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Indole-3-carbinol stimulates transcription of the interferon gamma receptor 1 gene and augments interferon responsiveness in human breast cancer cells

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Indole-3-carbinol (I3C), a naturally occurring compound of *Brassica* vegetables, has promising anticancer properties and activates an anti-proliferative pathway that induces a G_1 cell cycle arrest of human breast cancer cells. A microarray analysis of I3C treated versus untreated MCF-7 breast cancer cells revealed that I3C increased expression of the interferon gamma receptor 1 (IFNγR1). Western blot and RT–PCR analysis demonstrated that I3C strongly and rapidly stimulated IFNγR1 gene expression. Transfection of a series of 5′ deletion constructs of the IFNγR1 reporter plasmids revealed that I3C significantly stimulates the promoter activity of the IFNγR1 and uncovered an I3C-responsive region between −540 and −240 bp of the IFNγR1 promoter. I3C stimulation of the IFNγR1 expression suggests that indole treatment should enhance IFNγ responsiveness in breast cancer cells. A combination of I3C and IFNγ synergistically activated STAT1 proteins by increasing phosphorylation at the Tyr-701 site. In addition, I3C and IFNγ together more effectively induced a G_1 cell cycle arrest and stimulated expression of the $p21^{Waf1/Cip1}$ cell cycle inhibitor, compared with the effects of either agent alone. Our results suggest that one mechanism by which I3C mediates these anticancer effects is by stimulating expression of the IFNγR1 and augmenting the IFNγ response in MCF-7 human breast cancer cells.

Introduction

Breast cancer is one of the leading causes of death among females in North America (1). It is a complex disease in which several distinct classes of tumors can be produced that differs in their proliferative responses to hormonal and environmental signals. Although the exact mechanisms underlying the origin and the evolution of breast cancer are poorly understood, it is generally accepted that during the initial stages of tumor development, estrogens play a stimulatory role. Approximately one-third of breast cancers rely on estrogen for their growth, and for these patients, anti-estrogen therapy with the non-steroidal anti-estrogens tamoxifen and/or raloxifen, which will inhibit or slow the growth of estrogen-dependent tumors, remains a major option for treatment (2–5). Unfortunately, after prolonged treatment, estrogen-responsive breast cancers eventually become resistant to the inhibitory effects of anti-estrogens (6). For the nearly two-thirds of human breast tumors that are not responsive to anti-estrogens, the best currently available options for treatment are surgical removal of the tumors, general chemotherapy and/or radiation therapy. Thus, a critical problem in controlling breast cancer is the need to develop therapeutic strategies that would effectively target a wide variety of both estrogen-responsive and non-responsive mammary tumors.

One potential source of new classes of chemotherapeutic agents to control breast cancer with reduced side effects is compounds found in the diet. Considerable epidemiological evidence shows that an increased consumption of phytochemicals from whole grains, vegetables and fruits is directly associated with a decreased risk for breast cancer, and reduced mammary tumor incidence in experimental animals (7–13). Moreover, differences in the diet are thought to contribute to the low incidence rates of breast cancer found in Asian countries such as Japan, China, Indonesia, Korea and Singapore, and the high incidence rates (4–10-fold higher) in the USA and Canada (7). These studies implicate the existence of specific biologically active compounds from this class of dietary plants that represent a largely untapped source of potentially potent chemotherapeutic molecules. One such phytochemical is indole-3-carbinol (I3C), a naturally occurring component found in the family of *Brassica* vegetables, such as cabbage, broccoli and Brussels sprouts (11,14,15).

I3C has promising chemopreventive properties (16), and markedly reduces the incidence of spontaneous and carcinogen-induced tumors in rodents with low levels of toxicity (17,18). Studies by our group (19–21) and by others (22–25) have shown that I3C has both anti-proliferative and apoptotic effects on cultured human breast cancer cells that generally depend upon the concentration of this dietary indole used in the assays. In one study, I3C was shown to alter the level of BRCA1 gene expression, although the cellular significance of this observation remains unknown (26). As reported previously, we have discovered that the direct exposure of human breast cancer cells to I3C activates a novel anti-proliferative pathway that induces a G_1 cell cycle arrest accompanied by the selective and rapid down-regulation of CDK6 gene expression and strong stimulation of $p21^{Waf1/Cip1}$ gene expression (19–21,27). I3C was found to regulate CDK6 promoter activity by altering Sp1-promoter interactions (21,27). I3C mediated G_1 cell cycle arrest and gene expression changes occur through an estrogen receptor-independent pathway (19,20). Not all of the effects of I3C take place at the

Abbreviations: IFNγR1, interferon gamma receptor 1; I3C, indole-3-carbinol.

transcriptional level since treatment with this indole also inhibits CDK2-specific enzymatic activity without any effects on CDK2 protein expression (19,20). I3C treatment was also shown to alter the activity of several signal transduction components including Akt/PKB (28,29). In estrogen-responsive breast cancer cells, I3C synergizes with the anti-proliferative effects of tamoxifen, an anti-estrogen currently used in breast cancer therapies (20), suggesting that I3C acts through a distinct mechanism.

To further examine the cellular effects of I3C in human breast cancer cells, a microarray analysis of I3C treated and untreated MCF-7 breast cancer cells was carried out in order to identify additional molecular targets of I3C. This analysis revealed that I3C increases expression of the interferon gamma receptor (IFNγR1). We show that I3C stimulates the transcription of the IFNγR1 gene by regulating promoter activity, and augments the IFN response in human breast cancer cells. These results implicate the I3C control of the IFNγ receptor-dependent pathway as playing a key role in the anti-breast cancer properties of dietary indoles.

Materials and methods

Cell culture

MCF-7 human breast cancer cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine and 10 μg/ml insulin. Cells were propagated in a 37°C humidified chamber containing 5% CO_2 and the medium was changed every 48 h.

Microarray analysis

MCF-7 cells were treated with 200 μM I3C or DMSO as the vector control for 48 h. Poly-A-RNA was isolated by two cycles of purification on oligo-dT cellulose. Labeled cDNA probes were prepared using fluorescent cyanine 3 and cyanine 5 dyes for the treated and control samples, respectively, and hybridized simultaneously to microarrays (Incyte Genomics, Palo Alto, CA) of 960 selected cDNAs representing human genes involved in cell cycling, apoptosis, signal transduction, motility, adhesion and angiogenesis. Differential expression was measured by the ratio of the fluorescence intensity at the wavelengths corresponding to the two probes. The samples were spiked with known concentrations of various non-human cDNA to serve as positive controls and to correct for variations in hybridization efficiency.

Western blot analysis

MCF-7 cells were treated with DMSO or 200 μM I3C for 24, 48 and 72 h. After the indicated time points, cells were harvested in RIPA buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 μg/ml PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 1 mM DTT, 0.1 mM sodium orthovanadate and 0.1 mM β-glycerophosphate). Equal amounts of total cellular protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 ml/100 ml 2-mercaptoethanol, 10% bromophenol blue, 3.13% stacking gel buffer) and fractionated by electrophoresis on 10% polyacrylamide, 0.1% SDS resolving gels. Rainbow marker (Amersham Pharmacia Biotech, Piscataway, NJ) was used as the molecular weight standards. Proteins were electrically transferred to PVDF membranes and blocked for 2 h at 25°C with 5% non-fat dry milk in wash buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20). Blots were subsequently incubated with rabbit anti-IFNγR1 antibody (1:1000) or mouse anti-p21 antibody (1:200) for 18 h at 4°C. Immunoreactive proteins were detected after 1 h incubation with horseradish peroxidase-conjugated goat-anti rabbit IgG (1:3000) or with horseradish peroxidase-conjugated goat-anti mouse IgG (1:3000) at 25°C. The bands corresponding to IFNγR1 were visualized using ECL reagent (Perkin Elmer Life Sciences, Boston, MA) and exposure to BioMax MR film (Kodak, Rochester, NY). Equal loading was ascertained by Ponceau S staining of blotted membranes, and also by probing the membranes with mouse anti-tubulin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, 1:10 000). For detection of p-STAT1 by western blot analysis, cells were grown and treated in serum-free medium with or without 200 μM I3C for 48 h. Before harvesting, the cells were pulsed for 0, 15, 30, 60 and 90 min with 100 ng/ml IFNγ. The lysates were resolved by 8% SDS–PAGE and processed for western blot as described above, using the p-STAT1 antibody (Cell Signaling, Beverly, MA, 1:1000). STAT1 antibody (1:1000) was used as a loading control.

RNA extraction and RT–PCR

After treatment with DMSO, 200 μM I3C and 1 μg/ml actinomycin D, cells were lysed by the addition of Tri-reagent (Molecular Research Center, Cincinnati, OH) and chloroform was used for phase separation. The aqueous upper phase was collected and total RNA was precipitated by isopropanol, washed with 75% ethanol, and dissolved in DEPC treated water. Ten micrograms of total RNA was used to synthesize cDNA using a 15 mer oligo-dT primer (Promega, Madison, WI) and reverse transcriptase. For PCR amplification, 5 μl of the cDNA reaction product was used with 20 pM IFNγR1-specific primers (Upper-5′ CCAGGCATGCATACCGAAGACA 3′, Lower-5′ GCGATGCTGCCAGGTTCAGA 3′) and amplified for 24 cycles (95°C, 1 min/55°C, 2.5 min/72°C, 2 min. Primers for GAPDH were amplified under similar conditions and served as the loading control. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and photographed under UV. For p21 transcript detection, MCF-7 cells were treated with DMSO, 200 μM I3C, 100 ng/ml IFNγ and a combination of I3C and IFNγ. PCR using primers specific to p21 (Upper-5′ CCC GTG AGC GAT GGA ACT TC 3′, Lower-5′ CTG AGA GTC TCC AGG TCC AC 3′) was carried out as described above.

Transfection and luciferase assay

The luciferase reporter plasmids containing the IFNγR1 promoter and the 5′ deletion constructs were transiently transfected in MCF-7 cells using Fugene transfection reagent (Gibco, Carlsbad, CA). Fugene reagent (3 μl) and 1 μg DNA were mixed in plain DMEM medium, added to cells and incubated for 18 h at 37°C before treatment commenced. After treatment with DMSO, 200 μM I3C and 1 μg/ml actinomycin D for 24 h, cells were harvested by washing in PBS and lysed in 1× Reporter Lysis Buffer (Promega). Ten microliters of the cell lysate was added to 12 × 75 mm cuvettes and subsequently loaded into a luminometer (LUMAT LB 9507, EG&G Berthold, Germany). 100 μl of luciferase substrate [20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_25H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM D-luciferin sodium salt, 530 μM ATP disodium salt, pH 7.8] was injected automatically into each sample and luminescence was measured in relative light units. The luciferase specific activity was expressed as an average of relative light units produced per microgram of protein present in the corresponding cell lysates, as measured by the Lowry Assay (Bio-Rad, Hercules, CA).

Flow cytometry

MCF-7 cells were plated at 40 000 cells/well of 6-well tissue culture dishes and treated for 24, 48, 72, 96, 120, 144 and 168 h in complete medium. I3C was added to a final concentration of 200 μM and IFNγ to a final concentration of 100 ng/ml. Medium was changed every 24 h. Following treatment, cells were washed with phosphate-buffered saline and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). Cell debris was removed by filtration through 60-mm nylon mesh (Sefar America, Kansas City, MO). Nuclear-emitted fluorescence with wavelengths of 585 nm was measured with a Coulter, XL MCL instrument (Miami, FL). 10 000 nuclei were analyzed from each sample at a rate of 300–500 nuclei/s. The percentages of cells within the G_1, S and G_2/M phases of the cell cycle were determined by analysis with the WINCYCLE software (Phoenix Flow Systems, CA) in the Cancer Research Laboratory Flow Cytometry Facility of the University of California at Berkeley.

Quantification of autoradiography

Autoradiographic exposures were scanned with a Microtek ScanMaker 4 scanner, and band intensities were quantified using the NIH Image program. Autoradiographs from a minimum of three independent experiments were scanned per time point. The statistical differences between groups were determined using ANOVA and Student's *t*-test. The levels of significance are noted at level of $P < 0.05$. The results are expressed as means ± SE for at least three replicate determinations for each assay.

Results

I3C stimulates expression of IFNγR1 transcripts and protein in MCF-7 human breast cancer cells

Identification of I3C regulated gene expression changes between 48 h I3C treated and untreated human MCF-7 breast cancer cells was initially accomplished by cDNA microarrays using Incyte gene chips containing 960 selected cDNAs

representing human genes involved in cell cycling, apoptosis, signal transduction, motility, adhesion and angiogenesis. Expression profiling analysis revealed that I3C induced or repressed expression by at least 1.5–2.0-fold of several genes involved in signaling by the IFN family of cytokines. In particular, I3C up-regulated expression of IFNγR1, IFN receptor 2 and IFN-induced p56 whereas, this indole down-regulated expression of IFNβ, IFN-related developmental regulator-2 and 2′-5′-oligoadenylate synthetase-2. The I3C stimulation of IFNγR1 expression was the most significant of all of the gene array results, and therefore examined in more detail. To confirm the microarray results and examine in more depth the I3C regulation of IFNγR1 expression, MCF-7 cells were treated with or without 200 μM I3C for 6 h in the presence or absence of actinomycin D, an inhibitor of transcription. Expression of IFNγR1 transcripts was determined by RT–PCR. This optimal dose of I3C had shown previously significant growth-inhibitory effects in MCF-7 cells without any effects on cell viability (21). Oligonucleotide primers specific to the coding region of the IFNγR1 were used to detect the corresponding transcripts, whereas, GAPDH oligonucleotide primers were used as a loading control. As shown in Figure 1A, I3C strongly and rapidly induced IFNγR1 transcripts by a process that is rapid and dependent upon *de novo* RNA synthesis. The relatively short 6 h time point is well before steady state increase in transcript levels (data not shown). Actinomycin D treatment lowered the basal levels of IFNγR1 transcripts under conditions in which the level of GAPDH transcripts remained unaltered, suggesting that IFNγR1 transcripts have a relatively short half-life. These results are consistent with the I3C signaling pathway inducing transcription of the IFNγR1 gene, as determined by microarray analysis of I3C-treated versus untreated MCF-7 cells.

Western blot analysis of MCF-7 cells treated throughout a 72 h time course with or without 200 μM I3C revealed that the I3C stimulation of IFNγR1 transcripts results in a corresponding increase in the level of IFNγR1 protein (Figure 1B). A significant increase in IFNγR1 protein was observed after 24 h treatment with I3C, and by 72 h of indole treatment IFNγR1 protein was induced ~7-fold compared with DMSO-treated control cells. Under these conditions, there was virtually no change in tubulin production, which was used as the loading control.

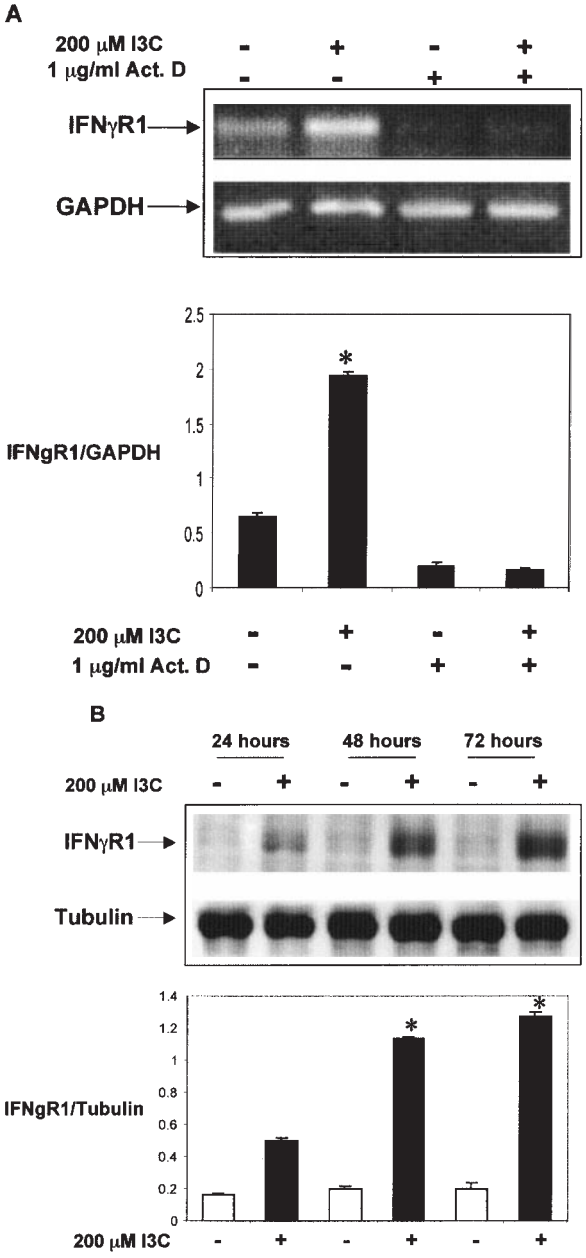

Fig. 1. I3C stimulation of IFNγR1 gene expression. (**A**) MCF-7 cells were treated with DMSO or 200 μM I3C for 48 h, with or without actinomycin D. RT–PCR was carried out using primers specific to the IFNγR1 or GAPDH, which served as a loading control. The PCR products were visualized by gel electrophoresis on a 1% agarose gel. *Significant difference with DMSO control as determined with Student's *t*-test with a $P \leq 0.05$. (**B**) MCF-7 cells were treated with or without 200 μM I3C for 24, 48 and 72 h. Cell lysates were fractionated by electrophoresis and transferred on a PVDF membrane. Blots were probed with anti-IFNγR1 antibody or tubulin antibody and the proteins were visualized using the enhanced chemiluminescence reagent. *Significant difference with DMSO control as determined with Student's *t*-test with a $P \leq 0.05$.

I3C activation of the IFNγR1-promoter activity and detection of an I3C-responsive region within the IFNγR1 gene promoter

The cloning and characterization of the IFNγR1 promoter has been reported previously (30). A series of IFNγR1 promoter-luciferase reporter plasmid constructs were used to determine whether or not the I3C stimulation of IFNγR1 transcripts is mediated by activation of the IFNγR1 gene promoter, and to functionally identify the *cis*-acting region of the IFNγR1 promoter that confers I3C transcriptional responsiveness. Serial 5′ deletions of the promoter were utilized (30) that start at −840, −540, −240, −160 and −128 bp of the IFNγR1 promoter, and which each end at +28 bp of IFNγR1 gene. Each of the IFNγR1-luciferase reporter plasmids were transiently transfected into MCF-7 cells, and cells were then treated with or without 200 μM I3C for 24 h. As illustrated in Figure 2, I3C up regulated the −840 bp IFNγR1-luciferase reporter plasmid, which implicates that I3C treatment stimulates IFNγR1 promoter activity. Treatment of the transfected cells with actinomycin D inhibited the I3C mediated activation of the IFNγR1 promoter (data not shown), which provided a control for the baseline transcriptional activity of the IFNγR1 promoter.

Transient transfection of the other IFNγR1 promoter-luciferase reporter plasmids revealed that the constructs containing the −840 and −540 bp promoter deletions remained

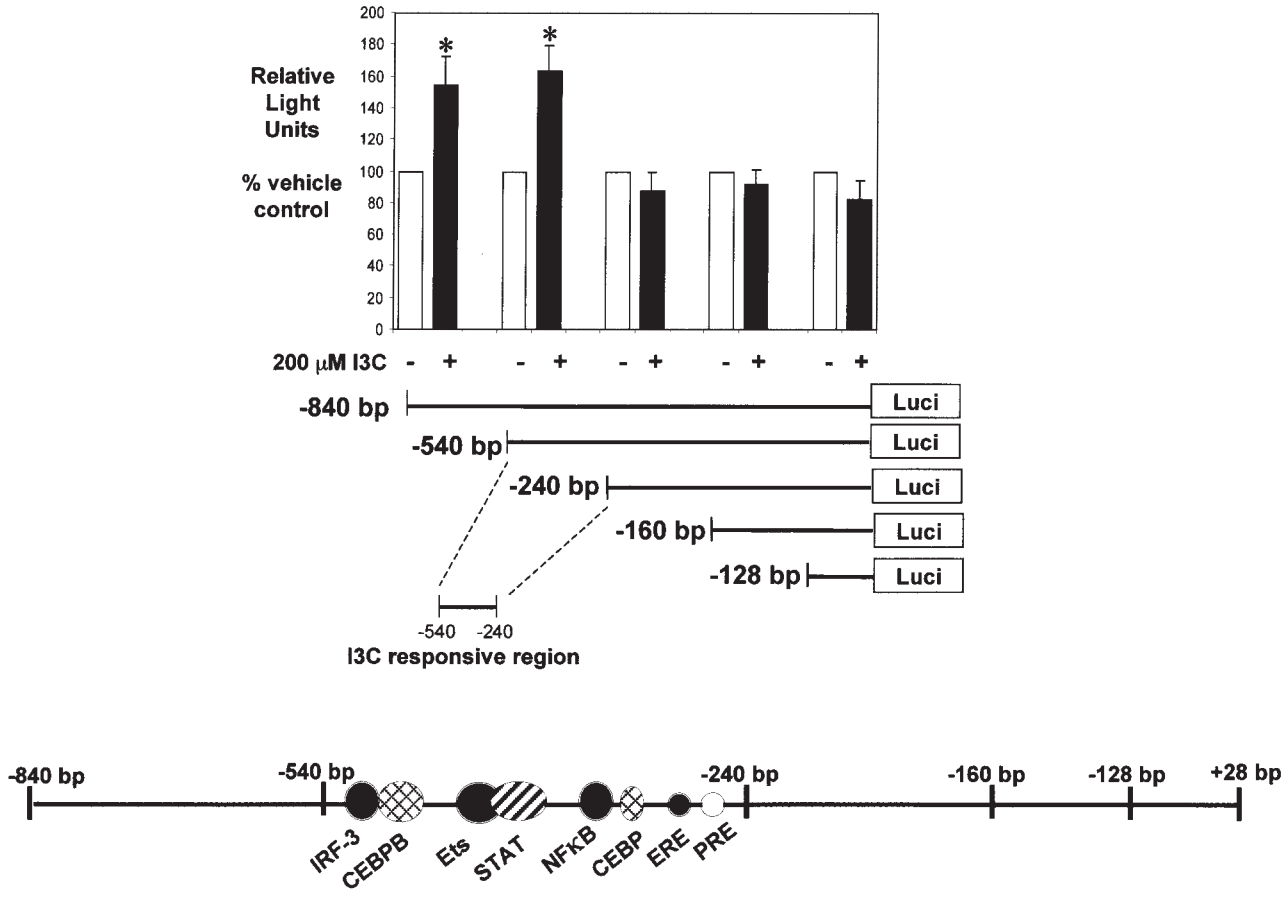

Fig. 2. Deletion analysis of the IFNγR1 promoter defines a 300 bp I3C-responsive region. MCF-7 cells were transiently transfected with the −840 bp IFNγR1-luciferase reporter plasmid and the 5′ deletion constructs, including −540, −240, −160 and −128 bp. Cells were treated with or without 200 μM I3C or 1 μg/ml actinomycin D for 48 h. The cells were harvested and assayed for luciferase activity. The luciferase-specific activity was determined as luciferase activity produced per microgram of protein present in the corresponding cell lysates relative to the vehicle control. The error bars represent the standard deviation and * indicates significant difference with DMSO control as determined with Student's *t*-test with a $P \leq 0.05$. The lower panel is a schematic representation of the IFNγR1 gene promoter, with potential transcription factor-binding sites within the I3C-responsive region, indicated by ovals and circles.

I3C responsive (Figure 2). In contrast, activity of the reporter plasmids containing the −240, −160 and the −128 bp IFNγR1 promoter fragments failed to be stimulated by I3C treatment. These results suggest that an I3C-responsive region can be localized within the 300 bp sequence located within the −540 to −240 bp region of the IFNγR1 promoter. Theoretical analysis of consensus transcription factor-binding sites by the TRANSFAC database search with MatInspector (31) revealed that the 300 bp I3C-responsive region of the IFNγR1 promoter contains DNA elements for the C/EBP binding site (324–342), NFκB (329–343), STAT (365–383), Ets family member FLI (389–405), CCAAT/enhancer binding protein beta (504–522) and the IFN regulatory factor 3 (523–537) transcription factors (Figure 2, lower panel). Interestingly, the presence of an Ets family member binding site, located between 389 and 405 bp, is generally consistent with our previous characterization of the transcriptional response to I3C in human breast cancer cells (21).

Pre-treatment with I3C increases IFNγ-induced phosphorylation of STAT1 at Tyr-701 without affecting the production of STAT1 protein levels

The I3C stimulation of IFNγ receptor expression predicts that I3C treatment should enhance IFNγ responsiveness in MCF-7 breast cancer cells. An early and critical IFNγR1 signaling event is the IFN mediated phosphorylation of STAT1 (signaling transducer and activator of transcription 1, consisting of Stat1α, a 91 kDa isoform and Stat1β, an 84 kDa splice variant) by the receptor-associated JAK kinases, which recruit and phosphorylate the STAT proteins (32). Phosphorylated STAT1 dimerizes, translocates to the nucleus and leads to transcription of specific target genes (33,34). Thus, phosphorylation of STAT1 is essential for induction of IFNγ-mediated biological response. Immunoblotting experiments with phospho-specific antibodies that recognize Tyr-701 phospho-STAT1 were carried out to examine the effect of I3C pre-treatment on phosphorylation at this residue. MCF-7 cells were pre-incubated with 200 μM I3C for 48 h in serum-free medium and then stimulated with 100 ng/ml IFNγ for 0, 15, 30, 60 and 90 min. Under these 48 h pre-treatment conditions, I3C has induced IFNγR1 gene productions to near maximal levels. The dose of IFNγ was shown in an earlier study to effectively suppress the growth of MCF-7 cells (35). Cell lysates were fractionated and immunoblotted with either the phospho-specific antibodies or with anti-STAT1 antibodies to detect total STAT-1 protein. STAT1α or STATβ was not phosphorylated when the cells were not stimulated with IFNγ (Figure 3, 0′ lanes), even when they were treated with I3C. Interestingly, the IFN-mediated phosphorylation on Tyr-701 of STAT1β was significantly increased compared with STAT1α in

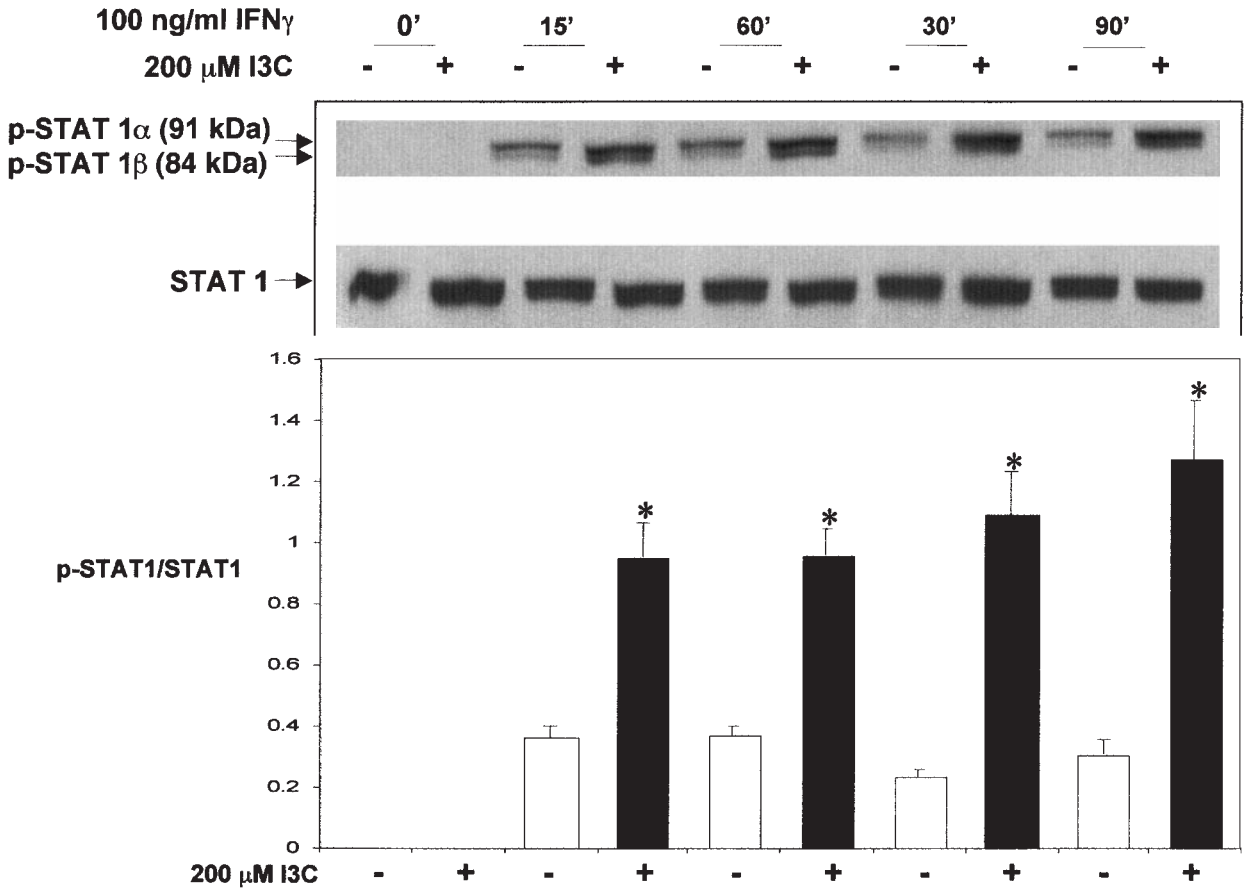

Fig. 3. Effect of I3C and IFNγ on the expression of STAT1 and phosphorylation on Tyr-701 of STAT1 in MCF-7 cells. MCF-7 cells were untreated (lanes 1, 3, 5, 7 and 9) or treated with 200 μM I3C for 48 h (lanes 2, 4, 6, 8 and 10) and then incubated with IFNγ (100 ng/ml) for varying time periods (0–90 min). Cell lysates (20 μg) were subjected to SDS–polyacrylamide gel electrophoresis and transferred onto a membrane. The membrane was incubated with an antibody against phospho-STAT1 (Tyr-701), or STAT1 as described under Materials and methods, and the proteins were visualized by enhanced chemiluminescence.
*Significant difference with DMSO control as determined with Student's *t*-test with a $P \leq 0.05$.

MCF-7 cells that were pre-treated with I3C and subsequently stimulated by IFNγ, compared with cells treated only with IFNγ (Figure 3, 15′, 30′, 60′ and 90′ – versus + lanes). This observation indicates that although I3C alone is unable to phosphorylate STAT1 at Tyr-701 residue, I3C pre-treatment significantly increases the magnitude of IFNγ-induced phosphorylation of STAT1. In contrast to the phosphorylated form of STAT1, lysates that were immunoblotted with an anti-STAT1 antibody did not show any significant differences in total STAT1 protein levels in I3C-pre-treated or untreated cells (Figure 3, lower panel).

The cooperative anti-proliferative effects of I3C and IFNγ in MCF-7 breast cancer cells

To test whether the anti-proliferative effects of I3C and IFNγ are enhanced when the breast cancer cells are exposed to both stimuli. MCF-7 cells were plated in 6-well tissue culture plates and treated daily with DMSO, 200 μM I3C, 100 ng/ml IFNγ, or a combination of I3C and IFNγ for up to 7 days. The cell culture media was changed every 24 h. At the indicated time points, the cells were hypotonically lysed in the presence of propidium iodide to stain the nuclear DNA. Flow cytometry profiles of nuclear DNA content revealed a significant time-dependent G_1 cell cycle arrest of cells treated with either I3C or IFNγ alone. As shown in Figure 4 (left panel), typical of growing MCF-7 cells, in the presence of DMSO 55.6% of cells displayed a G_1 DNA content, 33.6% of cells were in S phase, while 10.8% of cells contained a G_2/M DNA content. By 96 h treatment with either I3C or IFNγ, the growth-arrested cells were shifted to a significantly higher G_1 DNA content and cell number and reduced S phase DNA content with a decrease in cell number. A combination of I3C and IFNγ resulted in a significant enhancement of the number of breast cancer cells arrested in the G_1 phase of the cell cycle (93.2%), and loss of S phase cells (2.2%), accompanied by minor changes in G_2/M DNA content. The graph in the right panel in Figure 4 shows the overall time course of G_1 phase cells observed in breast cancer cells treated with combinations of I3C and IFNγ. The results appear to be additive and not synergistic, suggesting that I3C and IFNγ act by different mechanisms to block the cell cycle of MCF-7 cells.

Synergistic effects of I3C and IFNγ on expression of the p21 CDK inhibitory molecule

I3C regulates the expression of CDK6 and strongly inhibits CDK2 enzymatic activity (19–21). Thus, one potential mechanism by which I3C and IFNγ cooperatively induce a G_1 cell cycle arrest is the corresponding regulation of G_1-related cell cycle components. Here we show that co-treatment of I3C and IFNγ leads to an additive effect on the up regulation of p21 protein and transcript levels. Western blots revealed relatively small increases in p21 protein, in cells individually

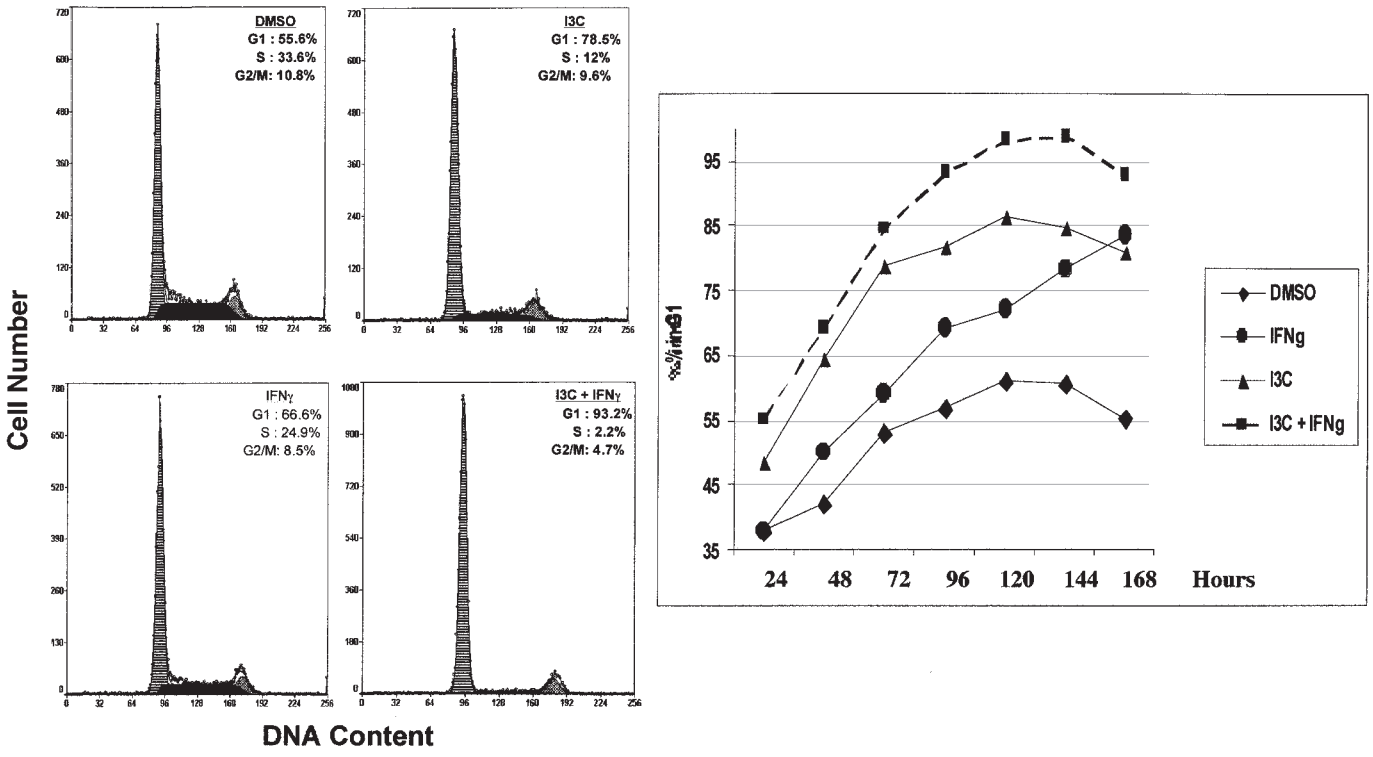

Fig. 4. Effects of a combination of I3C and IFNγ on DNA content of MCF-7 cells. MCF-7 cells were treated with DMSO, 200 μM I3C, 100 ng/ml IFNγ or a combination of I3C and IFNγ for 7 days. Cells were then lysed and stained with propidium iodide and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite Laser. The percentages of cells within the G_1 phase of the cell cycle are represented (right panel) and representative profiles of each condition at 96 h with percentages of cells in G_1, S and G_2/M are shown in the left panel.

treated with either I3C or IFNγ, whereas exposure to both stimuli caused a stronger induction of p21 protein (Figure 5, upper panel). Tubulin protein levels, which were used as the loading control, remained unaltered under each tested condition. RT–PCR analysis revealed that the cooperative effects of I3C and IFNγ on p21 production could be accounted for by corresponding changes in p21 transcript levels (Figure 5, lower panel). Parallel amplification of GAPDH transcripts was used as a loading control for this experiment.

Discussion

I3C is a promising chemotherapeutic agent for treatment of breast cancer because of its potent growth-inhibitory effects in both estrogen receptor positive and negative breast cancer cell lines (19,20,27). It has been suggested that I3C treatment may activate or repress multiple signal transduction pathways (27,28). A microarray analysis of I3C treated versus untreated breast cancer cells was used to uncover additional cellular anti-proliferative pathways that are activated by I3C. In this study, we demonstrate that in MCF-7 breast cancer cells, the IFNγR1 was up regulated as a result of I3C treatment. In addition to up regulating the expression of the IFNγR1 and augmenting the anti-proliferative effects of IFNγ, I3C by itself has potent cell cycle effects (19,20). IFNγ has been known to exhibit profound growth-inhibitory and apoptotic effects in human breast cancer (36), suggesting that IFNγ-activated pathways may play a key role in the anti-proliferative response to I3C. Our results have established that the anti-proliferative cascades initiated by I3C and IFNγ can cooperate to induce more stringent growth suppression in breast cancer cells than either agent alone. We propose that combined I3C and IFNγ-activated responses are mediated by converging signal transduction pathways, in which I3C enhances the IFNγ signaling pathway and renders the cells more responsive to the anti-proliferative effects of IFNγ. In a complementary pathway, I3C induces a G_1 cell cycle arrest by regulating cell cycle gene expression and function (Figure 6). I3C treatment can lead to apoptosis (37,38) and in human malignant T cells, drug-induced apoptosis is characterized by up regulation of the IFNγR1 expression (39). Hence, over-expression of the IFNγR1 could potentially be used to clinical advantage for treatment of breast cancer in humans through combination of the chemotherapeutic drug I3C, inhibiting cell proliferation and up-modulating IFNγR1 expression, followed by IFNγ administration.

IFNs are potent anti-proliferative cytokines that play an important role in immune response, apoptosis and antitumor activity (40). IFNγ is a product of activated T lymphocytes (41), and is used to treat various cancers (42). IFNγ can mediate potent anti-proliferative actions in epithelial tumors (43) and in cultured human cancer cells (44,45) including human breast cancer cells (36). At the cellular level, IFNγ causes cell growth arrest at the G_1 phase of the cell cycle (46,48). The biological activity of IFNγ is mediated via specific cell surface transmembrane receptors, which are internalized and degraded after binding to the ligand (42). The vast majority of human tumor cells derived from various tissue origins were found to express specific membrane receptors

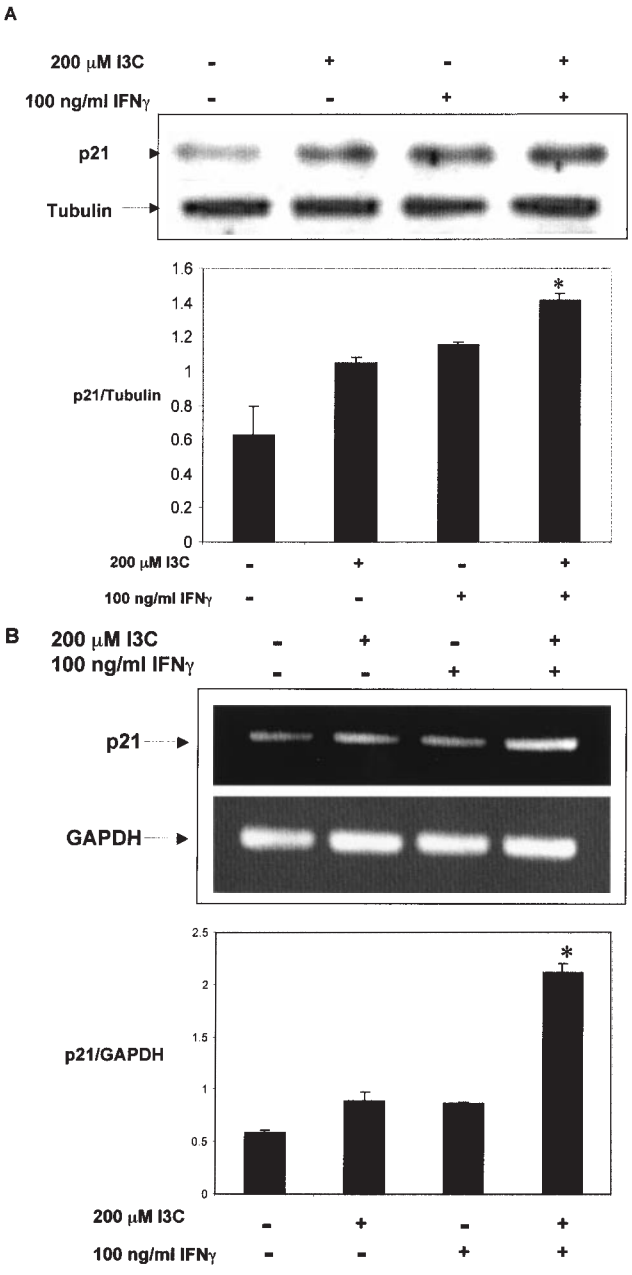

Fig. 5. I3C and IFNγ regulation of p21 gene expression in MCF-7 cells. (**A**) MCF-7 cells were treated with DMSO, 200 μM I3C, 100 ng/ml IFNγ or a combination of I3C and IFNγ for 96 h. Total cell extracts were electrophoretically fractionated in SDS–polyacrylamide gels. Western blots were probed with primary antibodies to $p21^{Waf1/Cip1}$ and tubulin, which served as a loading control. Immunoreactive products were visualized by enhanced chemiluminescence. *Significant difference with DMSO control as determined with Student's *t*-test with a $P \leq 0.05$. (**B**) MCF-7 cells were treated with DMSO, 200 μM I3C, 100 ng/ml IFNγ or with a combination of I3C and IFNγ. RT–PCR was carried out using primers specific to the $p21^{Waf1/Cip1}$ or GAPDH, which served as a loading control. The PCR products were visualized by 1% agarose gel electrophoresis. *Significant difference with DMSO control as determined with Student's *t*-test with a $P \leq 0.05$.

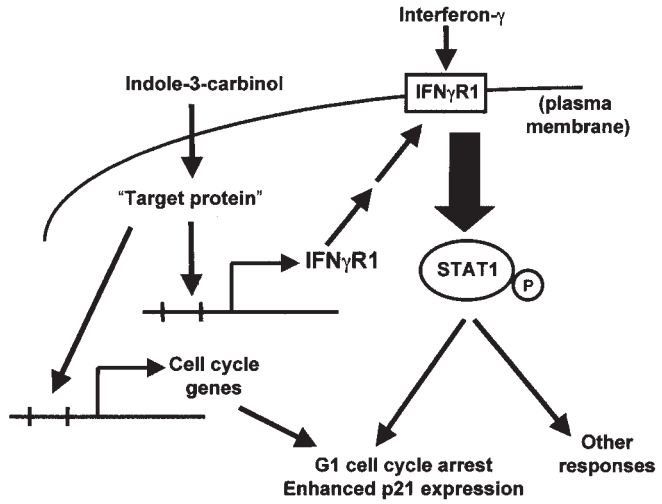

Fig. 6. Model depicting the anti-proliferative effects of I3C, which enhances IFNγ responsiveness in human breast cancer cells. I3C potentially mediates its effects through a putative cellular 'target protein' and stimulates the expression of IFNγR1, in addition to stimulating or repressing different cell cycle genes. Increased IFNγR1 renders the cells more responsive to the growth-inhibitory effects of IFNγ. I3C also increases IFNγ-induced phosphorylation of STAT1, a major event in IFNγ signaling. I3C and IFNγ signaling pathways eventually converge to induce more effective G_1 cell cycle arrest and enhance up regulation of the CDK inhibitor $p21^{Waf1/Cip1}$ levels in MCF-7 breast cancer cells.

for IFNγ (49–53). The contrasting ability of IFNγ to either stimulate the proliferation of malignant T cells or to induce their apoptosis, is determined by the low and high intensity of the IFNγ receptor expression, respectively (39). In addition, chemical carcinogen-induced tumor development increases in IFNγR deficient mice (54) or IFNγR knockout mice (33), emphasizing the importance of expression of the IFNγR in the prevention of cancer. However, when IFNγ signaling was restored in the tumor by expressing the IFNγR1, the tumor was rejected, since expression of the IFNγR on tumors renders them susceptible to the anti-proliferative effects of IFNγ. Hence, the current observations strongly implicate the importance of the expression of the IFNγR1 for the prevention of tumor development.

In this study, we have demonstrated by western blot and RT–PCR that I3C significantly up regulates expression of IFNγR1. This response to I3C is due to the activation of the IFNγR1 promoter and has established a direct link between I3C signaling and the control of IFNγR1 expression in MCF-7 cells. Deletion analysis revealed an I3C-responsive region between −540 and −240 bp in the IFNγR1 promoter. Within this region, there are a variety of consensus transcription factor DNA sites such as STAT, C/EBP, Ets family member FL1, IRF3, NFκB, PRE and ERE, any one of which could be a possible target for I3C signaling. I3C down regulates CDK6 promoter activity through a Sp1/Ets composite binding site (21), suggesting the potential importance of the Ets binding site within the I3C-responsive region of the IFNγR1 promoter. We are currently characterizing this region of the IFNγR1 promoter.

STAT1 phosphorylation by Jak on a single tyrosine residue (Tyr-701) is an important downstream event in the IFNγ signaling pathway (30,55,56). We have shown that I3C pretreatment leads to enhanced phosphorylation/activation of STAT1β by IFNγ in MCF-7 cells. Conceivably, this effect may be due to I3C prolonging the tyrosine kinase effects or repressing a phosphatase. In contrast, we do not observe major differences in phosphorylation of the STAT1α isoform. Earlier reports have established that STAT1α, but not STAT1β, is responsible for IFNγ-induced responsiveness (57). Hence,

I3C-mediated STAT1β phosphorylation suggests a distinct difference in signaling pathway between I3C and IFNγ. Interestingly, the anti-breast cancer drug tamoxifen is also known to increase the STAT1β isoform (58). Taken together, these data suggest that I3C mediates its anti-proliferative effects in part by enhanced activation of the IFNγ-induced STAT signaling pathway.

Although it is well established that I3C and IFNγ suppress the growth of certain types of tumor cells individually (19,59,60), combinations of I3C or IFNγ with other agents are known to produce significant additive or synergistic antitumor activities. For example, the growth-inhibitory effects of I3C are increased in combination with the polyamine putrescine in a colon tumor cell line (61). The growth-inhibitory activity of IFNs in different tumor types demonstrate that in solid tumors, IFNs lack single-agent activity (62,63). In addition, a combination of IFNγ and tamoxifen caused synergistic growth inhibition of human mammary xenografts in athymic nude mice (64), indicating the enhanced efficacy of combined treatment. Our results show that in addition to the I3C-induced increased expression of IFNγR1, I3C and IFNγ cooperate to exert significant growth-inhibitory effects in human breast cancer cells. I3C and IFNγ independently induce $p21^{Waf1/Cip1}$ CDK inhibitor protein levels in MCF-7 cells (19,65). When I3C and IFNγ are administered together, we observe an enhanced expression of both protein and transcript levels of $p21^{Waf1/Cip1}$. One of the primary events of the IFNγ signaling pathway is activation of STAT1. Recent studies have shown that $p21^{Waf1/Cip1}$ expression can be induced through activation of STAT signal transduction pathway (56). STAT proteins recognize and bind to the palindromic sequence TTCNNNGAA (66) and such sequences have been identified in the p21 promoter region. Activation of STAT1 in response to IFNγ correlates with up regulation of $p21^{Waf1/Cip1}$ expression and inhibition of cell growth in a number of cell types (67,68). In addition, hypermethylation at STAT1-binding sites in the p21 promoter region inhibits IFNγ signaling pathway (69). We are investigating the role of the STAT binding site in I3C stimulation of the IFNγR1 promoter, in order to unravel the precise mechanism of I3C regulation of the IFNγR1 promoter.

The oral administration of I3C, such as from dietary sources, has promising chemopreventive properties (16), although the precise concentration of indole that enters the tissues is not established. In addition, I3C is converted into several dimeric and trimeric acid catalyzed products in the low pH environment of the stomach (17,18). The minimum effective dose of orally administered I3C for breast cancer prevention is 300 mg/day in a capsule (70). Our previous studies have demonstrated that exposure of human breast cancer cells to 200 μM I3C is optimal for the growth-inhibition response without any effects on cell viability (19,20,27). We have shown that in cell lines treated with [^{3}H]I3C only ~0.3% of the extracellular indole enters the cells (27,71). Thus, the effective concentration of I3C that can induce a cell cycle arrest of human breast cancer cells is closer to 300–600 nM indole. Our current studies suggest that the direct exposure of reproductive tumors with I3C, and not the oral administration of I3C, will likely result in the type of response observed with cultured breast cancer cells, including the enhancement of IFN signaling. We are currently attempting to determine the effects of I3C treatment on IFN responsiveness in a physiological context.

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