3,3′-Diindolylmethane negatively regulates Cdc25A and induces a G2/M arrest by modulation of microRNA 21 in human breast cancer cells

Yucui Jin[a,b], Xianghong Zou[a,b] and Xiaoling Feng[b]

3,3′-Diindolylmethane (DIM) is a potential chemopreventive phytochemical derived from Brassica vegetables. In this study, we assessed the effects of DIM on cell cycle regulation in both estrogen-dependent MCF-7 and estrogen receptor negative p53 mutant MDA-MB-468 human breast cancer cells. In-vitro culture studies showed that DIM dose dependently inhibited the proliferation of both cells. In addition, in-vivo xenograft model showed that DIM strongly inhibited the development of human breast tumors. Fluorescence activated cell sorter analysis showed a DIM-mediated G2/M cell cycle arrest in MCF-7 and MDA-MB-468 cells. Western blot analysis showed that DIM downregulated the expression of cyclin-dependent kinases 2 and 4 and Cdc25A, which plays an important role in G2/M phase. Furthermore, treatment of MCF-7 cells with DIM, which increased microRNA 21 expression, caused a downregulation of Cdc25A, resulting in an inhibition of breast cancer cell proliferation. Taken together, our data show that DIM is able to stop the cell cycle progression of human breast cancer cells regardless of their estrogen-dependence and p53 status, by differentially modulating cell cycle regulatory pathways. The modulation of microRNA 21 mediates the DIM cell cycle regulator effect in MCF-7 cells. *Anti-Cancer Drugs* 21:814–822 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Breast cancer accounts for the highest incidence of cancer and cancer-related deaths in women in both developed and developing countries [1], suggesting that early diagnosis and prevention of this disease is urgently needed. As early-stage breast cancers are estrogen-dependent for their growth and survival, surgery, chemotherapy, hormone therapy, or a selective combination of modalities, have been used as the main therapeutic approach against the disease [2]. Clinical experience shows that the initial benefits of anti-estrogen treatment in breast cancer tend to decrease with time as hormone-independent cancer cells predominate, and monitoring seems to show that estrogen-dependent tumors eventually become estrogen-independent, which ultimately leads to patient mortality. At present, there is no generally effective treatment for estrogen-independent breast cancer, underscoring the need for the development of novel alternative therapeutic strategies.

Many studies show a connection between diet and breast cancer, with cancer incidence inversely correlated with consumption of fruit and vegetables [3–5]. Dietary glucosinolates present in Brassica species have been shown to protect against several types of cancer [6,7]. One of these phytochemicals, I3C, may prove to be a promising agent against cervical and breast cancers [8]. 3,3′-Diindolylmethane (DIM) is a stable condensation product of I3C, and it has uncovered an anti-proliferative pathway that implicates Sp1/Sp3-induced expression of p21 as a target for cell cycle control in human breast cancer cells [9]. In addition, results generated with in-vitro cell culture studies have shown that DIM inhibits the proliferation of a variety of cancer cell types, including prostate [10] and breast cancer cells [9], through the induction of cell cycle arrest and apoptosis. Despite studies that have suggested a use for DIM as a cancer chemopreventive agent, relatively little is known about the molecular mechanisms of DIM.

In mammalian cells, progression of the cell cycle is mainly regulated by the activities of the cyclin–cyclin-dependent kinase (CDK) complex [11]. p21Waf1/Cip1 and p27Kip1 are members of the CIP/KIP family, which can inhibit the cyclin–CDK complex [12]. In addition, activation of CDKs requires Cdc25, an important phosphatase. Cdc25 dephosphorylates Thr14 and Tyr15 of CDKs. In mammals, the Cdc25 family includes three homologs: Cdc25A, Cdc25B, and Cdc25C. In the past, Cdc25B and Cdc25C were regarded as mitotic regulators [13,14], whereas Cdc25A was regarded as a G1/S regulator. However, recent research shows that Cdc25A, in addition to its

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S-phase-promoting effect, physically and functionally interacts with the main mitosis-promoting cyclin–CDK complex and generates a rate-limiting stimulus for the G2/M transition; the lack of its activity can delay completion of the cell division cycle [15–17]. Schematic illustration of the cell cycle regulatory pathway has been depicted in Supplementary Fig. S1. Cdc25A activities are tightly regulated by multiple mechanisms during the cell cycle, and ubiquitin-mediated proteolysis is the major mechanism of Cdc25A turnover [18]. For example, hyperphosphorylation of Cdc25A by the ataxia telangiectasia and Rad3 related-Chk1 signaling leads to its degradation and contributes to a delay in the cell cycle, which allows either DNA repair or apoptosis, depending on the extent of DNA damage [18,19]. We have found that I3C induces Cdc25A degradation by Chk2 and inhibits the growth of human breast cancer cells Wu *et al.* [20].

Recently emerging evidence indicates the critical role of microRNAs (miRNA) in the regulation of various biological and pathologic processes. These small, noncoding molecules elicit their regulatory effects by imperfectly binding to the 3′-untranslated region (UTR) of target mRNA, either causing degradation of mRNA or inhibition of their translation to functional proteins [21]. The expression of miRNAs has been recognized as an integral component of many normal biological processes, such as those involving cell proliferation, differentiation, apoptosis, and stress resistance [22]. Natural agents including curcumin [23], isoflavone [24], indole-3-carbinol [25], and DIM [25] could alter miRNA expression profiles, leading to the inhibition of cancer cell growth, induction of apoptosis, reversal of epithelial mesenchymal transition, or enhancement of efficacy of conventional cancer therapeutics.

The studies presented here were performed in estrogen-dependent MCF-7 and estrogen receptor (ER) negative p53 mutant MDA-MB-468 human breast cancer cells to investigate whether DIM can target the more aggressive MDA-MB-468 cells. Both cell lines exhibited growth inhibition in response to DIM and the effects of DIM on cell cycle events were determined. Growth inhibition by DIM was accompanied by an arrest in the G1/S and G2/M phases of the cell cycle, reduction in Cdk levels, decrease in Cdc25A and Cdc25C, and increase in p21 levels in both cell lines, regardless of their ER and p53 status. In addition, we investigated the possibility that DIM could alter the expression profile of miRNAs and also assessed the cellular consequence. We found that the miRNA expression pattern was different between estrogen-dependent and estrogen-independent cells. The treatment of MCF-7 cells with DIM resulted in an increase in miR-21, and the DIM-mediated decrease of Cdc25A was reversed by anti-miR-21. These investigations provide the first evidence that DIM treatment activates miR-21 leading to cell cycle arrest through Cdc25A degradation in breast cancer cells.

Materials and methods

Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-468 were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium medium (Gibco, Rockville, Maryland, USA), supplemented with 10% FBS (fetal bovine serum), 1% penicillin/streptomycin, and 1 mmol/l glutamine in a 5% CO_2 atmosphere at 37°C.

Plasmid, site-directed mutagenesis, and transfection

Full-length Cdc25A was amplified and cloned into pcDNA 4/TO doxycycline-inducible promoter (Invitrogen, Carlsbad, California, USA). To generate phosphatase-site mutants of Cdc25A, a site-directed mutagenesis kit (Stratagene, Santa Clara, California, USA) was used. Sequences for Cdc25A and mutated-Cdc25A primer sets are listed in Supplementary Table 1. Finally, the correct mutation derivatives were verified by DNA sequencing. To establish cell lines with inducible expression of Cdc25A and its mutation derivatives in MCF-7, Tet-on cells were transfected using 5 μg of plasmid DNA (pCDNA4/TO-puro-Cdc25A) using 15 μl of Lipofectamine 2000 reagent according to the manufacturer's instruction (Invitrogen). The cells were selected with 1.5 μg/ml puromycin. To induce Cdc25A expression, cells were treated overnight with doxcycline (10 ng/ml) and then treated with DIM for 24–72 h.

Cell proliferation

To determine breast cancer cell proliferation, 5×10^5 MCF-7 and MDA-MB-468 cell lines were separately seeded in six-well plates and allowed to grow for 24 h. The cells were then treated with 0.1% dimethyl sulfoxide (DMSO), with 30 or 60 μmol/l DIM at 24–96 h. Fresh medium containing DMSO or DIM was changed each day. DIM was dissolved in DMSO as 1000× stock solutions for each treatment. Cell numbers were assessed at various time points by trypsinization and counting with a Coulter Z1 cell counter.

Cell colony formation assay

5×10^3 MCF-7 and MDA-MB-468 cells were separately seeded in six-well plates and allowed to grow for 24 h. The cells were then treated with 0.1% DMSO and 45 μmol/l DIM. After 2 weeks, breast cancer cells were washed with PBS twice, fixed with methanol and then stained with Giemsa.

MicroRNA target prediction

The miRNA targets were predicted using two algorithms, TargetScan 3 and PicTar 4.

Transfections

Transfection with 100 nmol/l anti-miR-21 or miRNA negative control (Ambion, Austin, Texas, USA) was done with Lipofectamine 2000 according to the manufacturer's

instructions. Cells plated at 50–60% confluence in six-well plates were transfected twice in 48 h and split into two six-well plates. The next day, the cells were either treated with DMSO or DIM as described above. Total proteins from each sample were extracted and subjected to western blot analysis as described below.

Growth inhibition assay

MCF-7 cells were transfected with anti-miR-21 or miRNA negative control for 4 days as described. Then, the transfected cells were seeded in six-well plates and treated with DMSO or 45 μmol/l DIM at 24–96 h. Fresh medium containing DMSO or DIM was changed each day. Cell numbers were assessed at various time points by trypsinization and counting with a Coulter Z1 cell counter.

MicroRNA real-time reverse transcription-PCR

To verify the alterations in the expression of specific miRNAs, TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, California, USA) was used. Total RNA (5 ng) from each sample was subjected to reverse transcription with a specific miRNA primer (Applied Biosystems). Real-time reverse transcription PCRs were then carried out in a total of 25 μl reaction mixture in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described by Li *et al.* [24]. The PCR program was initiated for 10 min at 95°C before 40 thermal cycles, each at 15 s at 95°C and 1 min at 60°C. Data were analyzed according to the comparative cycle threshold-method and normalized by U6 expression in each sample.

Real-time reverse transcription-PCR analysis

Total RNA was extracted using TRIZOL reagent as recommended by the manufacturer (Invitrogen). Total RNA (500 ng) was subjected to reverse transcription using the Superscript first-strand cDNA synthesis kit (Invitrogen). Real-time reverse transcription PCR reactions were carried out in a total of 25 μl of reaction mixture (1 μl of cDNA, 12.5 μl of 2 × SYBR Green PCR Master Mix (Austin, Texas, USA), 0.75 μl of each 10 μmol/l forward and reverse primers, and 10 μl of H_2O). The PCR program was initiated for 10 min at 95°C before 40 thermal cycles, each consisting of 15 s at 95°C and 1 min at 60°C. Data were analyzed according to the comparative cycle threshold method, with glyceraldehyde 3-phosphate dehydrogenase as a reference. Primer sequences for PCR are listed in Supplementary Table 2.

Western blot analysis

After the indicated treatments, the cells were washed with PBS and harvested in radioimmunoprecipitation assay buffer (150 mmol/l NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/l Tris), containing 10 mg/ml aprotinin, 5 mg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride. Protein concentrations were determined by the Bradford–Coomassie dye-binding assay. Equal amounts of protein were fractionated by electrophoresis on 4% polyacrylamide/0.1% SDS stacking gels and 15% polyacrylamide/0.1% SDS resolving gels. Proteins were electrotransferred to polyvinylidene fluoride Immobilon-P Transfer Membranes using a transfer buffer (25 mmol/l glycine, 25 mmol/l ethanolamine, and 20% methanol) and then blocked at room temperature for 1 h in 5% blocking buffer (1 × Tris-buffered saline, 0.1% Tween 20 with 5% w/v nonfat dry milk), and then incubated for 1 h at room temperature with primary mouse anti-human CDK2, CDK4, CDK6, p21 (Santa Cruz Biotechnology, Santa Cruz, California, USA), CyclinB1, Cdc2, Cdc25C (Cell Signaling Technology, Danvers, Massachusetts, USA), and Cdc25A monoclonal Mab Ab-3 (NeoMarkers Fremont, California, USA) antibody, and then washed with wash buffer (10 mmol/l Tris–HCl, pH 9.5,10 mmol/l NaCl, and 0.1% Tween 20). The membranes were incubated for 1 h with secondary antibody (1 : 3000). The proteins were visualized by chemiluminescence reagent and exposure to Kodak BioMax MR film (Rochester, New York, USA). Equal protein loading was confirmed by probing blots with antibody to β-actin (1 : 2000; Sigma, St Louis, Missouri, USA).

Flow cytometric analyses of DNA content

The breast cell lines were plated onto six-well tissue culture dishes (BD Biosciences, San Jose, California, USA). Cells treated with 0.1% DMSO or with 30 or 60 μmol/l DIM for 48 h were harvested by trypsinization, washed twice with PBS, and then fixed in 70% ethanol. Cells were treated with 100 U/ml ribonuclease A for 15 min at 37°C, resuspended and lysed in 1 ml of propidium iodide buffer (0.5 mg/ml propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). The percentages of cells within the G1, S, and G2/M phases of the cell cycle were determined by analysis with the Multicycle computer program (version 3.11) provided by Phoenix Flow Systems (San Diego, California, USA).

Human breast carcinoma xenograft study

A colony of 40 BALB/C female athymic (nu/nu) mice (4–5 weeks old) were housed in microisolator cages under standard conditions (12 : 12 h light/dark cycle, 50% relative humidity at 21°C) and given free access to a semi-purified AIN-76A diet and water. Half of them were implanted with a 60-day release of 0.72 mg estradiol pellet in the subscapular region. Then the mice were inoculated subcutaneously (s.c.) in the lateral flanks with 0.1 ml of PBS solution containing 3×10^6 MCF-7 cells. Another 20 mice were inoculated s.c. with 0.1 ml of PBS solution containing 3×10^6 MDA-MB-468 cells. The mice were divided into two groups: group 1 ($n = 2 \times 10$): without DIM treatment (DIM^-) and group 2 ($n = 2 \times 10$): with DIM treatment (DIM^+). Sesame seed oil was used to facilitate gavage and avoid irritation of the esophagus (safe method as also shown by earlier studies)

[26,27]. The mice in the intervention group were given DIM (5 mg/kg) by oral gavage once a day for 5 consecutive days, with another 2 days off weekly. The control mice received only sesame seed oil without DIM. The treatment with DIM or seed oil began on the next day of cell injection. The palpable tumor diameters were measured twice per week. Tumor volumes were calculated as $ab^2/2$ (where a is length and b is cross-sectional diameter) [28]. The treatment was terminated 7 weeks after cell injection, and all the mice were killed by CO_2 asphyxiation.

Statistical analysis

The statistical significance of differential findings between the experimental groups and control was determined by using Student's t-test, as implemented by Excel 2000 (Microsoft Corp., Redmond, Washington, USA), and a value of P less than 0.05 was considered significant.

Results

DIM inhibits proliferation of MCF-7 and MDA-MB-468 breast cancer cells

To assess the effects of DIM on cell growth, ER-positive, p53 wildtype MCF-7, and ER-negative, p53 mutant MDA-MB-468 cells were separately treated with 0.1% DMSO alone, or with 30 or 60 μmol/l DIM for 24–96 h. DIM inhibited the proliferation of MCF-7 and MDA-MB-468 cells in a concentration-dependent and time-dependent manner, with up to 50% inhibition of proliferation at 72 h treatment with 60 μmol/l DIM (Fig. 1a). A cell colony formation assay also showed that DIM treatment resulted in a dramatic reduction in the colony numbers of MCF-7 and MDA-MB-468 cells (Supplementary Fig. S2). These results suggest that DIM can directly reduce the proliferation of breast cancer cells irrespective of ER and p53 status.

DIM inhibits the growth of transplanted human breast carcinoma cells

As we observed an inhibition of in-vitro cell growth by DIM, we determined whether DIM could inhibit the growth of transplanted breast carcinoma cells in female athymic (nu/nu) mice. Mice were s.c. injected with MDA-MB-468 or MCF-7 cells in the bilateral flanks, as described earlier. DIM treatment led to a 60% decrease in tumor volume in both MCF-7 and MDA-MB-468 cells, compared with the untreated controls ($P < 0.05$) (Fig. 1b). These results indicate that DIM can inhibit the development of human breast tumors in a rodent xenograft model.

DIM induces cell cycle arrest at G1 and G2/M phases in breast cancer cells

On the basis of the growth-inhibitory responses of DIM in MCF-7 and MDA-MB-468 cells, we examined its effects on cell cycle regulation. Both cells treated with either 0.1% DMSO alone or with 30 or 60 μmol/l DIM for 48 h were analyzed by flow cytometry, as described earlier. Figure 2 shows representative histograms of cell cycle distribution in the control, 30 or 60 μmol/l DIM-treated MCF-7 and MDA-MB-468 cells. DIM treatment increased the cell population in the G1 phase and the G2/M phase of MCF-7 cells and caused G2/M arrest in MDA-MB-468 cells. However, after treating MCF-7 cells with 60 μmol/l DIM for 48 h, the apoptotic peak was showed by fluorescence activated cell sorter detection, which is not found in MDA-MB-468 cells.

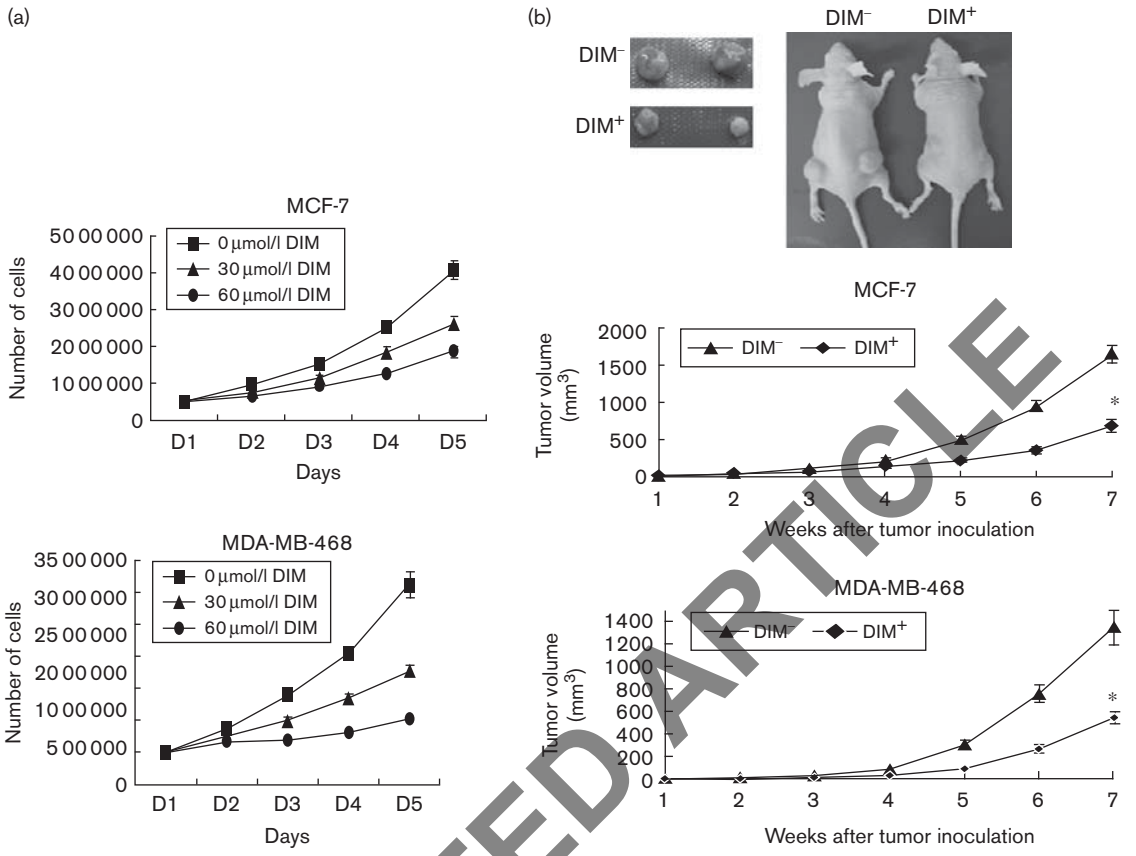
Effect of DIM on cell cycle regulatory molecules in MCF-7 and MDA-MB-468 cells

Our observation that DIM induces a G1 block in cell cycle progression of MCF-7 cells suggested that DIM might selectively regulate the activities of G1 cell cycle-regulating components. To examine this possibility, the expression of G1 cell cycle components was investigated by using western blot analysis. Results shown in Fig. 3 indicate that DIM treatment reduced the expression of CDK4 and CDK2 proteins, with no change in CDK6 protein (Fig. 3), and strongly increased p21Waf1/Cip1 expression (Supplementary Fig. S3). We also found that DIM downregulates CDK family members in MDA-MB-468 cells. However, the levels of CDK4 and CDK2 mRNA were not affected by DIM treatment (Supplementary Fig. S4). In addition, in both MCF-7 and MDA-MB-468 cell lines, we found that DIM can noticeably downregulate Cdc25A (Fig. 3), which plays an important role in the G1 and G2/M checkpoints [15–17]. We examined some G2/M-related molecules, such as CDC25C, CyclinB1, and CDC2 in MCF-7 cells, not in MDA-MB-468 cells. The results are referred to in Supplementary Fig. 5. These results indicate that DIM induces a cell cycle arrest in MCF-7 and MDA-MB-468 cells, which is accompanied by a downregulation of the expression of G1/S-related and G2/M-related kinases. A schematic illustration of how DIM is modulating the cell cycle has been depicted in Supplementary Fig. S6.

Ser^{76}, Ser^{82}, and Ser^{124} of Cdc25A are not required for DIM-induced Cdc25A degradation

Regulation of Cdc25A levels through the cell cycle requires phosphorylation at multiple sites by different kinases, and the presence of intact recognition motifs on Cdc25A, including Ser^{76}, Ser^{82}, and Ser^{124}, which are related to the degradation pathways mediated by Chk1, Smad3, and Chk2, respectively [18,19]. We developed a Tet-on system with doxycycline-inducible expression of human $Cdc25A^{WT}$ or the derivatives $Cdc25A^{S76A}$, $Cdc25A^{S82A}$, or $Cdc25A^{S124A}$ in the breast cancer cells, as described by Wu *et al.* [20]. The stability of the three mutation derivatives, $Cdc25A^{S76A}$, $Cdc25A^{S82A}$, and $Cdc25A^{S124A}$, was assayed in MCF-7 cells with or without DIM treatment by western blot. The results showed that all of them were rapidly degraded with DIM treatment

Fig. 1



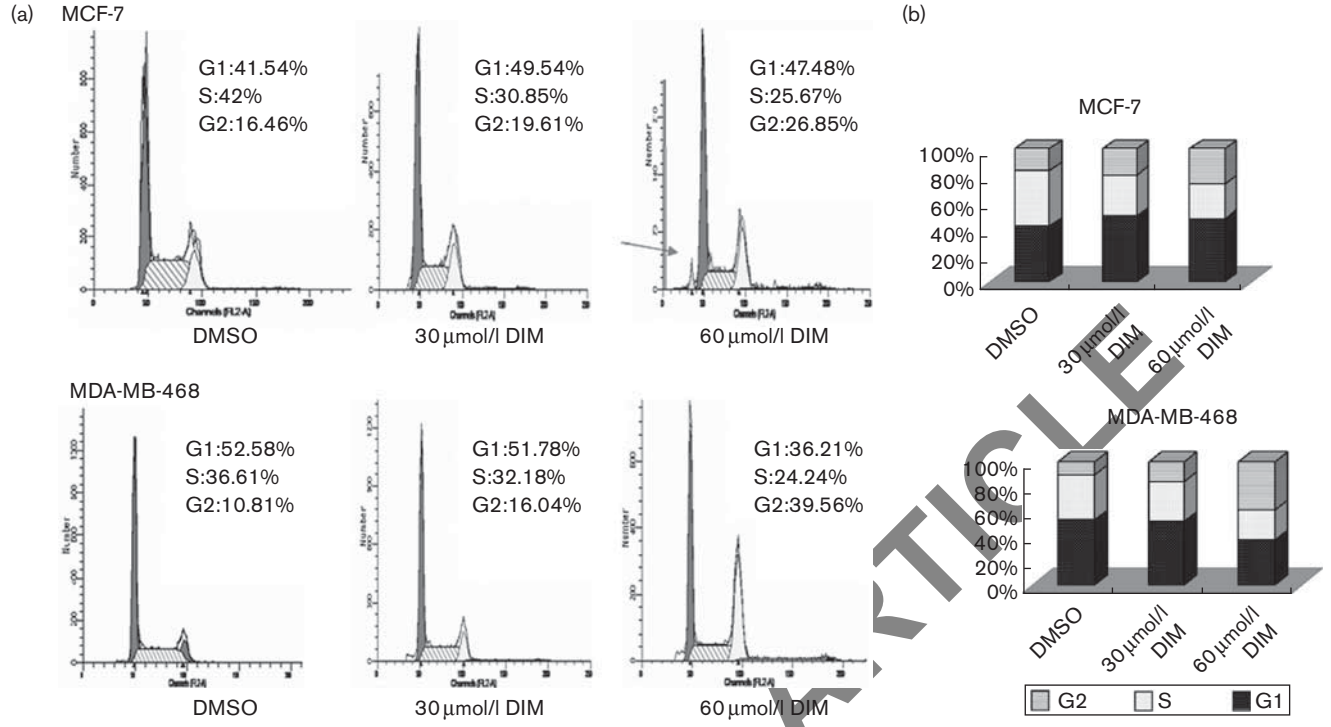
3,3′-Diindolylmethane (DIM) inhibits the breast cancer cell growth *in vitro* and *in vivo*. (a) The effect of DIM on MCF-7 and MDA-MB-468 proliferation. Plates were seeded in six-well plates with 5×10^5 cells and incubated overnight to allow attachment before starting the treatments. Treatments were either with dimethyl sulfoxide as a control, or with 30 or 60 μmol/l DIM. Cell numbers were assessed at various time points by trypsinization and counting using a Coulter Z1 cell counter. Values were expressed as mean ± SD. Results are representative of at least three independent experiments. (b) DIM strongly inhibits the development of human breast tumor in xenograft model. DIM treatment caused a 60% reduction in tumor volume in xenograft model. The representative figure of mice from each group, photographed at time of killing (from top). Statistical analysis for tumor volume from control and treatment group at different time points (from bottom). The mice were inoculated subcutaneously in the lateral flanks with 0.1 ml of PBS solution containing 3×10^6 MCF-7 or MDA-MB-468 human breast cancer cells. The mice in the intervention group ($n=10$) were DIM (5 mg/kg) by oral gavage five times weekly, and the majority of the implants began to appear as a tumor after the inoculation of each cell line for 1–2 week. The control mice ($n=10$) received only sesame seed oil without DIM. The palpable tumor diameters were measured and volumes were calculated twice per week. Tumor volumes were calculated as $ab^2/2$. * indicates the significant difference from control at a level of $P < 0.05$.

(Supplementary Fig. S7). These results suggest that the Ser^{76}, Ser^{82}, and Ser^{124} sites of Cdc25A are not required for the Cdc25A-mediated G2/M checkpoint after DIM treatment.

MiR-21 inhibits cell proliferation after DIM treatment through Cdc25A

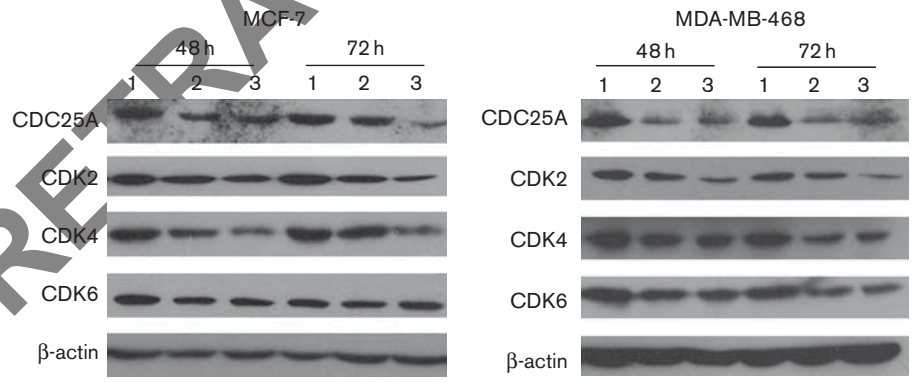
The miRNA real-time reverse transcription-PCR was conducted to determine whether the treatment of cells with DIM could alter the expression of miRNA compared with untreated cells. Interestingly, we found that miR-21 was upregulated under DIM treatment in MCF-7 cells (Fig. 4a), which is not observed in ER-negative, p53 mutant MDA-MB-468 cells. On closer inspection, a putative miR-21 binding site located in the 3′-UTR of the Cdc25A gene was predicted by two algorithms (Target Scan and PicTar; Fig. 4b). Importantly, this putative miR-21 binding site is 100% conserved in five species in the region that pairs with the seed sequence (Fig. 4b). In addition it has been proved that miR-21 regulates Cdc25A through the miR-21 binding site in its 3′-UTR, and that Cdc25A is a direct target of miR-21 [29]. We transfected anti-miR-21 into the MCF-7 cells and then treated the cells with DIM for 48 h. Transfection of anti-miR-21 elevated Cdc25A expression and restored Cdc25A expression of MCF-7 cells treated with DIM (Fig. 4c). Cdc25A is an important regulator of cell cycle progression during G1/S and G2/M transitions [15,17]. To evaluate whether miR-21 affects cell cycle progression, we compared the growth rate of cells transfected with

Fig. 2



3,3′-Diindolylmethane (DIM) induces cell cycle arrest at G1 or G2/M phases in breast cancer cells. (a) Histograms of distribution of DNA content in MCF-7 and MDA-MB-468 cells with either 0.1% dimethyl sulfoxide (DMSO) alone or with 30 or 60 μmol/l DIM treatment for 48 h by flow cytometry. Cells were lysed in 1 ml of propidium iodide solution. Nuclear fluorescence was measured and analyzed. The percentages of cells in G1, S, and G2 phases of the cell cycle were determined by analysis with the Multicycle computer software. The apoptosis peak is indicated by an arrow. (b) Distribution of cell population in G1, S, and G2 phases in control and in 30 or 60 μmol/l DIM-treated MCF-7 and MDA-MB-468 cells. Results are representative of at least three independent experiments.

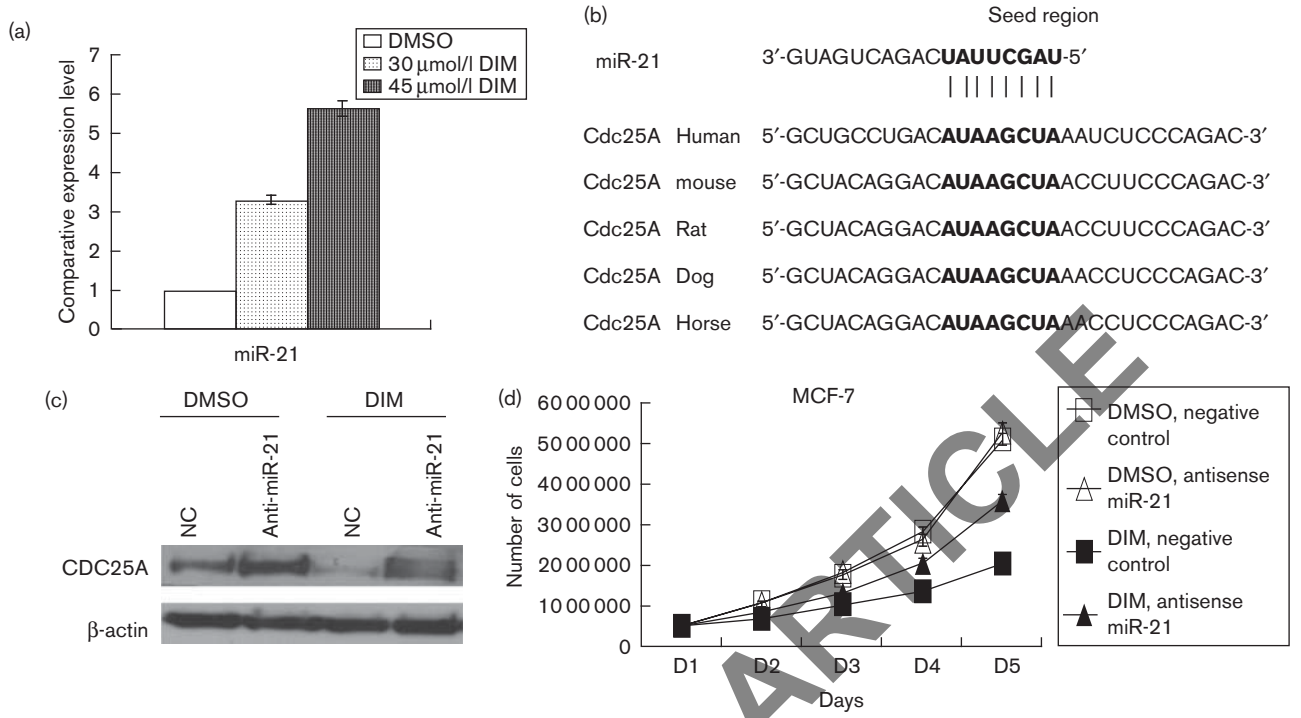
Fig. 3



3,3′-Diindolylmethane reduces the levels of cyclin-dependent kinase 4 (CDK4), CDK2, and of Cdc25A protein in both MCF-7 and MDA-MB-468 cells. Cell lysates were analyzed through western blotting with the indicated antibodies. Photographs of the chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified. 1: represents control cells treated with 0.1% dimethyl sulfoxide; 2 and 3: represent cells treated with 30 or 60 μmol/l DIM.

anti-miR-21 or miRNA-negative control, and cultured in a medium containing DMSO or 45 μmol/l DIM over a course of 5 days. The growth rate of cells transfected with negative control and anti-miR-21 was indistinguishable under DMSO treatment in the entire 5 days. However, anti-miR-21 transfected MCF-7 cells exhibited enhanced proliferation over control cells under DIM treatment (Fig. 4d).

Fig. 4



MiR-21 inhibits cell proliferation after 3,3′-Diindolylmethane (DIM) treatment through Cdc25A. (a) DIM induces miR-21 expression in MCF-7 cells. Cells were treated with DIM, as described in the 'Materials and methods' section. Total RNA was isolated, and real-time reverse transcription-PCR was performed. Levels of mature miR-21 expression were normalized to those of U6. Values were expressed as mean ± SD. Results are representative of at least three independent experiments. (b) Conserved miR-21 binding site in the 3′-untranslated region of Cdc25A. Schematic representation of Cdc25A transcript with its 3′-untranslated region. The predicted miR-21 binding sites in the *Cdc25A* gene of five species are shown with miR-21 targeting sequences aligned (GenBank accession numbers NM_201567.1,NM_007658.3,NM_133571.1, NM_001145215.1, and NM_001145213.1, respectively). The base-pairing nucleotides are in bold. (c) DIM-mediated decrease of Cdc25A was reversed by anti-miR-21. MCF-7 cells transfected with or without anti-miR-21 were treated with dimethyl sulfoxide (DMSO) or 45 μmol/l DIM and subjected to western blot analysis. NC, negative control. (d) MCF-7 cells transfected with anti-miR-21 or miRNA-negative control were cultured in a medium containing DMSO or 45 μmol/l DIM for 5 days. Cell numbers were determined by counting using a Coulter Z1 cell counter. Results are representative of at least three independent experiments.

Discussion

In spite of major advances in early detection and adjuvant therapy, advanced breast cancer remains a major clinical problem. Therefore, there is a pressing need for new therapeutic approaches. 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. DIM has been shown to be effective against the proliferation of breast cancer cells [30,31]; however, in spite of its known beneficial role in preventing breast cancer, the exact mechanism of the anti-cancer action of DIM has not been fully elucidated, and its regulation of the cell cycle in estrogen-dependent cells and efficacy against estrogen-independent breast cancer has not been investigated.

Here we show for the first time that DIM treatment inhibits breast cancer cell growth *in vitro* regardless of estrogen responsiveness and p53 status. Consistent with the inhibitory effect of DIM on breast cancer cell proliferation and function *in vitro*, it also inhibited human tumor xenograft growth *in vivo*. The potencies of DIM in our rodent assays of tumorigenesis were similar to published findings in which a DIM dose of 5 mg/kg body weight was reported to inhibit carcinogen-induced mammary carcinogenesis in rodents [32,33]. In our animal study, DIM effectively inhibited tumor growth at a dose as low as 5 mg/kg, and we did not observe any weight loss or toxicity to major organs of the animals.

We also found that DIM not only causes G1 arrest, but also causes G2/M arrest in MCF-7 and MDA-MB-468 cells. Cellular proliferation is driven by the periodic association of Cdks with their cyclin partners, and is controlled by kinase inhibitors. For the first time in breast cancer cells, we show that DIM can also cause G2/M arrest regardless of their estrogen-dependence and p53 status, mainly by degradation of Cdc25A protein to reduce the activity of cyclinB–Cdk1.

Cdc25A, a critical regulator of cell cycle progression and checkpoint response [34], whose overexpression is found in approximately 50% of breast cancer patients and is

associated with poor prognosis [35,36]. The major mechanism of rapid turnover of Cdc25 family proteins is regulated by ubiquitin-mediated proteolysis [19]. However, extensive effort in the mapping of phosphorylation sites in Cdc25A and the use of cells deficient in Chk2 or ataxia telangiectasia mutated indicates that many such sites are not required for Cdc25A-mediated G2/M checkpoint after DNA damage [37,38]. Our data also show that Ser^{76}, Ser^{82}, and Ser^{124} sites of Cdc25A are not required for Cdc25A-mediated G2/M checkpoint after DIM treatment. In addition, we provide a novel mechanism of Cdc25A protein turnover that the full extent of Cdc25A inactivation requires miR-21 in DIM-treated MCF-7 cells. An involvement of miR-21 in cell cycle progression after DIM treatment is supported by several recent studies, as it was induced by the chemotherapeutic drug 5-fluorouracil in colon cancer cells [39] and by UV irradiation in primary fibroblasts [40] or in colon cancer cells [29]. Interestingly, the elevated Cdc25A levels in anti-miR-21 transfected cells do not seem to affect proliferation under DMSO treatment but profoundly affect cell progression after DIM treatment, suggesting the importance of fully inactivating Cdc25A under these conditions. These results clearly suggest that DIM could inhibit proliferation of breast cancer cells, which is in part because of the upregulation of miR-21, and subsequent downregulation of Cdc25A.

Apoptosis is one of the most vital pathways through which chemopreventive agents inhibit the overall growth of cancer cells. When we treated MCF-7 cells with 60 μmol/l DIM for 48 h, the apoptotic peak was shown by fluorescence activated cell sorter detection. We also found that DIM can decrease some apoptosis associated gene expression, such as survivin and FOXM1 (Supplementary Fig. S8). The inhibition of FOXM1 expression by DIM may disrupt survivin–microtubule interactions and result in the loss of survivin's anti-apoptosis function. We conclude that induction of apoptosis by DIM enhances its inhibition efficacy in breast cancer cells in general.

In conclusion, the findings presented here show preclinical proof of efficacy of DIM in breast cancer cell lines, and thus DIM should be evaluated further as a potential agent for estrogen-dependent and estrogen-negative breast cancer.

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