migration while uPAR-silencing resulted in 16.9% less migration. Compared to 0  $\mu$ M B-DIM, 25  $\mu$ M B-DIM caused only 8.3% further reduction in migration in uPA-transfected cells and 9.1% reduction in uPAR-transfected cells. Comparing these numbers with 22.2% (as observed in non-specific silencing) suggests that silencing of uPA/uPAR significantly attenuated the inhibitory effect of B-DIM treatment. Also, in MCF-7 cells, the basal-level migration was elevated by uPA and uPAR transfections under all experimental conditions (see 0  $\mu$ M B-DIM; vector vs. uPA/uPAR-transfected cells and also 25  $\mu$ M B-DIM; vector vs. uPA/uPAR-transfected cells). To provide a visual representation of the effect of uPA/uPAR expression on the migration of breast cancer cells, representative

pictures are shown in Figure 5C. From our results it is clearly evident that silencing of uPA as well as uPAR in the highly aggressive and motile MDA-MB-231 cells results in the inhibition of migration while transfection of uPA and uPAR in MCF-7 cells, the cells with low migratory ability, results in a significant induction of migration. These results further establish the relevance of uPA and uPAR towards migration and aggressiveness of breast cancer cells.

## DISCUSSION

The perceived health benefits of micronutrient supplements and phytochemicals have generated considerable interest in recent years because of their potential role in cancer prevention and treatment. It has been estimated that up to 60–80% of cancer patients take micronutrients, phytochemicals or herbs as dietary supplements [Nam et al., 1999; Richardson et al., 2000]. DIM has been demonstrated to be effective against the proliferation of breast cancer cells [Rahman and Sarkar, 2005; Rahman et al., 2006a,b]; however, inspite of its known beneficial role, especially against breast cancer, the exact mechanism of anti-cancer action of DIM has not been fully elucidated. We have recently reported our findings on

the mechanistic role of uPA/uPAR in mediating the growth inhibitory effect of DIM (B-DIM) against prostate cancer cells [Ahmad et al., 2009], and since B-DIM is also known to be effective against breast cancer cells [Rahman and Sarkar, 2005; Rahman et al., 2006b; Wang et al., 2008], we have investigated the mechanism for the observed properties of B-DIM in two breast cancer cell lines with different levels of aggressiveness. The results from the MTT assays clearly showed that B-DIM could inhibit cell proliferation of estrogen receptor (ER)-positive MCF-7 cells as well as ER-negative MDA-MB-231 cells in a dose dependent manner. B-DIM, being a pleiotropic agent, has been shown to inhibit proliferation of breast cancer cells by acting on various pathways [Rahman and Sarkar, 2005; Rahman et al., 2006b; Wang et al., 2008] and this might explain its ability to inhibit growth of uPA/uPAR-positive as well as -negative breast cancer cells as observed in the current study.

The degree of sensitivity of breast cancer cells to B-DIM was found to differ between the two cell lines tested in the current study, showing an inverse correlation with their endogenous expression of uPA and uPAR. This led us to speculate the role of uPA and uPAR in breast cancer cell growth and proliferation. The MDA-MB-231 cells showed higher metastatic potential, and have high endogenous expression of uPA as well as its receptor uPAR, and we have found that B-DIM could significantly inhibit cell growth and colony formation of MDA-MB-231 cells. In addition, silencing of uPA and uPAR resulted in diminished sensitivity of MDA-MB-231 cells to the inhibitory effect of B-DIM treatment, suggesting that B-DIM-induced anti-proliferative activity against the highly aggressive MDA-MB-231 breast cancer cells is in part dependent on B-DIM mediated inactivation of uPA-uPAR system. In order to gain further insight in support of the role of uPA and uPAR, which play important role in the metastatic potential of breast cancer cells, we selected a less aggressive MCF-7 breast cancer cell line for our studies. MCF-7 cells express very low endogenous levels of uPA and uPAR and upon transfection with uPA and uPAR cDNA, the MCF-7 cells showed a significant increase in cell growth and anchorage-independent growth, suggesting the role of uPA-uPAR system in cancer cell aggressiveness.

The cell growth inhibition by B-DIM in uPA/uPAR-transfected MCF-7 cells was found to be significantly reduced compared with that observed in vector-transfected cells. These results strongly support the role of uPA and uPAR in cell growth and proliferation of breast cancer cells. Our study also demonstrates that in highly metastatic cells such as MDA-MB-231, wherein uPA and uPAR are over-expressed, B-DIM might be able to exert its anti-cancer properties by down-regulation of these proteins, suggesting that B-DIM mediated anti-proliferative effect requires the expression of uPA-uPAR system. Interestingly, breast cancer cells with low endogenous levels of uPA and uPAR, such as MCF-7 cells, showed that B-DIM could equally inhibit proliferation, which could be mediated via dysregulation of alternate pathways perhaps by directly inhibiting the levels of VEGF and MMP-9, which indeed could be mediated by inactivation of NF- $\kappa$ B as shown by our previous studies in prostate cancer [Kong et al., 2007]. Also, we have previously demonstrated inactivation of Akt and NF- $\kappa$ B and inhibition of nuclear translocation of NF- $\kappa$ B along with reduced phosphorylation of I $\kappa$ B as a mechanism of DIM-induced

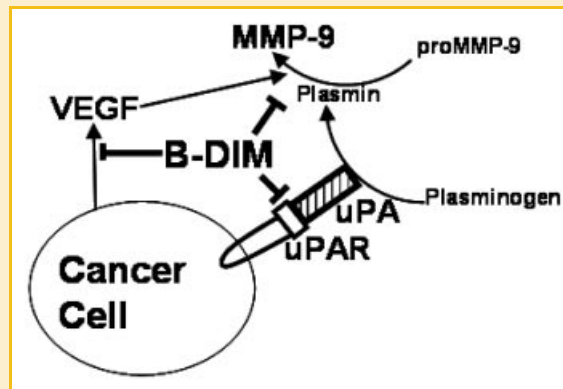


Fig. 6. Schematic representation of the effect of B-DIM on the factors that influence migration, invasion and metastasis of breast cancer cells. uPA binds to its receptor (uPAR) on the surface of cancer cells leading to the generation of active MMP-9 which takes part in the degradation of extracellular matrix leading to invasion and metastasis. Cancer cells also secrete VEGF (responsible for angiogenesis). B-DIM can inhibit uPA-uPAR thus causing an inhibition of MMP-9. It can also negatively regulate the production of VEGF to ensure a multi-targeted blockage of pathways responsible for the aggressiveness of breast cancers.

apoptosis in breast cancer cells [Rahman and Sarkar, 2005]. A model explaining the observed properties of B-DIM has been depicted in Figure 6, which suggests pleiotropic effects of B-DIM.

DIM has been shown to exert anti-proliferative effects and also induce apoptosis in MCF-7 as well as MDA-MB-231 cells by down-regulation of anti-apoptotic protein Bcl-2 [Hong et al., 2002a]. In addition, DIM is known to inhibit the invasion and metastasis of these cell lines through the down-regulation of chemokine receptor CXCR4 and its ligand, CXCL12 [Hsu et al., 2008]. DIM is known to act on multiple targets in MCF-7 cells which might explain its increased anti-proliferative effects against these cells, as observed in the current study. For instance, DIM has been shown to strongly inhibit the expression of ER- $\alpha$  and estrogen signaling [Okino et al., 2009] accounting for its inhibitory effects on the growth and proliferation of ER-positive MCF-7 cells. Moreover, additional mechanisms for the protective effects of DIM against tumorigenesis in MCF-7 cells have also been suggested such as increased interferon- $\gamma$  secretion [Xue et al., 2005], enhanced generation of mitochondrial reactive oxygen species leading to cell cycle arrest through increased expression of p21 [Gong et al., 2006] and suppression of cyclooxygenase-2 expression [Degner et al., 2009]. These observations further support the pleiotropic nature of DIM and thus DIM could be considered as a multi-targeting agent. Our results are in full agreement with the previously published data showing that MCF-7 cells are more sensitive to B-DIM treatment compared with MDA-MB-231 cells. However, it is important to note that our present study describes a mechanism for the action of B-DIM against more aggressive MDA-MB-231 cells which is through the modulation of uPA-uPAR system. MDA-MB-231 cells represent triple-negative breast cancer (TNBC) phenotype, which, in the absence of well-characterized receptors, is a deadly subtype of human breast cancers associated with poor prognosis. Our data, therefore, suggests that DIM could be useful for targeting TNBC.



The urokinase plasminogen activator system is involved in the proteolysis of plasmin and matrix metalloproteinase (MMP) leading to the observed aggressiveness of human cancers [Carmeliet et al., 1998; Koopman et al., 1998; Dass et al., 2008]. We were, therefore, interested in evaluating the role of VEGF and MMP-9 in B-DIM-induced inactivation of uPA-uPAR system. In the highly aggressive MDA-MB-231 cells, we found a significant down-regulation of secreted VEGF and MMP-9 in response to both B-DIM treatment as well as siRNA-mediated silencing of uPA/uPAR (Fig. 4). VEGF and MMP-9 down-regulation, achieved by the silencing of uPA/uPAR, was found to be similar to those observed by B-DIM treatment although the combined effect was not additive or synergistic. VEGF and MMP-9 assays in MCF-7 cells showed increased production of both VEGF and MMP-9 in response to over-expression of uPA and uPAR (Fig. 4). The inhibition of VEGF and MMP-9 production by silencing of uPA/uPAR should theoretically result in decreased motility and metastasis. To confirm this, we performed migration assays and found that silencing of uPA as well as uPAR led to a significantly diminished motility of highly aggressive MDA-MB-231 cells. Conversely, in MCF-7 cells, transfections with uPA/uPAR led to an increased production of VEGF and MMP-9 which led to significantly increased migration of these cells. These observations provided molecular evidence (increased production of VEGF and MMP-9 in response to increased uPA/uPAR) in support of the positive role of uPA/uPAR with tumor aggressiveness.

The relevance of uPA and its receptor, uPAR, in the prognosis [Pacheco et al., 2001; Giannopoulou et al., 2007] and progression of human cancers is increasingly being recognized [Dass et al., 2008]. In a recent report on the prognostic potential, it has been shown, using stromal tissue from 60 invasive breast carcinoma samples, that high uPA and uPAR levels can be positively correlated with shorter relapse-free survival and overall survival [Hildenbrand et al., 2009]. Another interesting study has suggested the role for uPA and uPAR in the stem cell tropism to malignant solid tumors [Gutova et al., 2008]. This study showed a dependence of stem cells on secreted uPA for their migration. Our results with highly aggressive MDA-MB-231 cells, using migration assays, support the notion that uPA as well as its receptor play a role in the migration of cancer cells, and thus their selective targeting might be a beneficial strategy to halt the process of metastasis. Moreover, studies have shown that the down-regulation of uPA and uPAR is associated with the inhibition of tumor invasion and angiogenesis in breast cancer cells [Subramanian et al., 2006; Kunigal et al., 2007]. Thus, the uPA-uPAR system represents a highly potential target for designing comprehensive strategies whereby dietary chemopreventive agents could be useful for prevention of tumor aggressiveness and/or could also be useful for chemo-sensitizing aggressive cancer cells to conventional therapeutic agents for better treatment outcome in breast cancer.

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