ABSTRACT

The current options for treating breast cancer are limited to excision surgery, general chemotherapy, radiation therapy, and, in a minority of breast cancers that rely on estrogen for their growth, antiestrogen therapy. The naturally occurring chemical indole-3-carbinol (I3C), found in vegetables of the Brassica genus, is a promising anticancer agent that we have shown previously to induce a G1 cell cycle arrest of human breast cancer cell lines, independent of estrogen receptor signaling. Combinations of I3C and the antiestrogen tamoxifen cooperatively inhibit the growth of the estrogen-dependent human MCF-7 breast cancer cell line more effectively than either agent alone. The more stringent growth arrest was demonstrated by a decrease in adherent and anchorage-independent growth, reduced DNA synthesis, and a shift into the G1 phase of the cell cycle. A combination of I3C and tamoxifen also caused a more pronounced decrease in cyclin-dependent kinase (CDK) 2-specific enzymatic activity than either compound alone but had no effect on CDK2 protein expression. Importantly, treatment with I3C and tamoxifen ablated expression of the phosphorylated retinoblastoma protein (Rb), an antiproliferative pathway are unknown and an understanding of the mechanism of I3C action in human breast cancer cells could potentially lead to a novel approach to control breast cancer. Currently, the only specific therapy for breast cancer is antiestrogen treatment, and this treatment is only effective on tumors that rely on estrogen for their growth. Because I3C has been shown to suppress the growth of both estrogen-dependent and estrogen-independent human breast cancer cell lines (6), a combination of these two growth suppressors may potentially provide beneficial effects for breast cancer patients.

Tamoxifen has been a clinically useful antiestrogen for breast cancer patients for >20 years (7–9). Tamoxifen, a nonsteroidal triphenyl ethylene, can adopt a structural conformation that resembles steroids in the nucleus and act as a competitive inhibitor of E2 binding to the estrogen receptor (10, 11). Although tamoxifen generally acts as an estrogen receptor antagonist in breast cancer cells, in certain other cell types, tamoxifen can act as an estrogen receptor agonist (reviewed in Ref. 12). Several mechanisms are proposed for the modulation of breast cancer cell proliferation by tamoxifen, including down-regulation of oncogenes, modulation of growth factor signaling, and regulation of the cell cycle machinery (13–15). Regulated changes in the expression and/or activity of cell cycle components that act within G1 have been closely associated with alterations in the proliferation rate of normal and transformed mammary epithelial cells (16). Regulation of these events by both antiproliferative and proliferative extracellular signals is well documented. For example, progesterone inhibition of breast cancer cell growth has been linked to the inactivation of G1 CDKs by modulating the components of the CDK complex (17). Also, the estrogen-induced activation of CDK4 and CDK2 during progression of human breast cancer cells between the G1 and S phases is accompanied by the increased expression of cyclin D1 and decreased association of the CDK inhibitors with the cyclin E-CDK2 complex (18).

The sequential activation of CDKs and subsequent phosphorylation of specific substrates govern progress through the cell cycle on multiple levels. CDKs are inactive in the absence of cyclin binding; therefore, cyclin abundance is a major determinant of cyclin-CDK activity (19–22). Each cyclin is typically present for only a restricted portion of the cell cycle, and alterations in cyclin abundance are sufficient to alter the rate of cell cycle progression (19, 21, 22). CDK activity is also regulated by a network of kinases and phosphatases (reviewed in Ref. 20), which can either activate or inactivate the complex. A further level of control results from the actions of two families of specific CDK-inhibitory proteins. Members of the p16INK4 family specifically target the kinases that associate with the D-type cyclins, CDK4 and CDK6 (21, 23, 24). Members of the p21 (WAF1, Cip1) family interact with a broader range of CDKs, including CDK2, CDK4, and CDK6 (25, 26). One of the key endogenous substrates of the G1 CDKs is the retinoblastoma protein (Rb). Its phosphorylation is an important step in the transition between the G1 and S phases of the cell cycle because, when sufficiently phosphory-
rlylated, Rb releases a transcription factor of the E2F family that drives cells into S phase (27).

Tamoxifen has been shown to decrease the activity of the estrogen receptor, and it does not have an antiproliferative effect on estrogen receptor-negative cell lines. In contrast, I3C can suppress the growth of cells regardless of estrogen receptor status (6). Taking into account the known features of the growth suppression cascades induced by I3C or tamoxifen, we tested the combinatorial effects of these two breast cancer cell growth suppressors in estrogen-responsive MCF-7 cells. Most significantly, a combination of tamoxifen and I3C displayed a more effective growth suppression response, a more stringent inhibition of CDK2 specific activity, and more endogenous Rb phosphorylation than either compound alone. Our results suggest the possibility of developing I3C and tamoxifen as a potential combinatorial therapy to control estrogen-responsive breast cancers.

MATERIALS AND METHODS

Materials. DMEM, FBS, calcium- and magnesium-free PBS, l-glutamine, and trypsin-EDTA were supplied by BioWhittaker (Walkersville, MD). Insulin (bovine) and tamoxifen (IZ-1-[\(\beta\]-diphenylaminoethoxyphenyl]-1,2-diphenyl-1-butene) citrate salt were obtained from Sigma Chemical Co. (St. Louis, MO). \([^{3}H]\)Thymidine (84 Ci/mmole) and \([\gamma^{32}P]ATP\) (3,000 Ci/mmole) were obtained from NEN Life Science Products (Boston, MA). I3C was purchased from Aldrich (Milwaukee, WI). I3C was recrystallized in hot toluene prior to use. The sources of other reagents used in the study are listed in the lowest below or were of the highest purity available.

Cell Culture. The MCF-7 human breast adenocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were grown in DMEM supplemented with 10% FBS, 10 \(\mu\)g/ml insulin, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 \(\mu\)l/ml-glutamine and maintained at subconfluency at 37°C in humidified air containing 5% CO2. I3C was obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in growth medium plated onto Corning six-well tissue culture dishes. Triplicate samples were treated with the indicated concentrations of I3C and tamoxifen. The medium was changed every 24 h. Cells were incubated for 96 h and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, and 0.05% Triton X-100). Nuclear emitted fluorescence with wave-length of \(>585\) nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Nuclei (10,000) were analyzed from each sample at a rate of 300-500 nuclei/s. 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 provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

Western Blot Analysis. After the indicated treatments, cells were harvested in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% NP40, 0.1% SDS, and 50 mM Tris) containing protease and phosphatase inhibitors (50 \(\mu\)g/ml phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml aprotinin, 5 \(\mu\)g/ml leupeptin, 0.1 \(\mu\)g/ml NaF, and 10 \(\mu\)g/ml \(\beta\)-glycerophosphate). Equal amounts of total cellular protein were mixed with loading buffer [25% glycerol, 0.075% SDS, 1.25 mM of 14.4 \(\beta\)-mercaptoethanol, 10% bromphenol blue, 3.13% 0.5 M Tris, and 0.4% SDS (pH 6.8)] and fractionated on 10% (7.5% for Rb) polyacrylamide/0.1% SDS resolving gels by electrophoresis. Rainbow marker (Amersham Life Sciences, Arlington Heights, IL) was used as the molecular weight standard. Proteins were electrically transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA) and blocked overnight at 4°C with Western wash buffer-5% NFDM [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20–3% nonfat dry milk]. Blots were subsequently incubated for 1 h at room temperature for rabbit anti-CDK2, CDK4, CDK6, p16, p21, and cyclin D1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and overnight at 4°C for mouse anti-Rb and cyclin E antibodies (PharMingen, San Diego, CA). The working concentration for all antibodies was 1 \(\mu\)g/ml in Western wash buffer. Immunoreactive proteins were detected after incubation with horseradish peroxidase-conjugated secondary antibody diluted to 3 \(\times\)10–4 in Western wash buffer-1% NFDM (goat antirabbit IgG (Bio-Rad, Hercules, CA); rabbit anti-mouse IgG (Zymed, San Francisco, CA)). Blots were treated with enhanced chemiluminescence reagents (NEN Life Science Products), and all proteins were detected by autoradiography. Equal protein loading was confirmed by Ponceau S staining of blotted membranes.

IP and CDK Kinase Assay. MCF-7 cells were cultured in growth medium with combinations of tamoxifen and I3C for the indicated times and then rinsed twice with PBS, harvested, and stored as dry pellets at –70°C. For the IP, cells were lysed for 15 min in IP buffer [50 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 0.1% Triton X-100] containing protease and phosphatase inhibitors (50 \(\mu\)g/ml phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml aprotinin, 5 \(\mu\)g/ml leupeptin, 0.1 \(\mu\)g/ml NaF, 10 \(\mu\)g/ml \(\beta\)-glycerophosphate, and 0.1 mM sodium orthovanadate). Samples were diluted to 500 \(\mu\)g of protein in 1 ml of IP buffer. Samples were precleared for 30 min at 4°C with 20 \(\mu\)l of a 1:1 slurry of protein A-Sepharose beads (Pharmacia Biotech, Sweden) in IP buffer and 1 \(\mu\)g of rabbit IgG. After a brief centrifugation to remove precleared beads, 0.5 \(\mu\)g of substrate was added to 4 \(\mu\)l of IP buffer. Samples were incubated for 1 h at 4°C with 6 \(\mu\)l of normal rabbit IgG or 6 \(\mu\)l of rabbit IgG.NovaZyme IgG (Zymed, San Francisco, CA) was used as a negative control. After 1 h of incubation at 4°C, samples were centrifuged for 5 min at 12,000 rpm, and supernatants were loaded onto 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Blots were treated with enhanced chemiluminescence reagents (NEN Life Science Products), and all proteins were detected by autoradiography. Equal protein loading was confirmed by Ponceau S staining of blotted membranes.

Crystal Violet Staining of Low Confluency Cultures. MCF-7 cells were plated onto 100-mm Corning tissue culture dishes (10,000 cells per plate). The cells were treated with the indicated concentrations of I3C and tamoxifen for 8 days. At the end of the treatment, the cells were washed with PBS and incubated in a solution of 0.5% crystal violet and 10% formalin for 10 min and then rinsed with water. The integrated density of the colonies on each plate was determined using NIH Image software.

Flow Cytometric Analyses of DNA Content. MCF-7 cells (4 \(\times\)104) were plated onto Corning six-well tissue culture dishes. Triplicate samples were treated with the indicated concentrations of I3C and tamoxifen. The medium was changed every 24 h. Cells were incubated for 96 h and hypotonically lysed in 1 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, and 0.05% Triton X-100). Nuclear emitted fluorescence with wave-length of \(>585\) nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Nuclei (10,000) were analyzed from each sample at a rate of 300–500 nuclei/s. 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 provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

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anti-CDK2 or anti-CDK6 antibody was added to each sample and incubated on a rocking platform at 4°C for 2 h. Then, 20 μl of protein A-Sepharose beads were added to each sample, and the slurries were incubated on the rocking platform at 4°C for 30 min. The beads were then washed five times with IP buffer and twice with kinase buffer (50 mM HEPES, 10 mM MgCl₂, 5 mM MnCl₂, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, and 0.1 mM sodium orthovanadate). Half of the immunoprecipitated sample was checked by Western blot analysis to confirm the IP.

For the kinase assay, the other half of the sample was resuspended in 25 μl of kinase buffer containing 20 mM ATP, 5 mM DTT, 0.21 mM of Rb COOH-terminal domain protein substrate (Santa Cruz Biotechnology), and 10 μCi of [γ-3P]ATP (3000 Ci/mmol). Reactions were incubated for 15 min at 30°C and stopped by the addition of an equal volume of 2× loading buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 6.25 mM Tris-HCl [pH 6.8], and bromphenol blue). Reaction products were boiled for 10 min and then electrophoretically fractionated in SDS-10% polyacrylamide gels. Gels were stained with Coomassie blue to monitor loading and destained overnight with 3% glycerol in 10% acetic acid. Subsequently, gels were dried and quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and visualized by autoradiography.

Quantitation of Autoradiography. Autoradiographic exposures were scanned with a UMAX UC630 scanner, and band intensities were quantified using the NIH Image program. Autoradiographs from a minimum of three independent experiments were scanned per time point.

RESULTS

I3C and Tamoxifen Cooperate to Arrest the Growth of MCF-7 Cells. To determine the potential combinatorial effects of I3C and tamoxifen on the growth of an estrogen-dependent breast cancer cell line, MCF-7 cells were grown in medium supplemented with 10% FBS, which contains enough estrogen to support the proliferation of these cells. The cells were treated with 100 μM I3C, 1 μM tamoxifen, or a combination of I3C and tamoxifen over a 96-h time course (Fig. 1). These concentrations of I3C and tamoxifen were previously shown to decrease cell growth without affecting viability (6, 29). The cells were then pulse-labeled with [3H]thymidine for 3 h at each time point to provide a measure of their proliferative state. Analysis of [3H]thymidine incorporation revealed that tamoxifen caused a steady decrease in DNA synthesis over the time course with a 60% inhibition after 96 h of treatment. I3C treatment also resulted in a time-dependent decrease in DNA synthesis with a 90% inhibition after 96 h. The combination of I3C and tamoxifen yielded statistically similar results as I3C alone for the 24- and 48-h time points. However, by the 72- and 96-h time points, the combination of I3C and tamoxifen resulted in a more effective growth suppression than either agent alone, resulting in a 95% inhibition after 96 h of treatment.

Because both I3C and the antiestrogen tamoxifen inhibited the growth of estrogen-responsive breast cancer cells, we wanted to determine whether I3C has any effect on estrogen receptor ligand binding. An in vitro competition binding assay for receptor-ligand interactions was used to examine the relative affinities of I3C and tamoxifen for the estrogen receptor. As a control and point of reference for the relative ligand affinity, unlabeled E2 was shown to effectively compete with [3H]E2 binding to the estrogen receptor, with half-maximal competition occurring at ~3 nM (Fig. 2). As expected, tamoxifen has a relatively high affinity for the estrogen receptor with a half maximal [3H]E2 displacement of ~200 nM. In contrast, I3C caused no significant displacement of [3H]E2 binding to the estrogen receptor, even at 1 μM. For the remainder of this study, the highest concentration of tamoxifen used was 1 μM, which is within the range of E2 competition, and the highest concentration of I3C used was 100 μM, which does not compete with E2 for receptor binding.

Effects of I3C and Tamoxifen on Anchorage-independent Cell Growth. To characterize the inhibitory effects of I3C and tamoxifen on adherent cell growth, MCF-7 cells were plated at low confluency (10,000 single cells per 100-mm plate) and grown for 8 days in medium containing the vehicle control or various doses of each agent alone or in combination. To visualize the cell colonies, the cells were stained and fixed in crystal violet/formaldehyde. Representative plates of vehicle control, tamoxifen (100 μM) or I3C (100 μM) and the combination of I3C and tamoxifen are shown in Fig. 3A. The average integrated density of replicate areas on each plate was determined by NIH Image and normalized by dividing that value by the area that was measured on each plate. This measurement takes into account both the number and size of the colonies and is representative of the number of cells on each plate. Treatment with the high doses of I3C or tamoxifen alone inhibited cell colony formation by 80 and 65%, respectively, whereas
that were treated with increasing doses of I3C or tamoxifen lead to a dose-
to-stain the nuclear DNA. Flow cytometry profiles revealed that
96 h and then hypotonically lysed in the presence of propidium iodide
were treated with the indicated concentrations of each compound for
combinations of I3C and tamoxifen on the cell cycle, MCF-7 cells
observed with the highest doses of either I3C or tamoxifen alone.
shown in Fig. 4A, middle) induced the G1 shift to approximately
the same extent as observed with the highest concentrations of either
compound alone. As shown graphically in Fig. 4B, the I3C- and
tamoxifen-mediated shift in number of G1 cells (top) appeared to
result from a decrease in S-phase cells (middle), whereas the G2-M
phase values did not significantly change (bottom).

The expression levels of specific cell cycle proteins that are respon-
sible for progression through G1 and/or transition into S phase were
examined in cells treated for 96 h with combinations of 100 \( \mu M \) I3C
and/or 1 \( \mu M \) tamoxifen. Western blot analysis revealed that I3C
selectively decreased the level of CDK6 protein and increased the
level of the p21 CDK inhibitor at this time point (Fig. 5). In contrast,
tamoxifen had no effect on each of these cell cycle proteins under our
cell culture conditions. Our previous studies show that I3C also
slightly increases the level of the p27 CDK inhibitor protein (6),
whereas tamoxifen has no effect on p27 protein levels (data not
shown). The expression of the other cell cycle proteins tested, such as
CDK2, CDK4, cyclin D1, and cyclin E, were not affected by either
I3C or tamoxifen. The p16 CDK inhibitor was not detectable in the
MCF-7 cells used for our study.

I3C and Tamoxifen Cooperate to Decrease the in Vitro Activity of CDK2 and the Phosphorylation of Endogenous Rb. The control
of G1 CDK enzymatic activity is critical for regulating cell cycle
progression (22). The activity of specific CDKs is regulated, in part,
by the composition of the holoenzyme, which includes the appropriate
CDK inhibitory proteins. Therefore, although the levels of
CDK2 and CDK4 remain unaltered after I3C and/or tamoxifen

treatment, we examined the potential effects of I3C and tamoxifen on

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Fig. 3. Effects of I3C and tamoxifen on low confluency colony number in MCF-7 cells on plastic and in soft agar. A, four representative 100-mm plates of MCF-7 cells cultured
for 8 days in the presence of the indicated concentrations of I3C and tamoxifen (Tam). At the end of the treatment the cells were fixed and stained as described in “Materials and
Methods.” B, quantification of the amount and size of the colonies on each plate treated with the indicated combination of I3C and tamoxifen was determined by calculating the
integrated density using NIH Image. Data shown represent a detailed dose response. Columns, average of triplicate samples, expressed as integrated density in pixel value/area in mm2;
bars, SE. C, 500 MCF-7 cells were cultured in 0.3% soft agar as described in “Materials and Methods.” After 4.5 weeks at the indicated concentrations of I3C and/or tamoxifen, colonies
that were >50 \( \mu \)m in diameter were counted. Columns, average of triplicate samples; bars, SE.
CDK specific activities. Because one of the key endogenous substrates for the G₁ CDKs is the Rb protein, we determined the ability of the individual G₁ CDKs to phosphorylate Rb in vitro. MCF-7 cells were treated for 48 h with I3C and/or tamoxifen and then CDK2, CDK4, or CDK6 were immunoprecipitated from total cell extracts. For the kinase assays, half of each immunoprecipitated sample was incubated with the COOH-terminal domain of Rb fused to GST and [γ-32P]ATP. The electrophoretically fractionated reaction products were quantitated on a PhosphorImager and then visualized by autoradiography. The other half of the immunoprecipitated samples were analyzed by Western blot and densitometry to confirm the efficiency and specificity of each IP. The CDK-specific activity was calculated by dividing the in vitro kinase activity by the corresponding protein expression. As shown in the CDK2 specific activity graph, increasing doses of I3C alone or tamoxifen alone resulted in dose-dependent decreases in CDK2-specific activity (Fig. 6). Treatment with combinations of these two growth inhibitors resulted in a more stringent decrease in CDK2-specific activity than either agent alone.

Fig. 4. Effects of I3C and tamoxifen on DNA content of MCF-7 cells. A. MCF-7 cells were treated with the indicated combinations of concentrations of I3C and tamoxifen (Tam) for 96 h. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite Laser. A total of 10,000 nuclei were analyzed from each sample, and the percentages of cells within G₁, S, and G₂-M were determined as described in “Materials and Methods.” Representative profiles are shown for each condition, and the numbers in the upper right corner of the profiles are average of triplicate samples. B. Results from the flow cytometry profiles were converted into bar graphs. Top, G₁; middle, S; bottom, G₂-M, after the indicated treatments of doses of I3C and tamoxifen (Tam) for 96 h. Bars, SE.
In contrast, I3C inhibited CDK6 protein expression, but the specific activity of the residual CDK6 protein remained unaltered. Tamoxifen had no effect on CDK6 protein expression or specific activity (Fig. 6). Thus, the decrease in CDK6 activity appears to result from the I3C-mediated decrease in CDK6 protein levels and not due to an effect on CDK6 enzymatic activity. CDK4-specific activity was not affected by either tamoxifen or I3C (data not shown).

It was important to determine whether the in vitro kinase assay results reflected the phosphorylation status of endogenous Rb after treatment with the antiproliferative agents. Therefore, the levels of phosphorylated and hypophosphorylated Rb were examined in MCF-7 cells treated for 48 h with I3C, tamoxifen, or a combination of both agents. The extent of Rb phosphorylation was determined by probing Western blots with an Rb-specific antibody and analyzing the characteristic mobility shift of the hyperphosphorylated Rb protein. As shown in Fig. 7, I3C treatment, and to a lesser extent tamoxifen treatment, caused a decrease in total Rb protein levels and an increase in the relative levels of hypophosphorylated Rb (pRb). Most significantly, a combination of I3C and tamoxifen virtually ablated the expression of the hyperphosphorylated form of Rb (ppRb), which likely explains the more potent growth arrest observed in the presence of these two growth suppressors.

DISCUSSION

A wide range of extracellular signaling molecules either inhibit or stimulate the proliferation of mammalian cells through pathways that ultimately target specific components within G1 (19, 22, 30). It is well

Fig. 5. Effects of I3C on expression of G1 cell cycle proteins. MCF-7 cells were treated with 100 μM I3C and/or 1 μM tamoxifen (Tam) for 96 h, and the protein production of the G1 cell cycle components was determined by Western blot analysis using specific antibodies. The same cell extracts were used for the analysis of each cell cycle protein, and equal sample loading was confirmed by Ponceau S staining of the Western blot membrane.

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ity. However, within the 24-h time point when CDK2 activity is affected, neither p21 protein levels nor p21’s ability to coimmuno-precipitate with CDK2 changes (data not shown). Thus, p21 does not appear to be responsible for I3C’s ability to decrease CDK2 activity.

In addition to the differential cell cycle effects of I3C and tamoxifen, treatment with both compounds virtually eliminated colony formation in soft agar. This suggests the possibility that I3C could enhance the effectiveness of tamoxifen to control the growth of breast tumors in vivo. Tamoxifen has been shown to act as an estrogen agonist or antagonist, depending on cell type (reviewed in Ref. 12). The use of tamoxifen to treat estrogen-responsive breast cancers has extended the lives of many women (7–9). In addition, the preliminary

Fig. 6. Effects of I3C and tamoxifen on in vitro CDK2 and CDK6 kinase activity in MCF-7 cells. MCF-7 cells were treated with I3C and/or tamoxifen (Tam) for 48 h. CDK2 or CDK6 was immunoprecipitated from cell lysates and assayed for in vitro kinase activity using the COOH terminus of the Rb protein as a substrate (GST-Rb). One control immunoprecipitation (no IP) contained rabbit anti-IgG only in vehicle control-treated MCF-7 cell lysates. The kinase reaction mixtures were electrophoretically fractionated, and the level of [33P]Rb (pGST-Rb) was quantitated by PhosphorImager analysis and visualized by autoradiography. The efficiency of the IP for each sample was confirmed and quantitated by Western blot analysis as described in “Materials and Methods.” To normalize for IP efficiency, the specific activity was determined by dividing the values for the activity by the values for the protein level. Columns, average results of at least three kinase assays for each condition; bars, SE.

Fig. 7. Effects of I3C and tamoxifen on phosphorylation of endogenous Rb in MCF-7 cells. MCF-7 cells were treated with 100 μM I3C and/or 1 μM tamoxifen (Tam) for 48 h, the cell extracts were electrophoretically fractionated, and Western blots were probed with anti-Rb antibodies. The extent of endogenous Rb phosphorylation was determined by the characteristic migration of the hyperphosphorylated (ppRb) and hypophosphorylated (pRb) forms of Rb.

Fig. 8. Model for the comparison of the antiproliferative effects of I3C and tamoxifen in breast cancer cells. I3C potentially mediates its effects through a putative cellular target (“target”). I3C-induced breast cancer cell growth suppression is correlated with a rapid inhibition of CDK6 expression and activity and a decrease in the activity of CDK2. The inhibition of the activity of these two G1-acting CDKs are most likely responsible for the decreased retinoblastoma protein (Rb) phosphorylation and thereby results in the observed G1 block in cell cycle progression. In contrast, tamoxifen mediates its effects by inhibiting the activity of the estrogen receptor, which prevents the estrogen-stimulated growth of breast cancer cells through a pathway that eventually converges on the activity of CDK2.
results of a clinical trial using tamoxifen as a preventative treatment for women that are at a high risk for breast cancer are very encouraging (34). However, approximately two-thirds of breast cancer patients have estrogen receptor-positive tumors, only half of which respond to tamoxifen therapy (35). Moreover, after 12–18 months of treatment, resistance to tamoxifen develops in all patients (36), and tamoxifen has been shown to actually stimulate the growth of breast cancer cells after prolonged treatment (37, 38). The molecular mechanism underlying the development of acquired resistance to antiestrogens is unclear, but continued exposure of cells to tamoxifen may select for cells able to grow without estrogen stimulation (39).

Our results suggest that I3C, in combination with tamoxifen, could overcome some of the drawbacks of tamoxifen therapy while capitalizing on the positive effects of this proven therapy. Lower doses and/or pulses of tamoxifen are two of the proposed methods of circumventing tamoxifen resistance (40). In this regard, our results showed that lower doses of tamoxifen and I3C inhibited MCF-7 cell growth and CDK2-specific activity to the same extent as higher doses of either agent added individually. In principle, this response could be exploited to circumvent acquired drug resistance to sustained high doses of tamoxifen. Alternatively, patients could conceivably receive intermittent pulses of tamoxifen while undergoing I3C treatment. I3C has been shown to reduce the formation of both spontaneous and carcinogen-induced mammary tumors in rodents with no apparent side effects (4, 41–43). Human subjects who ingested I3C also had no side effects (44, 45). To extend our current studies, we plan to examine the in vivo effectiveness of combinations of I3C and tamoxifen on the growth of tumors derived from estrogen-responsive and estrogen-nonresponsive breast cancer cell lines and to determine the mechanism by which the I3C and tamoxifen pathways cooperate to block cell cycle progression.

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Indole-3-Carbinol and Tamoxifen Cooperate to Arrest the Cell Cycle of MCF-7 Human Breast Cancer Cells
