

Therapeutic intervention of experimental breast cancer bone metastasis by indole-3-carbinol in SCID-human mouse model

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Abstract

Several lines of experimental evidence have suggested that chemokine receptor CXCR4, a metastasis-promoting molecule, may play important roles in breast cancer bone metastasis. There is emerging evidence linking CXCR4 to matrix metalloproteinases (MMP) as well as their regulator nuclear factor- κ B (NF- κ B), a key transcription factor, which is known to activate metastasis-promoting molecules for many types of malignancies, including breast cancer. A recent study also showed that promoter region of CXCR4 has several NF- κ B-binding sites, suggesting that there may be a cross-talk between CXCR4 and NF- κ B. We have shown previously that indole-3-carbinol (I3C), a natural compound present in vegetables of the genus *Brassica*, can inhibit NF- κ B in breast cancer cells. However, there are no reports in the literature showing any effect of I3C on CXCR4 expression *in vitro* and *in vivo*. We therefore examined whether I3C could inhibit bone metastasis of breast cancer by inhibiting CXCR4 and MMP-9 expression mediated via the inhibition of the NF- κ B signaling pathway. Here, we have modified the severe combined immunodeficient (SCID)-human mouse model of experimental bone metastasis for use with the MDA-MB-231 breast cancer cell line. In this animal model, we found that I3C significantly inhibited MDA-MB-231 bone tumor growth, and our results were correlated with the down-regulation of NF- κ B. Moreover, we found that I3C significantly inhibited the expression of multiple genes involved in the control of metastasis and invasion *in vitro*

and *in vivo*, especially the expression of CXCR4 and MMP-9 along with pro-MMP-9, with concomitant decrease in Bcl-2 and increase in the proapoptotic protein Bax. From these results, we conclude that the CXCR4/NF- κ B pathway is critical during I3C-induced inhibition of experimental breast cancer bone metastasis. These results also suggest that I3C could be a promising agent for the prevention and/or treatment of breast cancer bone metastasis in the future. [Mol Cancer Ther 2006;5(11):2747–56]

Introduction

Metastasis is a nonrandom process, and each cancer type has its own preferred sites of metastasis (1, 2). Metastasis of cancer cells is a complex process involving multiple steps, including invasion, angiogenesis, intravasation, trafficking of cancer cells through blood vessels, extravasations, organ-specific homing, and growth (3, 4). Bone is one of the most common sites of metastasis for human breast cancer (5). A recent study has shown that a chemokine receptor, CXCR4, is highly expressed in breast cancer cells but not in normal breast tissue, whereas its ligand, stromal-derived factor-1 α (SDF-1 α ; also called CXCL12), is expressed in those organs where breast cancer metastasis is frequently found (6). Thus, it is quite possible that CXCR4 and/or SDF-1 α signaling may be involved in attracting and homing breast cancer cells in the bone.

There are some protease systems that are required for extracellular matrix degradation for the growth of cancer cells at the metastatic sites. Matrix metalloproteinases (MMP) are a group of enzymes required for extracellular matrix degradation for the growth of cancer cells at the metastatic sites (3, 4). These enzymes are secreted as pro-proteins and usually need to be activated by urokinase-type plasminogen activators (uPA; 3, 4). There is increasing evidence connecting CXCR4 to MMPs, IL-8, and uPA (7). MMPs and uPA as well as their regulator nuclear factor- κ B (NF- κ B), a key transcription factor, are known to activate metastasis-promoting molecules for many types of malignancies, including breast cancer (5, 6, 8–13). NF- κ B was also found to be constitutively activated in human breast cancer (14, 15). A recent study and TESS analysis (TRANSFAC 4.0 database version) also showed that the promoter region of CXCR4 has several NF- κ B-binding sites, suggesting that there may be a cross-talk between NF- κ B and CXCR4 (16–18). Thus, NF- κ B may regulate the expression and function of CXCR4, which may, in part, be activated via up-regulation of the expression of IL-8, uPA, and MMP-9 (19). Taken together, CXCR4 may be the point of convergence of many mediators of metastatic processes at the metastatic sites.

Indole-3-carbinol (I3C) and its stable condensation product, 3,3'-diindolylmethane, are compounds that are abundant

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in cruciferous vegetables and have been shown to possess inhibitory effects on the growth and metastatic abilities of several prostate and breast cancer cell lines (20–29). It has also been shown that I3C and 3,3'-diindolylmethane inhibit cell growth and induce apoptotic cell death by their pleiotropic effects on the regulation of multiple genes, such as *p21*, *p27*, *cyclin-dependent kinase*, *survivin*, *Bax/Bcl-2*, *cytochrome P450 1A1*, and *GADD153* (28, 30). Interestingly, our recent studies have shown that the effect of I3C and 3,3'-diindolylmethane is mediated by the inactivation of NF- κ B (23–27, 29). Thus, agents that directly block the expression of CXCR4 signaling, partly due to inhibition of NF- κ B, may have great therapeutic potential for treating metastatic breast cancer. I3C also inhibits the *in vitro* invasive potential of human breast cancer cell lines, suggesting that I3C could inhibit the metastatic growth of breast cancer (31). Collectively, these studies suggest that NF- κ B may play a pivotal role in controlling breast cancer metastasis, a concept supported by many other lines of evidence, in breast cancer and other types of malignancies (23–27, 29). We report here that I3C treatment of MBA-MB-231 breast cancer cells results in decreased CXCR4 mRNA expression. However, the role of I3C in the inhibition of bone metastasis of human breast cancer has not been documented, perhaps due to a lack of appropriate animal models of experimental breast cancer bone metastasis. We hypothesize that I3C-induced inhibition of NF- κ B will inhibit CXCR4 and other NF- κ B targeted genes that could be mechanistically responsible for the inhibition of experimental breast cancer bone metastasis in a suitable animal model.

There are some reports in the literature about the spontaneous animal model of breast cancer (32, 33). A mouse model of human prostate cancer metastasis [severe combined immunodeficient (SCID)–human (SCID-hu)] using human prostate cancer cells grown in the human bone implanted into SCID mice has been developed by Nemeth et al. (34). But there are no other reports in the literature about suitable animal models that could be used faithfully for studying human breast cancer bone metastasis using human breast cancer cell lines that grow in the marrow of human bone environment implanted s.c. into mice. We believe that the advantage of SCID-hu model may provide a more clinically relevant model for growth of human breast cancer in a human bone microenvironment. For these reasons, here, we have adapted the SCID-hu model of experimental bone metastasis for use with the MDA-MB-231 breast cancer cell line and determined the effect of I3C *in vivo* in this model to test our hypothesis as stated above. We found that I3C could inhibit the growth of MDA-MB-231 cells in a SCID-hu model with concomitant inhibition of CXCR4, MMP-9, and Bcl-2 mediated by the down-regulation of NF- κ B.

Materials and Methods

Two-Step Real-time Quantitative Reverse Transcription-PCR Analysis of Gene Expression in I3C-Treated MDA-MB-231 Breast Cancer Cells

Total RNA was isolated in I3C-treated MDA-MB-231

breast cancer cell line by Trizol (Invitrogen, Carlsbad, CA). One microgram of total RNA was subjected to first-strand cDNA synthesis using Taqman reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a total volume of 50 μ L, including 6.25 units MultiScribe reverse transcriptase and 25 pmol random hexamers. Reverse transcription reaction was done with 25°C for 10 minutes followed by 48°C for 30 minutes and 95°C for 5 minutes. The primers were checked by running a virtual PCR, and primer concentration was optimized to avoid primer dimer formation. In addition, dissociation curves were checked to avoid nonspecific amplification. Real-time PCR amplifications were undertaken in Mx4000 Multiplex QPCR System (Stratagene, La Jolla, CA) using 2 \times SYBR Green PCR Master Mix (Applied Biosystems). One microliter reverse transcriptase reaction was used for a total volume of 25 μ L quantitative PCRs. The thermal profile for SYBR real-time PCR was 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Animal Care and Human Bone Implantation

Female homozygous CB17 scid/scid mice, ages 4 weeks, were purchased from Taconic Farms (Germantown, NY). The mice were maintained according to the NIH standards established in the "Guidelines for the Care and Use of Experimental Animals," and all experimental protocols were approved by the Animal Investigation Committee of Wayne State University (Detroit, MI). Human fetal bone tissue was obtained by a third party, nonprofit organization (Advanced Bioscience Resources, Alameda, CA), and written informed consent was obtained from the donor, consistent with regulations issued by each state involved and the federal government. Human fetal femurs and humeri of 16 to 22 weeks of development were divided in half longitudinally and then again in half transversely into four fragments \sim 1 cm long and 3 or 4 mm in diameter (34). After 1 week of acclimatization, these bone fragments were implanted s.c. in the flank through a small skin incision with the opened marrow cavity against the mouse muscle. Isoflurane anesthesia was used during all surgical procedures.

Production of Breast Cancer Bone Tumors and I3C Treatment

Suspensions of MDA-MB-231 cells (2×10^5 cells in a volume of 20 μ L PBS) were injected using a 27-gauge needle through the mouse skin directly into the marrow of implanted fetal bone. The mice were then divided into two groups: control ($n = 10$) and intervention ($n = 10$) groups (Fig. 2). Sesame seed oil was used to facilitate gavage and avoid irritation of the esophagus and was safe as shown also by others (35, 36). The mice in the intervention group were given I3C (1 mg/d/mouse) by oral gavage everyday for 5 weeks as soon as the majority of the bone implants began to enlarge (now called a "bone tumor") as determined by caliper measurements (23rd day after cancer cell injection). The control mice received only sesame seed oil without I3C. The volume of the bone tumor in each group was determined by twice-weekly caliper measurements according to the formula $ab^2 / 2$, where a is the length and b is the

shortest measurement. Percentage (%) reduction in tumor volume at the end of the treatment was deduced by the formula: volume of tumor in experimental group / volume in control mice \times 100. The statistical significance of differential findings between the experimental groups and control was determined by Student's *t* test as implemented by Excel 2000 (Microsoft Corp., Redmond, WA). The mice were sacrificed 3 months after cell injection. Bone tumors were subjected to *ex vivo* imaging on a Lo-Rad M-IV mammography unit (Karmanos Cancer Institute, Detroit, MI) using a magnified specimen technique. Images were developed using a Kodak 2000 screen and radiography film (Kodak, Rochester, NY). On sacrifice, tumor tissue from each mouse was harvested and cut into two pieces; one part was frozen for molecular analysis, and the other part of the tissue was fixed in formalin and embedded in paraffin for histologic evaluation and immunohistochemistry.

Tissue Collection, Fixation, and H&E Staining

Freshly harvested tumors grown in the implanted bones were fixed in 10% buffered formalin for 48 hours and decalcified with 10% EDTA, embedded, and sectioned. Samples were then washed with tap water and soaked in a graded series of 50%, 60%, 70%, 80%, and 90% ethanol for 30 minutes and then in 90% and 100% ethanol for 1 hour. They were then held in a solution of 100% ethanol and xylene at a 1:1 ratio for 30 minutes before being embedded in paraffin and held at 60°C for 1 hour to make paraffin blocks. Transverse sections (5 μ m) were taken from the blocks and prepared for histochemical and immunohistochemical staining. H&E staining was used for histologic observation.

Electrophoretic Mobility Shift Assay for Measuring NF- κ B Activity

MDA-MB-231 cells were plated at a density of 1×10^6 in 100-mm dishes and cultured for 24 hours. Subsequently, the cultures were treated with 60 and 100 μ mol/L I3C or DMSO for 24, 48, and 72 hours. I3C (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO (final concentration, 0.1%) to make a 10 mmol/L stock solution and was added directly to the culture medium at different concentrations. Nuclear extracts were prepared from control and I3C-treated breast epithelial cells as previously described (25, 29, 37) and subjected to analysis for NF- κ B DNA-binding activity as measured by electrophoretic mobility shift assay. Using frozen tumor tissue, nuclear proteins were also extracted as described previously (25, 29, 37). Briefly, tissues were minced and incubated on ice for 30 minutes in 0.5 mL ice-cold buffer A composed of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT, 10% NP40, 0.1% IGEPAL CA-630, and 0.5 mmol/L phenylmethylsulfonyl fluoride. The minced tissue was homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) followed by centrifugation at $5,000 \times g$ at 4°C for 10 minutes. The supernatant (cytosolic proteins) was collected for Western blot analysis and kept at -70°C until use. The crude nuclear pellet was suspended in 200 μ L buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 4 μ mol/L leupeptin] and

incubated on ice for 30 minutes. The suspension was centrifuged at $16,000 \times g$ at 4°C for 30 minutes. The supernatant (nuclear proteins) was collected and kept at -70°C until use. The protein concentration was determined using bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Electrophoretic mobility shift assay was done by preincubating 8.0 μ g nuclear extract with a binding buffer containing 20% glycerol, 100 mmol/L MgCl₂, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.25 mg/mL poly(deoxyinosinic-deoxycytidylic acid) for 10 minutes. After the addition of IRDye 700-labeled NF- κ B oligonucleotide, samples were incubated for an additional 20 minutes. The DNA-protein complexes were electrophoresed in an 8.0% native polyacrylamide gel and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

Western Blot Analysis for Measuring the Protein Levels of Bcl-2, Bax, CXCR4, and MMP-9 Activity

MDA-MB-231 cells were plated on culture dishes and allowed to attach for 24 hours followed by the addition of 60 or 100 μ mol/L I3C and incubated for 24, 48, and 72 hours. Control cells were incubated in the medium with DMSO for similar times. Total cell lysates were prepared using the method as described previously (25, 29, 37). Using frozen tumor tissue, nuclear proteins were extracted using the method as described previously (25, 29, 37). Briefly, tissues were minced and incubated on ice for 30 minutes in 0.5 mL ice-cold buffer A, composed of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT, 10% NP40, 0.1% IGEPAL CA-630, and 0.5 mmol/L phenylmethylsulfonyl fluoride. The minced tissue was homogenized using a Dounce homogenizer followed by centrifuging at $5,000 \times g$ at 4°C for 10 minutes. The supernatant (cytosolic proteins) was collected for Western blot analysis. Protein content was quantified with a bicinchoninic acid protein assay, and equal amounts of proteins were resolved by 10%, 12%, and 14% SDS-PAGE. Immunoblot was done with antibodies to Bcl-2 (Calbiochem, La Jolla, CA), Bax and CXCR4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), MMP-9 (Santa Cruz Biotechnology), and β -actin (Sigma-Aldrich). A representative blot from three independent experiments was presented.

Immunohistochemical Staining for CXCR4 and MMP-9

Freshly harvested tumors grown in the implanted bones were fixed in 10% buffered formalin, decalcified, embedded, and sectioned. The paraffin sections of tumor tissues were deparaffinized then rehydrated through a graded alcohol series. Slides were placed in 10 mmol/L citrate buffer (pH 6.0) and heated in a microwave for 3 minutes. Nonspecific sites were blocked by incubation with Superblock (ScyTek, Logan, UT). Sections were incubated with antibodies to MMP-9 (Calbiochem) and CXCR4 followed by staining with appropriate horseradish peroxidase-conjugated secondary antibodies followed by color development. The stained slides were dehydrated and mounted in per mount and visualized using an Olympus microscope (Olympus, Center Valley, PA). Images were captured with an attached camera linked to a computer.

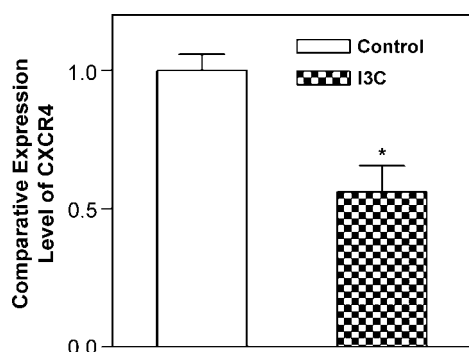


Figure 1. Real-time reverse transcription-PCR analysis of CXCR4 genes. Comparative analysis shows the down-regulation of CXCR4 mRNA expression in MDA-MB-231 cells grown in culture after 72 h of treatment with I3C compared with control. *, $P < 0.05$.

MMP-9 Activity Assay

MDA-MB-231 cells were seeded in a six-well plate (1.0×10^5 per well) and incubated at 37°C . After 24 hours, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium supplemented with 60 or $100 \mu\text{mol/L}$ I3C for 72 hours. MMP-9 activity in the conditioned medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay kit (R&D System, Inc., Minneapolis, MN) according to the manufacturer's protocol.

Invasion Assay

The invasive activity of MDA-MB-231 cells with different treatments was tested by using BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) according to the manufacturer's protocol with minor modification. Briefly, MDA-MB-231 cells (5×10^4) with serum-free medium supplemented with 60 or $100 \mu\text{mol/L}$ I3C were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium and the same reagent treatment as the upper chamber. After 48 hours of incubation, the cells in the upper chamber were removed, and the cells, which invaded through the Matrigel matrix membrane, were stained with $4 \mu\text{g/mL}$ calcein AM in Hank's buffered saline at 37°C for 1 hour. Then, fluorescence of the invaded cells was read in ULTRA Multifunctional microplate reader (TECAN, Research Triangle Park, NC) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

Statistical Analysis

The statistical significance was determined using Student's t test, and $P < 0.05$ was considered significant.

Results

Regulation of CXCR4 mRNA Expression by I3C Treatment

A recent study has indicated that the MDA-MB-231 cell line highly expresses CXCR4 (17), and I3C has been shown to possess inhibitory effects on the growth and metastatic

abilities of several cancers, including breast cancer (24–27, 29, 38–43). To confirm the alternations of CXCR4 expression after I3C treatment in MDA-MB-231 breast cancer cells, we conducted real-time reverse transcription-PCR analysis for the CXCR4 gene (Fig. 1). The altered mRNA expression of CXCR4 was observed as early as 24 hours after I3C treatment and was significantly more evident after 72 hours treatment. The results of reverse transcription-PCR analysis for CXCR4 gene along with published *in vitro* results suggest that I3C could regulate the transcription of genes involved in angiogenesis, tumor cell invasion, and metastasis (27–30, 38, 44–46). However, we asked the most important question whether I3C could affect tumor growth in the bone environment, which is answered by the following experiments.

Inhibition of Bone Tumor Growth by I3C

We report here, for the first time, the use of the SCID-hu animal model (34) as an experimental model for breast cancer bone metastasis and the effect of I3C *in vivo* in impeding tumor growth and metastasis. The experimental design and treatment schedule is depicted in Fig. 2. Currently, there are no reports in the literature documenting any effect of the host (i.e., mouse) environment on the bone xenograft (34, 47). Under our experimental conditions, administration of I3C by gavage treatment caused 50% reduction in tumor volume (Fig. 3A) compared with control group. The statistical analysis indicated that compared with the control group, bone tumor growth was significantly lower in the intervention ($P < 0.05$) group. The experiment was terminated 3 months after I3C treatment. We found that I3C (1 mg I3C/d/mouse) significantly inhibited breast cancer bone tumor growth (Fig. 3A and B) and osteolysis, documenting the efficacy of I3C in inhibiting breast cancer cell growth in the experimental model of bone metastasis. Figure 3B indicates *ex vivo* bone tumor X-ray showing bone osteolysis and

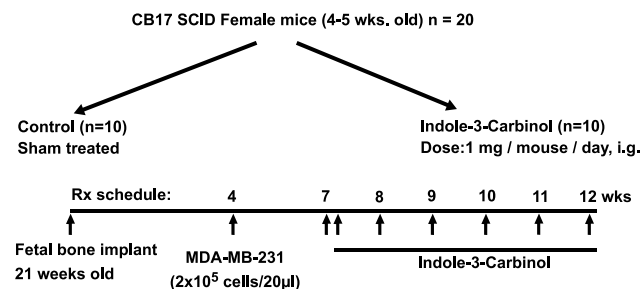


Figure 2. Flow chart representation of *in vivo* experimental design and treatment schedule. The human fetal bone was implanted s.c. in the right flank of female homozygous CB17 SCID mice through a small skin incision with the open marrow cavity against the mouse muscle. Four weeks after the bone implantation, suspensions of MDA-MB-231 cells (2×10^5 cells) were injected through the mouse skin directly into the marrow of implanted fetal bone. The mice in the intervention group were given I3C (1 mg/d/mouse) by oral gavage everyday for 5 wks as soon as the majority of the bone implants began to enlarge (now called a "bone tumor") as determined by caliper measurements (23rd day after cancer cell injection). The control mice received only saline without I3C. All animals were sacrificed at 12 wks after the start of the experiment.

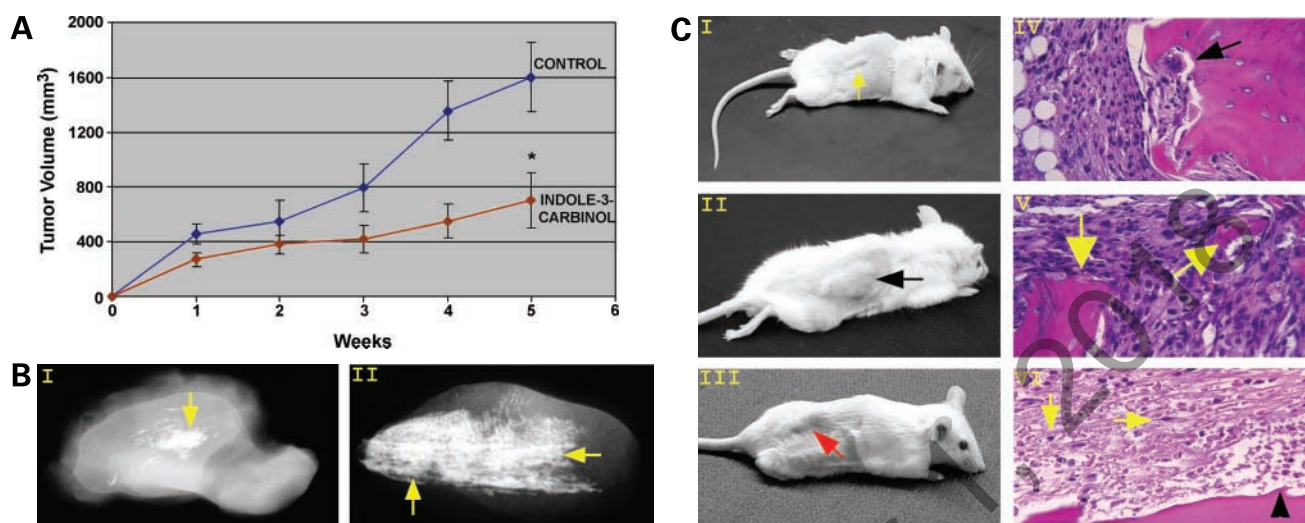


Figure 3. **A**, inhibitory effects of I3C on the growth of xenograft bone tumors of MDA-MB-231 cells in SCID-hu mice. A total of 20 mice were divided into two groups. Under the experimental conditions, administration of I3C by gavage treatment caused 50% reduction in tumor volume compared with control group. *, $P < 0.05$. **B**, *ex vivo* bone tumor X-ray showing bone osteolysis and tumor growth of MDA-MB-231 cells in the control and I3C-treated group. Arrow, less residual bone in the control group (I) relative to I3C-treated group (II). We found that treatment with I3C inhibited tumor growth and osteolytic bone destruction resulting in more residual bone volume compared with control group (I). **C**, typical osteolytic bone metastasis of MDA-MB-231 cells in SCID-hu model. I, human fetal bone s.c. implanted in SCID mice. II, MDA-MB-231 cells (2×10^6) were injected directly into the marrow of implanted bone. Black arrow, the site of significant progression of tumor growth in representative untreated control mice. III, representative figure of mice from treatment group photographed at time of sacrifice showing significant regression of tumor growth (red arrow) by I3C treatment. IV, MDA-MB-231 cells were injected into the implanted human fetal bone showing invasion (black arrow). V, continuous osteolytic and invasive process resulting in gradual loss of the bone structure. Yellow arrows, residual bone surrounded by invasive tumor cells. VI, histologic evaluation showing I3C treatment resulted in pronounced death of tumor cells (yellow arrows) and preservation of the bone structure (black arrowhead).

tumor growth of MDA-MB-231 cells in the control and I3C treatment group. Arrow indicates less residual bone in the control group (Fig. 3B, I) relative to I3C-treated group (Fig. 3B, II). We found that treatment with I3C significantly inhibited cellular growth of breast cancer cells in a bone environment and also inhibited bone osteolysis in SCID-hu mice, showing an inhibitory effect of I3C in an *in vivo* model of experimental breast cancer bone metastasis. At autopsy, all tumors were found localized at the site of injection with essentially spread to no other organs. Our treatment conditions did not cause any weight loss of the animals, suggesting that I3C did not induce any deleterious effects under the present experimental conditions. These results show, for the first time, the efficacy of I3C in inhibiting tumor growth in an experimental breast cancer bone metastasis model.

Tumor Histology and Inhibition of Osteolysis by I3C

H&E histology evaluation showed typical osteolytic bone metastasis of MDA-MB-231 cells in SCID-hu animals (Fig. 3C). Human fetal bones s.c. implanted in SCID mice, as shown by representative figure, were randomly selected from each group (Fig. 3C, I). Suspensions of MDA-MB-231 cells (2×10^5 cells in a volume of 20 μ L PBS) were injected using a 27-gauge needle through the mouse skin directly into the marrow of implanted fetal bone. Black arrow represents the site of significant progression of tumor growth as shown by representative figure from untreated group (Fig. 3C, II). Figure 3C (III) shows representative

figure of mice from treatment group photographed at the time of sacrifice. Red arrow represents the site of significant regression of tumor growth in I3C treatment group (Fig. 3C, III). In the control group, continuous osteolytic and invasive growth into the adjacent bone resulted in gradual loss of the bone structure (Fig. 3C, IV, black arrow). Yellow arrows indicate residual bone surrounded by invasive tumor cells (Fig. 3C, V). In contrast, the group receiving I3C treatment showed pronounced death of tumor cells (yellow arrows) and preservation of the bone structure (Fig. 3C, VI, black arrowhead). To further show whether the antitumor effect observed by I3C treatment could be due to inactivation of NF- κ B, we did the following experiments.

Inhibition of NF- κ B Activation by I3C

To answer the most important question, whether treatment of animals with I3C could effectively target a specific signaling molecule, such as NF- κ B in tumor tissues, nuclear extract from frozen tumor tissues (randomly selected from control and treatment groups) was subjected to analysis for NF- κ B DNA-binding activity as measured by electrophoretic mobility shift assay. The results are shown in Fig. 4B, which clearly show that I3C was effective in down-regulating NF- κ B DNA-binding activity in animal tumors receiving I3C compared with control tumors. These *in vivo* results were similar to our *in vitro* findings (Fig. 4A), including our previously published data (27, 29), suggesting that the inactivation of NF- κ B is at least one of the molecular mechanisms by which I3C induced antitumor activity in our

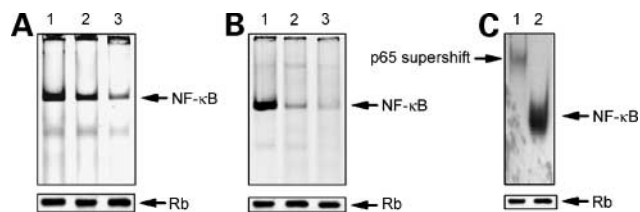


Figure 4. I3C abrogates NF- κ B DNA-binding activity. **A**, I3C abrogates NF- κ B DNA-binding activity in MDA-MB-231 breast cancer cells. Cells were treated with 60 or 100 μ mol/L I3C (lanes 2 and 3, respectively) for 72 h. Nuclear extracts were prepared from control and I3C-treated cells and subjected to analysis for NF- κ B DNA-binding activity as measured by electrophoretic mobility shift assay. **B**, MDA-MB-231 cells were grown in the marrow surface of the previously implanted bone in SCID animal. After the termination, tumors were removed and the nuclear proteins were subjected to analysis of the DNA-binding activity of NF- κ B as measured by electrophoretic mobility shift assay. Gel shift assay for NF- κ B done on randomly selected frozen tumor tissues obtained from each treatment groups of animals. Group 1 (lane 1, control animal; lanes 2 and 3, animal received I3C). Results showed that I3C was effective in down-regulating NF- κ B in treated animals relative to control tumors. Arrow, NF- κ B DNA-binding activity. **C**, the specificity of NF- κ B DNA-binding activity was confirmed by supershift assays. Retinoblastoma (Rb) protein level served as nuclear protein loading control.

experimental animal model. The specificity of NF- κ B DNA-binding activity was confirmed by supershift assays (Fig. 4C). Retinoblastoma protein level served as nuclear protein loading control. These data also provide proof of principle and suggest that I3C could be an effective antitumor agent in an animal model of breast cancer bone metastasis, which is mediated by inactivation of NF- κ B DNA-binding activity (27, 29). The inactivation of NF- κ B may decrease the expression of NF- κ B downstream genes, such as CXCR4 and Bcl-2 proteins with concomitant increase in the proapoptotic protein Bax in addition to the alterations in the expression of genes which are involved in the osteolytic and apoptotic processes.

Effect of I3C on the Expression of Bcl-2, Bax, CXCR4, and MMP-9

The effect of CXCR4 expression in tumor tissue (tissue randomly selected from control and treatment groups) was studied by Western blot analysis, and we found a significant down-regulation in the expression of CXCR4 and MMP-9 in tumors of the I3C-treated group (Fig. 5B, lane 2) compared with the untreated group (Fig. 5B, lane 1). These *in vivo* results were similar to our *in vitro* findings (Fig. 5A, lanes 2 and 3). It is known that CXCR4 protein family significantly increases the viability of CD34(+) T-cell precursors by modulating the expression of Bcl-2 and Bax genes and consequently stimulating the proliferation of CD34(+) thymic precursor cells (48). We found that I3C caused a decrease in the expression of Bcl-2 and an increase in the expression of Bax in I3C-treated tumors (Fig. 5B) and MDA-MB-231-cells (Fig. 5A). These results are consistent with our previously published *in vitro* data on Bax and Bcl-2 expression in cells treated with I3C (24, 25). Moreover, recent evidence indicates that deregulation of MMP expression contributes to invasion, metastasis, and tumor angiogenesis (49–55). For example, overexpression of MMP-2 and MMP-9 has been reported to

be positively associated with the progression of human cancers (49–58). Our present results suggest that I3C can inhibit metastasis of breast cancer cells mediated by the inhibition of MMP-9 expression. These results also provide evidence in support of our hypothesis, implicating the role of CXCR4 in metastatic tumor cells by controlling tumor cell migration. Remarkably, we show that CXCR4 may protect tumor cells by reducing apoptotic processes, resulting in increased tumor cell growth and viability. This protective effect involves both the down-regulation of the proapoptotic Bax protein and the up-regulation of the antiapoptotic Bcl-2 protein. The level of Bcl-2 expression was found to be significantly down-regulated in I3C-treated animal tumors and MDA-MB-231 cells (Fig. 5A and B). In contrast, the expression of Bax was found to be significantly up-regulated in I3C-treated animals and MDA-MB-231 cells (Fig. 5A and B). Collectively, our results implicate the role of CXCR4/NF- κ B pathway as a critical event during I3C-induced apoptosis and inhibition of tumor cell proliferation. These preclinical data suggest that I3C could have beneficial effects in patients with breast cancer bone metastasis.

Analysis of CXCR4 and MMP-9 Expression by Immunohistochemistry

Previous studies have shown overexpression of chemokine receptor CXCR4 and/or SDF-1 α in metastatic breast cancer cells (6), and these chemokine protein families are also known to activate other metastasis-promoting molecules, such as MMPs, in breast cancer cells (17). Thus, agents that directly block the expression of CXCR-4 could also down-regulate MMP-9 expression, which could also be partly due to inactivation of NF- κ B, and this could have great therapeutic potential for treating metastatic breast cancer. To verify the alteration of CXCR4 and MMP-9 by I3C treatment, we conducted immunohistochemical analysis for CXCR4 and

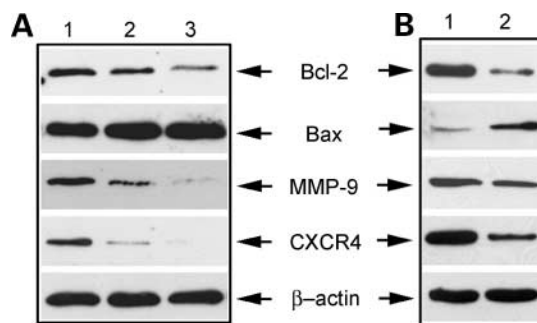
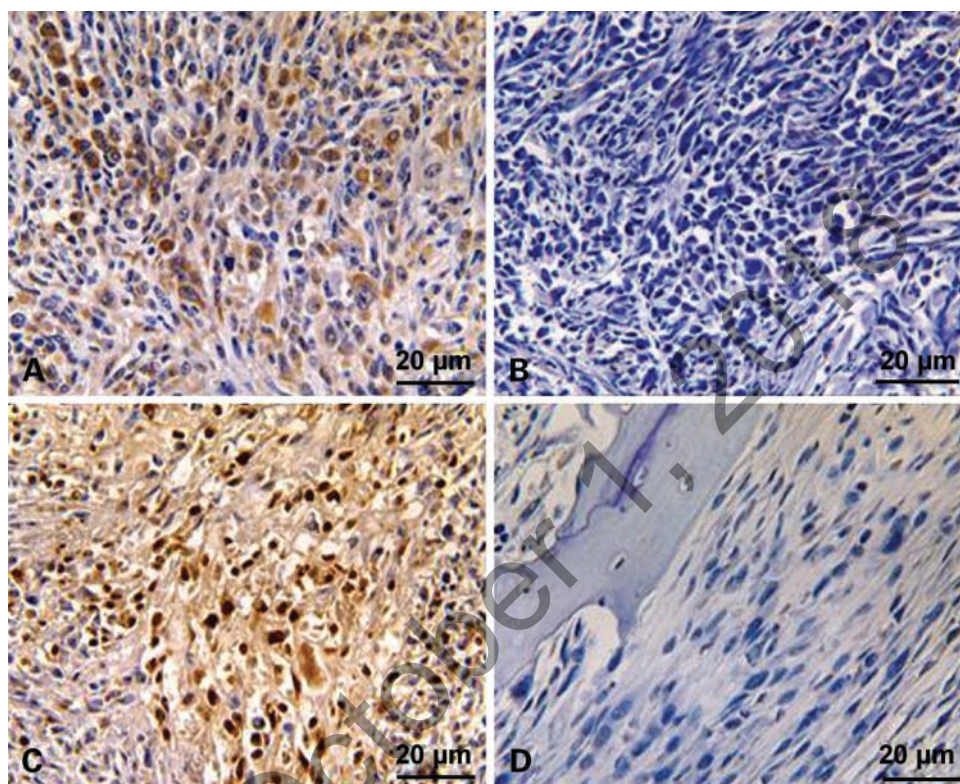


Figure 5. I3C-induced alterations in metastasis-promoting and apoptosis-related genes. **A**, I3C down-regulated metastasis-related protein, such as Bcl-2, Bax, CXCR4, and MMP-9 expression in total cell lysate. Control, MDA-MB-231 cells treated with DMSO; day 3, cells treated with 60 or 100 μ mol/L I3C (lanes 2 and 3) for 72 h. Whole-cell lysates were prepared and proteins were subjected to Western blot analysis. **B**, Western blot analysis was done on randomly selected frozen tumor tissues obtained from each treatment group of animals. Group 1 (lane 1, control animal; lane 2, animal received I3C). Results from Western blot analysis showed that the levels of Bcl-2, Bax, CXCR4, and MMP-9 were significantly down-regulated in I3C-treated MDA-MB-231 cells and in animal tumors compared with control group.

Figure 6. Immunohistochemical staining for CXCR4 and MMP-9 in I3C-treated and untreated SCID-hu model and animal tumors done on randomly selected tumor tissues. Tumor cells in untreated control group show intensive staining of CXCR4 (A) and MMP-9 (C). In contrast, tumor cells in I3C-treated SCID-hu mice show much weaker staining of CXCR4 (B) and MMP-9 (D). Results from immunohistochemical staining showed that the expression of CXCR4 and MMP-9 was significantly decreased in MDA-MB-231 bone tumors in SCID-hu mice receiving the I3C-containing diet.



MMP-9 protein. The expression of CXCR4 (Fig. 6, top) and MMP-9 (Fig. 6, bottom), two important molecules of tumor cell survival and metastasis that are downstream of NF- κ B, was significantly decreased in MDA-MB-231 bone tumors in SCID-hu mice receiving the I3C (Fig. 6B and D) compared with control (Fig. 6A and C). Overall, our results suggest that the inactivation of NF- κ B may indeed reduce the levels of CXCR4 and MMP-9 in the tumor microenvironment resulting in antitumor activity of I3C in our experimental model of breast cancer bone metastasis.

Regulation of the Expression and Activity of MMP-9 by I3C

Treatment with I3C showed down-regulation of MMP-9 by Western blot analysis. Furthermore, we found that I3C significantly inhibited the activity of MMP-9 in conditioned medium in MDA-MB-231 cell culture (Fig. 7A). Together, these results clearly suggest that I3C down-regulates MMP-9; thus, I3C could enhance the antitumor and antimetastatic activity in MDA-MB-231 breast cancer cells. To further support the role of MMP in breast cancer metastasis, we conducted cell invasion assay.

I3C Inhibits Cancer Cell Invasion

It has been well known that MMP-9 is an important molecule involved in cancer cell invasion and metastasis. Because I3C inhibited the expression and activity of MMP-9, we tested the effects of I3C on cancer cell invasion. We found that I3C inhibited invasion of MDA-MB-231 cells through Matrigel matrix membrane compared with control (Fig. 7B and C).

Discussion

The importance of this study lies in the use of a SCID-hu mouse model system that resembles human disease and may also be more relevant to clinical breast cancer than other animal models. Typical osteolytic bone metastasis of MDA-MB-231 cells in a SCID-hu model showed continuous osteolytic and invasive processes resulting in the loss of the bone structure and increased tumor growth, which are believed to be associated with activation of CXCR4/NF- κ B signaling pathways (6, 17), similar to those observed in many human breast cancers (6, 17, 59). Our present observations are also in direct agreement with reports showing a significant elevation of CXCR4 in models of metastatic breast cancer with immunodeficient mice (60). Thus, our SCID-hu animal model of breast cancer bone metastasis has offered us an experimental model, in which we have tested the effects of I3C as a chemopreventive or therapeutic agent against breast cancer.

I3C has been shown to target multiple pathways of tumorigenesis, including proliferation, apoptosis, angiogenesis, invasion, and tumor-induced immunosuppression in various breast tumor cell lines (23–27, 29, 38–43, 61–64). Several studies have suggested that chemokine receptor CXCR4 is overexpressed in metastatic breast cancer cells (6). However, no reports exist in the literature elaborating how I3C inhibits CXCR4, a metastatic promoting molecule *in vivo* model of breast cancer cells. In the current report, we show, for the first time, that I3C is an effective agent in the inhibition of cellular growth of breast

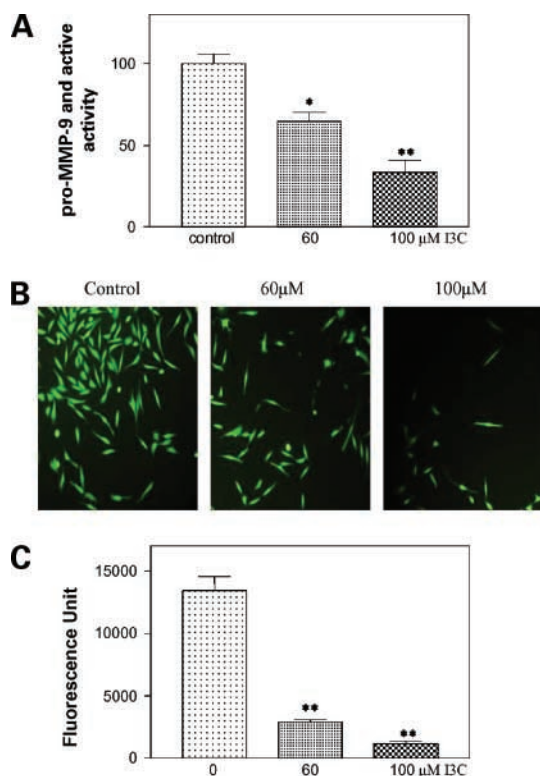


Figure 7. **A**, MMP-9 expression was down-regulated by I3C. MMP-9 activity assay showed that MMP-9 was down-regulated by I3C in the conditioned medium of MDA-MB-231 breast cancer cells. **B**, I3C induced inhibition of MDA-MB-231 cell invasion. Invasion assay showed that I3C significantly inhibited the invasion of MDA-MB-231 cells through Matrigel matrix membrane. **C**, value of fluorescence from the invaded MDA-MB-231 breast cancer cells. The value indicates the comparative amount of invaded MDA-MB-231 breast cancer cells. *, $P < 0.05$; **, $P < 0.01$.

cancer in a bone environment using an animal model of experimental breast cancer bone metastasis. The antitumor activity of I3C was regulated by the inhibition of CXCR4, which is known to regulate the organ-specific trafficking and invasion of metastatic tumor cells mediated by inactivation of NF- κ B.

Chemokines and chemokine receptors are believed to play important roles in metastatic breast cancer (6, 59). The most common sites of breast cancer metastases include the lung, liver, lymph node, and bone, in which CXCR4 was found to be highly expressed (6). Moreover, NF- κ B, a key transcription factor, may regulate the expression and function of CXCR4, which in turn may activate other metastasis-promoting molecules, such as MMP-9 in breast cancer cells (17). A recent study also showed that the promoter region of CXCR4 has several NF- κ B-binding sites, suggesting that there may be a cross-talk between NF- κ B and CXCR4 (17). In models of metastatic breast cancer, the administration of neutralizing antibodies or peptide antagonists of CXCR4 substantially reduced lung metastases (6, 65). CXCR4 expression was found to be up-regulated in murine lung metastases in a

NF- κ B-dependent fashion (17), again suggesting that CXCR4 signaling is an important determinant of metastatic breast cancer. Moreover, a significant correlation between the relative expression levels of CXCR4 and the extent of lymph node metastases was found in invasive ductal carcinomas of the breast in humans. The expression of CXCR4 was also found to be increased in invasive ductal carcinoma specimens compared with ductal carcinoma *in situ* and normal breast epithelium. CXCR4 expression by normal breast stromal cells adjacent to malignant cells was also increased (66). We anticipated that CXCR4 in the tumor microenvironment may function to promote breast cancer proliferation, migration, and invasion, and our current data suggest that I3C could interrupt this signaling pathway, resulting in tumor growth inhibition of breast cancer bone metastasis.

Our present data recapitulate recent *in vitro* results in breast cancer cell lines, showing that I3C induces apoptosis by blocking NF- κ B activation (27, 29). Moreover, NF- κ B, a key transcription factor, may regulate the expression and function of CXCR4 and activate other metastasis-promoting molecules (uPA and MMPs) in breast cancer cells (17). Decreased expression of CXCR4 was associated with decreased expression of NF- κ B, which in turn reduced the levels of MMP-9 in tumors treated with I3C. This could be one of the mechanism(s) underlying enhanced tumor cell apoptosis and reduced tumor cell proliferation *in vivo*. Taken together, our results suggest multiple functions of NF- κ B in the growth, migration, and organ-specific metastasis of breast cancer cells, which, in part, appear to be mediated through the induction of CXCR4. Moreover, the CXCR4/NF- κ B pathways activated MMP-9 expression in breast cancer bone metastasis and were also abrogated by I3C, thus suggesting that our model could be useful to fully evaluate the role of CXCR4/NF- κ B pathways *in vivo* as well as its mechanistic role in the inhibition of tumor cell invasion and metastasis (Fig. 8).

Several other potential mechanism(s) could also explain the regulation of CXCR4 associated with breast cancer metastasis (Fig. 8). One potential mechanism that has been associated with CXCR4-related inhibition of apoptosis is that CXCR4 reduces the apoptotic rate by increasing the level Bcl-2 expression and decreasing the level of Bax expression (48). Our *in vivo* and *in vitro* data support this concept because inhibiting NF- κ B production by targeting CXCR4 activity in the SCID-hu tumors led to a decrease in Bcl-2 protein levels, a concomitant increase in the proapoptotic protein Bax, and decreased MMP-9 expression. These results are consistent with our previously published *in vitro* data on Bax/Bcl-2 expression (24, 25) and provide support for our hypothesis, implying the role of CXCR4 in metastatic tumor cells, suggesting that I3C could be beneficial in patients with breast cancer bone metastasis.

In addition to these mechanism(s), MMP-9 has also been reported to be involved in cancer invasion and metastasis (52, 53, 55). Several studies have shown that MMPs play prominent roles in metastasis (49–53, 55). MMP-9 has been

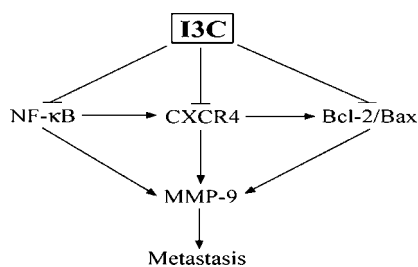


Figure 8. Hypothetical diagram showing the role of CXCR4, MMP-9, and/or other genes in breast cancer cells in a metastatic model and further showing that these molecules could be inhibited by I3C mediated by the inactivation of the NF- κ B pathway.

implicated in metastasis because of its role in the degradation of basement membrane collagen. In addition, MMP activity is known to play a role in both normal and cancer-induced bone remodeling. It has been reported that NF- κ B and MMPs are involved in the processes of tumor invasion and metastasis (67). In this study, we found that I3C inhibited the expression, secretion, and activation of MMP-9, suggesting that I3C could prevent bone matrix degradation and reduce breast cancer cell growth in human bone implanted in SCID mice. The down-regulation of MMP-9 by I3C could be mediated by the down-regulation of NF- κ B whose binding site has been found in the promoter of MMP-9 (67, 68). These results suggest that I3C could promote the antitumor and antimetastasis activities in SCID-hu model of breast cancer bone metastasis partly through the down-regulation of MMP expression (Fig. 8). Because we observed that I3C down-regulated MMP-9, we tested the effects of I3C on the invasion of MDA-MB-231 breast cancer cells and found that I3C inhibited the invasion of MDA-MB-231 breast cancer cells. These results corresponded with MMP-9 data, showing that I3C could inhibit cancer cell invasion partly through down-regulation of MMP-9.

In summary, I3C treatment may exert its antiproliferative, antiangiogenic, and proapoptotic effects by decreasing CXCR4 and MMP-9 expression, which was associated via the down-regulation of the NF- κ B pathway. In this report, we have shown that reduced expression of CXCR4 by I3C limits cellular growth of breast cancer cells in a bone environment and prevents breast cancer bone metastasis. These results further extend the potential therapeutic application of I3C for metastatic breast cancer.

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