[CANCER RESEARCH 59, 1244–1251, March 15, 1999]

Indole-3-Carbinol and Tamoxifen Cooperate to Arrest the Cell Cycle of MCF-7 Human Breast Cancer Cells[1]

Carolyn M. Cover, S. Jean Hsieh, Erin J. Cram, Chibo Hong, Jacques E. Riby, Leonard F. Bjeldanes, and Gary L. Firestone[2]

Department of Molecular and Cell Biology and The Cancer Research Laboratory [C. M. C., S. J. H., E. J. C., G. L. F.] and Department of Nutritional Sciences [C. H., J. E. R., L. F. B.], The University of California at Berkeley, Berkeley, California 94720-3200

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 G_1 cell cycle arrest of human breast cancer cell lines, independent of estrogen receptor signaling. Combinations of I3C and the antiestrogen tamoxifen cooperate to inhibit the growth of the estrogen-dependent human MCF-7 breast cancer cell line more effectively than either agent alone. This more stringent growth arrest was demonstrated by a decrease in adherent and anchorage-independent growth, reduced DNA synthesis, and a shift into the G_1 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 endogenous substrate for the G_1 CDKs, whereas either agent alone only partially inhibited endogenous Rb phosphorylation. Several lines of evidence suggest that I3C works through a mechanism distinct from tamoxifen. I3C failed to compete with estrogen for estrogen receptor binding, and it specifically down-regulated the expression of CDK6. These results demonstrate that I3C and tamoxifen work through different signal transduction pathways to suppress the growth of human breast cancer cells and may, therefore, represent a potential combinatorial therapy for estrogen-responsive breast cancer.

INTRODUCTION

I3C[3] is a naturally occurring compound found in vegetables of the *Brassica* genus, such as broccoli and Brussels sprouts (1, 2), and it has been shown to reduce the incidence of spontaneous and carcinogen-induced mammary tumors in rodents (3, 4). When ingested, the stomach acid catalyzes the conversion of I3C into a number of derivatives. Two of the most prevalent derivatives that have been identified are 3,3′-diindolymethane and indolo[3,2-*b*]carbazole (5). These compounds are thought to be responsible for the long-term antiestrogenic biological activities of ingested I3C that may contribute to protection against mammary tumor formation. We have documented that the treatment of MCF-7 human breast cancer cells with I3C but not with its acid-catalyzed derivatives rapidly suppresses cell growth. This reversible G_1 cell cycle arrest is preceded by a decrease in the expression of CDK6 and is independent of estrogen receptor signaling (6). However, many of the molecular details of this 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 G_1 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 are well documented. For example, progesterone inhibition of breast cancer cell growth has been linked to the inactivation of G_1 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 G_1 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 G_1 CDKs is the retinoblastoma protein (Rb). Its phosphorylation is an important step in the transition between the G_1 and S phases of the cell cycle because, when sufficiently phospho-

Received 7/15/98; accepted 1/13/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Department of Defense Army Breast Cancer Research Program Grant DAMD17-96-1-6149 (to L. F. B.) and by University of California Breast Cancer Research Program Grant 31B-0110 (to G. L. F.). C. M. C. is a recipient of a predoctoral fellowship supported by NIH National Research Service Grant CA-09041. E. J. C. is a recipient of a predoctoral fellowship supported by the United States Army Medical Research and Materiel Command Breast Cancer Research Program Award BC971062.

[2] To whom requests for reprints should be addressed, at Department of Molecular and Cell Biology, 591 LSA, University of California at Berkeley, Berkeley, CA 94720-3200. Phone: (510) 642-8319; Fax: (510) 643-6791; E-mail: glfire@uclink4.berkeley.edu.

[3] The abbreviations used are: I3C, indole-3-carbinol; CDK, cyclin-dependent kinase; E2, β-estradiol; FBS, fetal bovine serum; IP, immunoprecipitation; GST, glutathione *S*-transferase.

rylated, 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 ([*Z*]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) citrate salt were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Thymidine (84 Ci/mmol) and [γ-^{33}P]ATP (3,000 Ci/mmol) 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 either listed 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 μg/ml insulin, 50 units/ml penicillin, 50 units/ml streptomycin, and 2 mM L-glutamine and maintained at subconfluency at 37°C in humidified air containing 5% CO_2. I3C and tamoxifen were dissolved in DMSO (99.9% high-performance liquid chromatography grade; Aldrich) at concentrations that were 1000-fold higher than the final medium concentration. In all experiments, 1 μl of the concentrated agent was added per 1 ml of medium, and for the vehicle control, 1 μl of DMSO was added per 1 ml of medium.

Estrogen Receptor Binding. Rat uterine cytosol was prepared as described (28). Briefly, 2.5 g of uterine tissue from five 12-week-old Sprague Dawley rats were excised and homogenized for 1 min with 30 ml of ice cold TEDG buffer [10 mM Tris, 1.5 mM EDTA, 1 mM DTT, and 10% glycerol (pH 7.4)], using a Polytron homogenizer at medium speed. The homogenate was centrifuged at 2800 rpm for 10 min at 4°C, transferred to a new tube, and centrifuged at 39,000 rpm for 90 min at 4°C. The supernatant was quickly frozen in a dry ice/ethanol bath and stored at −80°C.

For the competitive binding assay, 1 μl of competitor (100× solution in DMSO) was added to 5 μl of [^{3}H]E2 mixture [20 nM [^{3}H]E2 (NEN) in 50% ethanol, 5 mM Tris (pH 7.5), 5% glycerol, 0.5 mg/ml BSA, and 0.5 mM DTT] and incubated at room temperature with 95 μl of uterine cytosol (3 mg/ml) for 2 h. The range of concentrations of competitive ligand were 0.1 nM–1 μM for E2, 10 nM–10 μM for tamoxifen, and 10 μM–1 mM for I3C. The reaction was then incubated on ice for 15 min with 100 μl of 50% HAP slurry (hydoxylapatite; Bio-Rad BioGel HTP) rinsed and swelled overnight in TE buffer [50 mM Tris and 1 mM EDTA (pH 7.4)] and then adjusted to 50%. The slurry was vortexed to resuspend HAP every 5 min. Next, 1.0 ml of ice-cold wash buffer [40 mM Tris (pH 7.4), 100 mM KCl, 1 mM EDTA, and 1 mM EGTA] was added, and the mixture was vortexed and centrifuged for 5 min at 10,000 rpm at 4°C. The pellet was washed twice more with ice-cold wash buffer and then resuspended in 400 μl of ethanol and transferred to a scintillation vial for measurement. Relative binding affinities were calculated using the concentration of competitor needed to reduce [^{3}H]E2 binding by 50%, as compared to the concentration of unlabeled estrogen needed to achieve the same result.

[^{3}H]Thymidine Incorporation. MCF-7 cells were plated onto 24-well Corning tissue culture dishes. Triplicate samples of asynchronously growing mammary cells were treated for the indicated times with either vehicle control (1 μl of DMSO per 1 ml of medium) or varying concentrations of I3C and/or tamoxifen. The cells were pulsed for 3 h with 3 μCi of [^{3}H]thymidine (84 Ci/mmol), washed three times with ice-cold 10% trichloroacetic acid, and lysed with 300 μl of 0.3 N NaOH. Lysates (150 μl) were transferred into vials containing liquid scintillation cocktail, and radioactivity was quantitated by scintillation counting. Triplicates were averaged and expressed as cpm per well.

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.

Soft Agar Colony Formation. Two layers of Difco agar with different concentrations were set in individual wells of a 24-well plate. The lower layer contained 0.5 ml of 0.6% soft agar in medium with the indicated combinations of I3C and tamoxifen. The upper layer was composed of 0.5 ml of 0.3% soft agar in medium with MCF-7 cells (500 cells/well) and the corresponding combinations of I3C and tamoxifen in triplicate. After 4.5 weeks, all of the colonies that were <50 μm in diameter were counted.

Flow Cytometric Analyses of DNA Content. MCF-7 cells (4×10^4) 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 wavelength 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 G_1, S, and G_2-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 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, and 10 μg/ml β-glycerophosphate). Equal amounts of total cellular protein were mixed with loading buffer [25% glycerol, 0.075% SDS, 1.25 ml of 14.4 M β-mercaptoethanol, 10% bromphenol blue, 3.13% 0.5 M Tris-HCl, 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 20–5% 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 μg/ml in Western wash buffer. Immunoreactive proteins were detected after incubation with horseradish peroxidase-conjugated secondary antibody diluted to 3×10^{-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 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 μg/ml NaF, 10 μg/ml β-glycerophosphate, and 0.1 mM sodium orthovanadate). Samples were diluted to 500 μg of protein in 1 ml of IP buffer. Samples were precleared for 30 min at 4°C with 20 μl of a 1:1 slurry of protein A-Sepharose beads (Pharmacia Biotech, Sweden) in IP buffer and 1 μg of rabbit IgG. After a brief centrifugation to remove precleared beads, 0.5 μg of

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 $MgCl_2$, 5 mM $MnCl_2$, 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 μg of Rb COOH-terminal domain protein substrate (Santa Cruz Biotechnology), and 10 μCi of [γ-^{33}P]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.

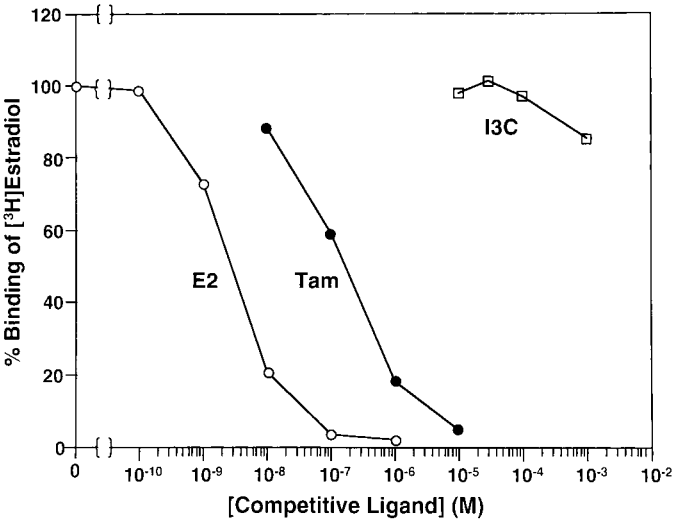

Fig. 2. Competitive binding to the estrogen receptor. The binding of [^{3}H]E2 (1 nM) to the estrogen receptor from rat uterine cytosol was measured in the presence of the unlabeled competitive ligands, E2 (○), Tam (●), and I3C (□), at the indicated concentrations as described in "Materials and Methods." The results are reported as the percentage of binding in the absence of competitors. Relative binding affinities were calculated using the concentration of competitor needed to reduce [^{3}H]E2 binding by 50% as compared to the concentration of unlabeled E2 to achieve the same result.

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 [^{3}H]thymidine for 3 h at each time point to provide a measure of their proliferative state. Analysis of [^{3}H]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.

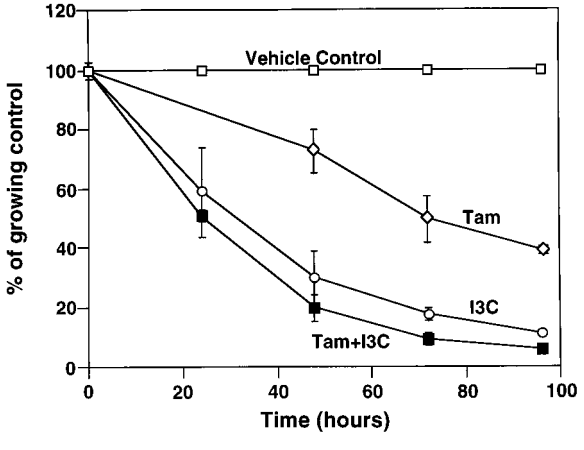

Fig. 1. Time course effects of I3C and tamoxifen on DNA synthesis in MCF-7 cells. MCF-7 cells cultured in 24-well dishes were treated for the indicated time with vehicle control (*Vehicle Control*; □), 1 μM tamoxifen (*Tam*; ◇), 100 μM I3C (*I3C*; ○), or a combination of tamoxifen and I3C (*Tam+I3C*; ■). Cells were labeled with [^{3}H]thymidine for 3 h, and the [^{3}H]thymidine incorporation into DNA was determined by acid precipitation as described in "Materials and Methods." The data are expressed as the percentage of growing control. This was calculated by dividing the value of the vehicle control-treated sample at each time point by the value of the drug-treated sample at the same time point. *Data points*, average of triplicate samples from six different experiments; *bars*, SE.

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 [^{3}H]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 [^{3}H]E2 displacement of ~200 nM. In contrast, I3C caused no significant displacement of [^{3}H]E2 binding to the estrogen receptor, even at 1 mM. 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 Adherent Cell Growth and 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/formalin. Representative plates of vehicle control, high doses of I3C (100 μM) or tamoxifen (1 μM) and the combination of I3C and tamoxifen are shown in Fig. 3*A*. 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

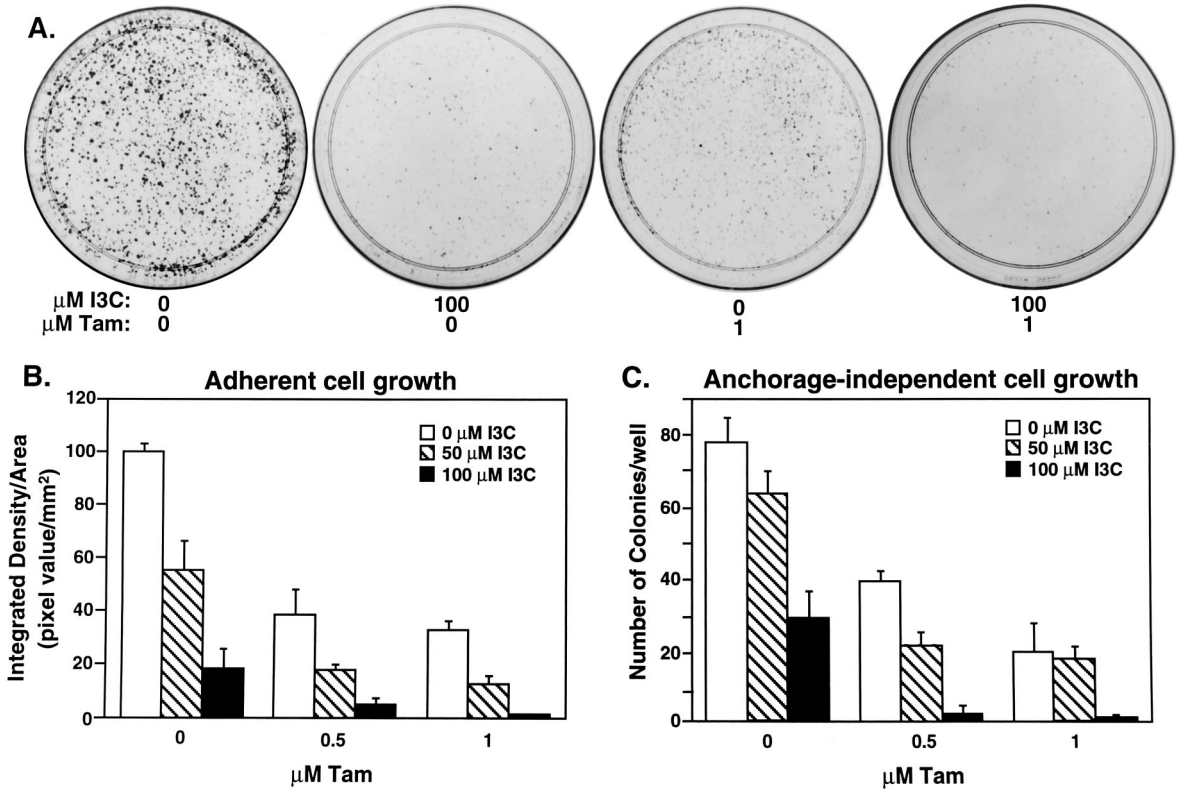

Fig. 3. Effects of I3C and tamoxifen on low confluency cell colony number in MCF-7 cells on plastic and in soft agar. *A*, four representative 100-mm plates of MCF-7 cells cultured for 8 days in the presence of the indicated concentrations of I3C and tamoxifen (*Tam*). At the end of the treatment the 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 mm^2; *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 μm in diameter were counted. *Columns*, average of triplicate samples; *bars*, SE.

a combination of both agents inhibited adherent cell growth by >95%. An expanded analysis of the dose-dependent suppression of adherent cell growth by I3C and tamoxifen is shown in Fig. 3*B*.

The effects of combinations of I3C and tamoxifen on the anchorage-independent growth of MCF-7 cells in soft agar was examined. Cells were cultured for 4.5 weeks in 0.3% agar and complete medium containing the vehicle control, I3C, tamoxifen, or a combination of both agents. Cell colonies that were >50 μm in diameter were counted. As shown in Fig. 3*C*, increasing doses of I3C or tamoxifen caused a decrease in cell colony formation, and consistent with the adherent cell growth properties, a combination of both agents caused a more pronounced decrease in cell colony formation. For both growth conditions, the combined inhibitory effect of suboptimal concentrations of I3C (50 μM) and tamoxifen (0.5 μM) approximated that observed with the highest doses of either I3C or tamoxifen alone.

Cell Cycle Effects of I3C and Tamoxifen. To assess the effect of combinations of I3C and tamoxifen on the cell cycle, MCF-7 cells were treated with the indicated concentrations of each compound for 96 h and then hypotonically lysed in the presence of propidium iodide to stain the nuclear DNA. Flow cytometry profiles revealed that treatment with increasing doses of I3C or tamoxifen lead to a dose-dependent shift in percentage of cells with a G_1-like DNA content. As shown in Fig. 4*A*, I3C or tamoxifen altered the DNA content of the MCF-7 cell population from an asynchronous population of growing cells in all phases of the cell cycle (61.4% in G_1, 29.0% in S, and 9.7% in G_2-M) to one in which most of the treated breast cancer cells were in the G_1 phase of the cell cycle (Fig. 4*A*, *top right* for I3C and *bottom left* for tamoxifen). Consistent with the cell growth studies, a combination of both agents caused a more striking shift to G_1 (Fig. 4*A*, *bottom right*). Incubation with suboptimal concentrations of both I3C and tamoxifen (Fig. 4*A*, *middle*) induced the G_1 shift to approximately the same extent as observed with the highest concentrations of either compound alone. As shown graphically in Fig. 4*B*, the I3C- and tamoxifen-mediated shift in number of G_1 cells (*top*) appeared to result from a decrease in S-phase cells (*middle*), whereas the G_2-M phase values did not significantly change (*bottom*).

The expression levels of specific cell cycle proteins that are responsible for progression through G_1 and/or transition into S phase were examined in cells treated for 96 h with combinations of 100 μM I3C and/or 1 μM 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 G_1 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 cyclin and/or 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

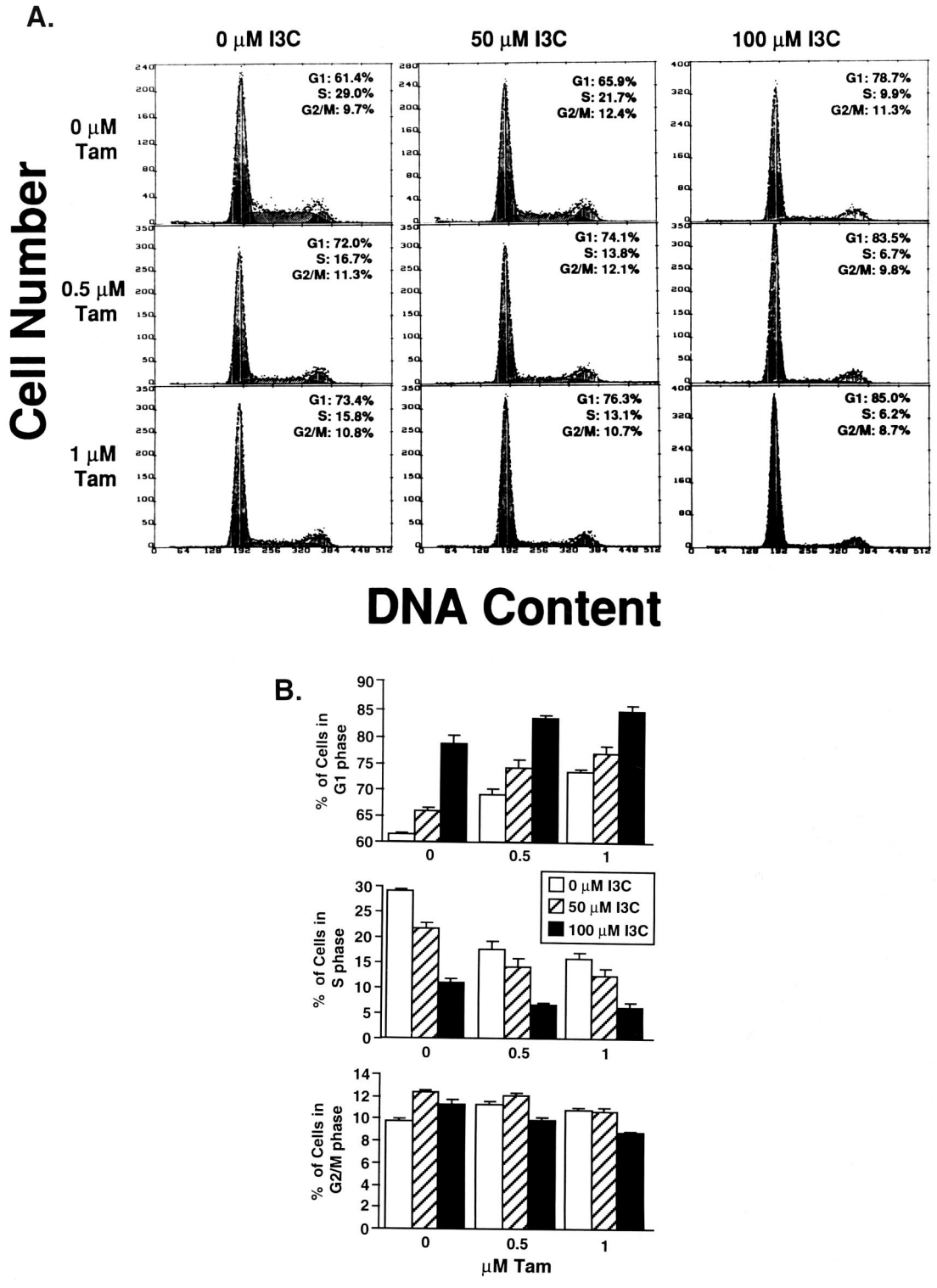

Fig. 4. Effects of I3C and tamoxifen on DNA content of MCF-7 cells. *A*, MCF-7 cells were treated with the indicated combinations of concentrations of I3C and tamoxifen (*Tam*) for 96 h. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite Laser. A total of 10,000 nuclei were analyzed from each sample, and the percentages of cells within G_1, S, and G_2-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 an average of triplicate samples. *B*, results from the flow cytometry profiles were converted into bar graphs. *Top*, G_1; *middle*, S; *bottom*, G_2-M, after the indicated treatments of doses of I3C and tamoxifen (*Tam*) for 96 h. *Bars*, SE.

G_1 CDK specific activities. Because one of the key endogenous substrates for the G_1 CDKs is the Rb protein, we determined the ability of the individual G_1 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-^{33}P]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.

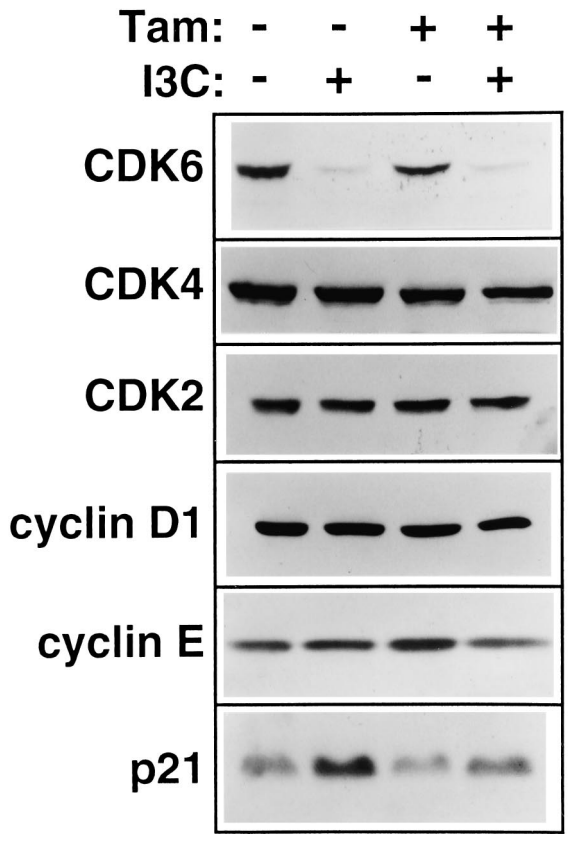

Fig. 5. Effects of I3C on expression of G_1 cell cycle proteins. MCF-7 cells were treated with 100 μM I3C and/or 1 μM tamoxifen (*Tam*) for 96 h, and the protein production of the G_1 cell cycle components was determined by Western blot analysis using specific antibodies. The same cell extracts were used for the analysis of each cell cycle protein, and equal sample loading was confirmed by Ponceau S staining of the Western blot membrane.

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 enyzmatic 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 a 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 G_1 (19, 22, 30). It is well established that tamoxifen can exert its growth-inhibitory effects in human breast cancer cells by antagonizing the estrogen receptor stimulation of cell cycle progression (18). We have recently documented that the dietary indole I3C can induce a G_1 block in cell cycle progression of breast cancer cell lines in the absence of estrogen receptor signaling (6). Depending on the antiproliferative pathways, cell cycle progression can be more effectively blocked through the coordinate actions of specific networks of cell cycle-regulated gene products (31). Our results have established that the antiproliferative cascades initiated by I3C and tamoxifen can cooperate to induce a more stringent growth suppression in breast cancer cells treated with a combination of both agents compared to treatment with either I3C or tamoxifen alone. We propose that the I3C- and tamoxifen-activated antiproliferative responses are mediated by distinct signal transduction pathways (see Fig. 8) that converge on the inhibition of CDK2-specific activity as a common target with a subsequent decrease in endogenous Rb phosphorylation.

The distinguishing feature of the I3C antiproliferative pathway is the rapid down-regulation of CDK6 expression (6), which accounts for the inhibition of total CDK6 activity in breast cancer cells. Tamoxifen had no apparent effect on either CDK6 protein levels or enzymatic activity. In contrast to tamoxifen, I3C did not compete with E2 for binding to the estrogen receptor and has been shown to exert its growth-suppressive effects in breast cancer cells irrespective of estrogen receptor status, such as in estrogen receptor-deficient breast cancer cells (6). The immediate cellular target for I3C or a specific metabolite is unknown. We propose (Fig. 8) that this extracellular signal binds to a target molecule that mediates the growth-inhibitory cascade. The sustained growth suppression of colonies in soft agar in the absence of a medium change for 4.5 weeks implies that I3C could be working through a stable compound that binds to a specific cellular component. I3C regulates several G_1-acting cell cycle components, including the down-regulation of CDK6 expression and the inhibition of CDK2-specific activity. Tamoxifen inhibited MCF-7 cell growth through its antiestrogenic effects because the cells were cultured in medium supplemented with 10% FCS, which contains enough endogenous estrogen to maintain the cells in a proliferative state. Other studies have shown that tamoxifen interferes with the estrogen stimulation of the cell cycle, which includes a disruption in the stimulation of CDK2 activity and Rb phosphorylation (32). Our results are consistent with these observations and we propose that the inhibition of CDK2-specific activity and Rb phosphorylation are common targets for both the I3C and tamoxifen pathways (Fig. 8) and, thereby, provides one mechanistic explanation for the more stringent growth suppression observed with both agents.

Several potential mechanisms could account the inhibition of CDK2-specific activity by the I3C and/or the tamoxifen pathways. For example, treatment with either agent could alter the composition of CDK2 holoenzyme complex resulting in inactive complexes that do not support the phosphorylation of Rb. Alternatively, because full activation of CDK2 requires phosphorylation on specific residues and removal of phosphates from other residues (17, 20, 33), the CDK2-specific kinases and phosphatases may be targets for either the I3C or tamoxifen pathways. The unique features of each pathway likely play a role in the more stringent inhibition of growth observed in cells treated with both I3C and tamoxifen. The I3C down-regulation of CDK6 expression could release other cell cycle factors contained within the CDK6 protein complexes and, thereby, shift the cellular equilibrium of these components in manner that alters the composition of the other G_1 CDK protein complexes. For example, enough CDK-inhibitory molecules could conceivably be released from the CDK6 protein complexes to negatively modulate the specific activity of CDK2. Furthermore, longer I3C treatments increase the level of the p21 CDK inhibitor, which could also influence CDK2-specific activ-

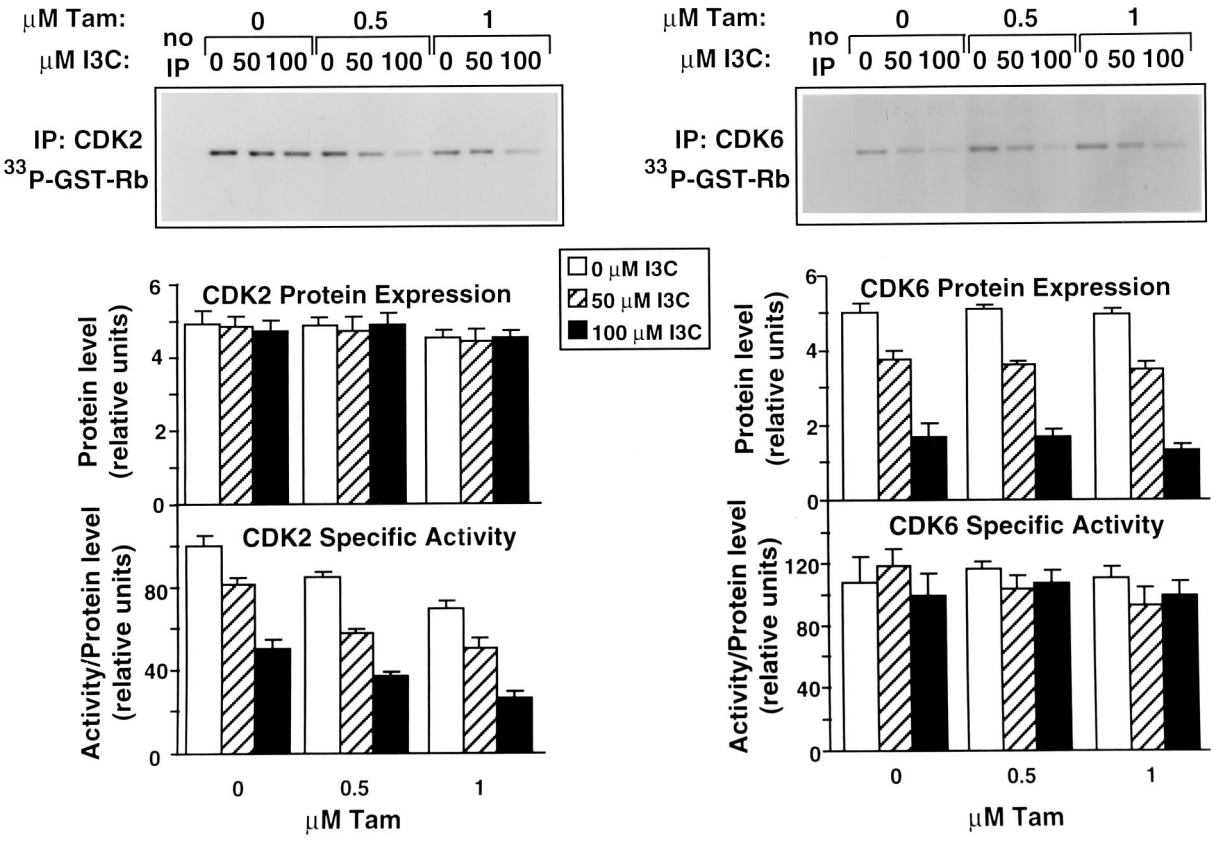

Fig. 6. Effects of I3C and tamoxifen on *in vitro* CDK2 and CDK6 kinase activity in MCF-7 cells. MCF-7 cells were treated with I3C and/or tamoxifen (*Tam*) for 48 h. CDK2 or CDK6 was immunoprecipitated from cell lysates and assayed for *in vitro* kinase activity using the COOH terminus of the Rb protein 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 [^{33}P]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.

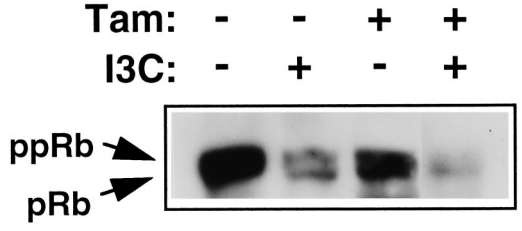

Fig. 7. Effects of I3C and tamoxifen on phosphorylation of endogenous Rb in MCF-7 cells. MCF-7 cells were treated with 100 μM I3C and/or 1 μM tamoxifen (*Tam*) for 48 h, the cell extracts were electrophoretically fractionated, and Western blots were probed with anti-Rb antibodies. The extent of endogenous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

ity. However, within the 24-h time point when CDK2 activity is affected, neither p21 protein levels nor p21's ability to coimmunoprecipitate 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

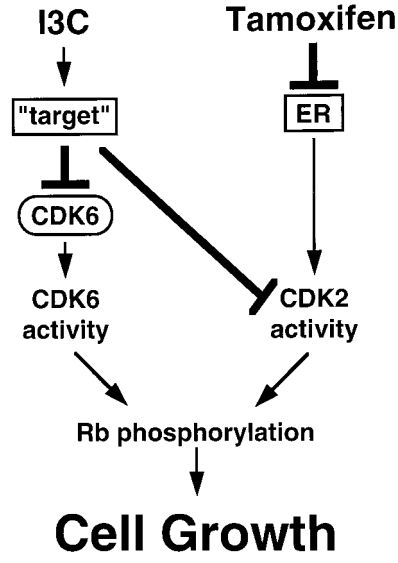

Fig. 8. Model for the comparison of the antiproliferative effects of I3C and tamoxifen in breast cancer cells. I3C potentially mediates its effects through a putative cellular target ("*target*"). I3C-induced breast cancer cell growth suppression is correlated with a rapid inhibition of CDK6 expression and activity and a decrease in the activity of CDK2. The inhibition of the activity of these two G_1-acting CDKs are most likely responsible for the decrease in retinoblastoma protein (Rb) phosphorylation and thereby results in the observed G_1 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.

ACKNOWLEDGMENTS

We express our appreciation to members of both the Firestone and Bjeldanes laboratories for their helpful comments throughout the duration of this work. We also thank Jerry Kapler for his excellent photography and Peter Schow, Meredith L. Leong, Minnie Wu, and Tran Van for their technical assistance.

REFERENCES

1. Loub, W. D., Wattenberg, L. W., and Davis, D. W. Aryl hydrocarbon hydroxylase induction in rat tissues by naturally occurring indoles of cruciferous plants. J. Natl. Cancer Inst. (Bethesda), *54:* 985–988, 1975.
2. Wattenberg, L. W., and Loub, W. D. Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. Cancer Res., *38:* 1410–1413, 1978.
3. Morse, M. A., LaGreca, S. D., Amin, S. G., and Chung, F. L. Effects of indole-3-carbinol on lung tumorigenesis and DNA methylation induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and on the metabolism and disposition of NNK in A/J mice. Cancer Res., *50:* 2613–2617, 1990.
4. Bradlow, H. L., Michnovicz, J., Telang, N. T., and Osborne, M. P. Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. Carcinogenesis (Lond.), *12:* 1571–1574, 1991.
5. Bjeldanes, L. F., Kim, J. Y., Grose, K. R., Bartholomew, J. C., and Bradfield, C. A. Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: comparisons with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Proc. Natl. Acad. Sci. USA, *88:* 9543–9547, 1991.
6. Cover, C. M., Hsieh, S. J., Tran, S. H., Hallden, G., Kim, G. S., Bjeldanes, L. F., and Firestone, G. L. Indole-3-carbinol inhibits the expression of cyclin-dependent kinase-6 and induces a G_1 cell cycle arrest of human breast cancer cells independent of estrogen receptor signaling. J. Biol. Chem., *273:* 3838–3847, 1998.
7. Pennisi, E. Drug's link to genes reveals estrogen's many sides. Science (Washington DC), *273:* 1171, 1996.
8. Powles, T. J. Efficacy of tamoxifen as treatment of breast cancer. Semin. Oncol., *24* (Suppl. 1)*:* S1-48–S1-54, 1997.
9. Forbes, J. F. The control of breast cancer: the role of tamoxifen. Semin. Oncol., *24* (Suppl. 1)*:* S1-5–S1-19, 1997.
10. Skidmore, J. R., Walpole, A. L., and Woodburn, J. Effect of some triphenylethylenes on oestradiol binding *in vitro* to macromolecules from uterus and anterior pituitary. J. Endocrinol., *52:* 289–298, 1972.
11. Jordan, V. C., and Prestwich, G. Binding of [^{3}H] tamoxifen in rat uterine cytosols. A comparison of swinging bucket and vertical tube rotor sucrose density gradient analysis. Mol. Cell. Endocrinol., *8:* 179–188, 1977.
12. Gallo, M. A., and Kaufman, D. Antagonistic and agonistic effects of tamoxifen: significance in human cancer. Semin. Oncol., *24* (Suppl. 1)*:* S1-71–S1-80, 1997.
13. Tsai, L. C., Hung, M. W., Yuan, C. C., Chao, P. L., Jiang, S. Y., Chang, G. G., and Chang, T. C. Effects of tamoxifen and retinoic acid on cell growth and c-*myc* gene expression in human breast and cervical cancer cells. Anticancer Res., *17:* 4557–4562, 1997.
14. Lindner, D. J., Kolla, V., Kalvakolanu, D. V., and Borden, E. C. Tamoxifen enhances interferon-regulated gene expression in breast cancer cells. Mol. Cell. Biochem., *167:* 169–177, 1997.
15. Otto, A. M., Paddenberg, R., Schubert, S., and Mannherz, H. G. Cell-cycle arrest, micronucleus formation, and cell death in growth inhibition of MCF-7 breast cancer cells by tamoxifen and cisplatin. J. Cancer Res. Clin. Oncol., *122:* 603–612, 1996.
16. Hamel, P. A., and Hanley, H. J. G1 cyclins and control of the cell division cycle in normal and transformed cells. Cancer Invest., *15:* 143–152, 1997.
17. Musgrove, E. A., Swarbrick, A., Lee, C. S. L., Cornish, A. L., and Sutherland, R. L. Mechanisms of cyclin-dependent kinase inactivation by progestins. Mol. Cell. Biol., *18:* 1812–1825, 1998.
18. Prall, O., Sarcevic, B., Musgrove, E. A., Watts, C., and Sutherland, R. L. Estrogen-induced activation of Cdk4 and Cdk2 during G_1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J. Biol. Chem., *272:* 10882–10894, 1997.
19. Hunter, T., and Pines, J. Cyclins and cancer. II: cyclin D and CDK inhibitors come of age. Cell, *79:* 573–582, 1994.
20. Morgan, D. O. Principles of CDK regulation. Nature (Lond.), *374:* 131–134, 1995.
21. Sherr, C. J., and Roberts, J. M. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev., *9:* 1149–1163, 1995.
22. Sherr, C. J. Cancer cell cycles. Science (Washington DC), *274:* 1672–1677, 1996.
23. Hirai, H., Roussel, M. F., Kato, J. Y., Ashmun, R. A., and Sherr, C. J. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol. Cell. Biol., *15:* 2672–2681, 1995.
24. Reynisdottir, I., and Massague, J. The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. Genes Dev., *11:* 492–503, 1997.
25. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinases. Nature (Lond.), *366:* 701–704, 1993.
26. Alessandrini, A., Chiaur, D. S., and Pagano, M. Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation. Leukemia (Baltimore), *11:* 342–345, 1997.
27. Slansky, J. E., and Farnham, P. J. Introduction to the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol., *208:* 1–30, 1996.
28. Santell, R. C., Chang, Y. C., Nair, M. G., and Helferich, W. G. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. J. Nutr., *127:* 263–269, 1997.
29. Bardon, S., Vignon, F., Derocq, D., and Rochefort, H. The antiproliferative effect of tamoxifen in breast cancer cells: mediation by the estrogen receptor. Mol. Cell. Endocrinol., *35:* 89–96, 1984.
30. Draetta, G. F. Mammalian G1 cyclins. Curr. Opin. Cell. Biol., *6:* 842–846, 1994.
31. Van heusden, J., Wouters, W., Ramaekers, F. C., Krekels, M. D., Dillen, L., Borgers, M., and Smets, G. The antiproliferative activity of all-*trans*-retinoic acid catabolites and isomers is differentially modulated by liarozole-fumarate in MCF-7 human breast cancer cells. Br. J. Cancer, *77:* 1229–1235, 1998.
32. Planas, S. M., and Weinberg, R. A. Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. Mol. Cell. Biol., *17:* 4059–4069, 1997.
33. Morgan, D. O. The dynamics of cyclin dependent kinase structure. Curr. Opin. Cell Biol., *8:* 767–772, 1996.
34. Smigel, K. Breast Cancer Prevention Trial shows major benefit, some risk. J. Natl. Cancer Inst. (Bethesda), *90:* 647–648, 1998.
35. Legha, S. S. Tamoxifen in the treatment of breast cancer. Ann. Intern. Med., *109:* 219–228, 1988.
36. Couillard, S., Gutman, M., Labrie, C., Belanger, A., Candas, B., and Labrie, F. Comparison of the effects of the antiestrogens EM-800 and tamoxifen on the growth of human breast ZR-75–1 cancer xenografts in nude mice. Cancer Res., *58:* 60–64, 1998.
37. Gottardis, M. M., and Jordan, V. C. Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. Cancer Res., *48:* 5183–5187, 1988.
38. Osborne, C. K., Coronado-Heinsohn, E. B., Hilsenbeck, S. G., McCue, B. L., Wakeling, A. E., McClelland, R. A., Manning, D. L., and Nicholson, R. I. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. J. Natl. Cancer Inst. (Bethesda), *87:* 746–750, 1995.
39. Wiebe, V. J., Osborne, C. K., Fuqua, S. A., and DeGregorio, M. W. Tamoxifen resistance in breast cancer. Crit. Rev. Oncol. Hematol., *14:* 173–188, 1993.
40. Lykkesfeldt, A. E. Mechanisms of tamoxifen resistance in the treatment of advanced breast cancer. Acta Oncol., *35* (Suppl. 5)*:* 9–14, 1996.
41. Baldwin, W. S., and LeBlanc, G. A. The anti-carcinogenic plant compound indole-3-carbinol differentially modulates P450-mediated steroid hydroxylase activities in mice. Chem. Biol. Interact., *83:* 155–169, 1992.
42. Bradfield, C. A., and Bjeldanes, L. F. Effect of dietary indole-3-carbinol on intestinal and hepatic monooxygenase, glutathione *S*-transferase and epoxide hydrolase activities in the rat. Food Chem. Toxicol., *22:* 977–982, 1984.
43. McDanell, R., McLean, A. E., Hanley, A. B., Heaney, R. K., and Fenwick, G. R. The effect of feeding *Brassica* vegetables and intact glucosinolates on mixed-function-oxidase activity in the livers and intestines of rats. Food Chem. Toxicol., *27:* 289–293, 1989.
44. Michnovicz, J. J., and Bradlow, H. L. Induction of estradiol metabolism by dietary indole-3-carbinol in humans. J. Natl. Cancer Inst. (Bethesda), *82:* 947–949, 1990.
45. Bradlow, H. L., Michnovicz, J. J., Halper, M., Miller, D. G., Wong, G. Y., and Osborne, M. P. Long-term responses of women to indole-3-carbinol or a high fiber diet. Cancer Epidemiol. Biomark. Prev., *3:* 591–595, 1994.