A New Indole-3-Carbinol Tetrameric Derivative Inhibits Cyclin-dependent Kinase 6 Expression, and Induces G1 Cell Cycle Arrest in Both Estrogen-dependent and Estrogen-independent Breast Cancer Cell Lines

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ABSTRACT

Indole-3-carbinol (I3C), a natural product of glucosinolates present in cruciferous vegetables, has been shown to inhibit the growth of human cancer cells in vitro and possesses anticarcinogenic activity in vivo. Because I3C is unstable and may be converted into many polymeric products in the digestive tract, it is not yet clear whether the biological activity observed can be attributed to I3C or some of its polymeric products.

In this study, we synthesized a stable I3C cyclic tetrameric derivative and investigated its effects on a panel of human breast cancer cell lines. The I3C tetramer suppressed the growth of both estrogen receptor (ER)-positive (MCF-7, T47D, and BT474) and ER-negative (BT20, MDA-MB-231, and BT53) human breast cancer cell lines, and it was found to induce G1 cell cycle arrest in a dose-dependent manner without evidence of apoptosis, suggesting a growth arrest via a cytostatic mechanism. At the molecular level, the tetramer inhibited cyclin-dependent kinase (CDK) 6 expression and activity, induced an increase in the level of p27kip1, and reduced the level of retinoblastoma protein expression. Contrarily to CDK6, the level of CDK4, the other kinase involved in the G1 phase of the cell cycle, remains unchanged. Interestingly, the tetramer resulted about five times more active than I3C in suppressing the growth of human breast cancer cells. On the whole, our data suggest that the I3C tetrameric derivative is a novel lead inhibitor of breast cancer cell growth that may be a considered a new, promising therapeutic agent for both ER+ and ER− breast cancer.

INTRODUCTION

One of the most important sources of compounds for chemoprevention are vegetables. In particular, those of the genus Brassica (e.g., cauliflower, broccoli, and brussel sprouts) have been shown to slow, if not to prevent, carcinogenesis, thus offering protection against various types of cancer, including breast cancer (1–3), one of the leading causes of female death related to cancer in developed countries (4). These plants contain significant quantities of isothiocyanates, which are strong inducers of so-called “phase-2 enzymes,” that are enzymes involved in cellular mechanisms of detoxification of toxic and/or mutagenic compounds (5). Furthermore, I3C, 1 a product of the autoxidation of glucosinolates present in the species of the genus Brassica, has been found to exert a good antiproliferative activity in human breast cancer cells (6–8) and to significantly inhibit tumor autolysis of glucosinolates present in the species of the genus Brassica.

The activity of CDK is modulated by cell cycle regulatory proteins, named cyclins, and CDK activation is specific for a given phase of the cell cycle. In the early G1 phase cyclin D complexes with CDKs phosphorylate pRb. pRb in the hypophosphorylated form binds and inactivates the E2F transcription factor, whereas after hyperphosphorylation E2F is released and transactivates a series of genes involved in cell cycle progression (13, 14).

The loss of normal cell cycle control has been implicated in tumor development (15). In particular, in breast cancer, elevated activity of CDK6 results in Rb phosphorylation, loss of E2F inhibition, and, as a consequence, an abnormally high percentage of cells enter the G1-S transition phase of the cell cycle.

The development of I3C as a therapeutic agent for breast cancer prevention or treatment in humans is limited by several factors. First, I3C is unstable and may be easily converted into many polymeric products (16) the activity of which needs to be additionally investigated. Indeed, whereas several studies reported that the biological activity of I3C may be because of the action of one or more of its acid-catalyzed derivatives, such as 2-(Indol-3-ylmethyl)-3,3-diindolylmethane (17), 3,3-diindolylmethane (18–20), and indolo(3,2-b)carbazole (18), an other study showed that I3C but not 3,3-diindolylmethane and indole(3,2-b)carbazole inhibits the growth of breast cancer cells (6). Second, the concentrations of I3C needed to inhibit the expression of CDK6 and to induce cell cycle arrest in breast cancer cell is relatively high, ranging from 60 to 200 μM (6–8, 21). Third, when administered at high doses after treatment with a carcinogen, I3C acts as a potential tumor promoter (22–24). Thus, molecules with a high stability and showing antitumor activity at lower doses than I3C need to be developed to overcome these limitations.

The aim of this study was to synthesize a stable I3C derivative and to test its activity on a panel of human breast cancer cells. Here we report the preparation and characterization of an I3C cyclic tetrameric derivative, named tetramer. This new molecule is able to inhibit the growth of both ER-positive and ER-negative breast cancer cells. At the molecular level, tetramer inhibits CDK6 expression and activity, and induces an increase in the level of p27kip1 and a decrease in pRb expression and Rb phosphorylation. Interestingly, tetramer resulted more active (~5-fold) than I3C in suppressing the growth of human breast cancer cells and displayed a preferential activity against this cell type.

MATERIALS AND METHODS

Materials. DMEM, insulin, I3C, Hoechst 33342, and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, MO). FCS was from...
Mascia Brunelli (Milan, Italy). [3H]Thymidine (25 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). Iodine, acetic acid, the other oxidizing agents, and solvents were of the highest grade available, and were used without additional purification.

**Synthesis and Characterization of the I3C Cyclic Tetrameric Derivative.** Fourier Transform Infra Red (FT-IR) spectra were performed in Nujol mull and recorded on Nicolet Impact 400. Mass spectrometry spectra were made at an ionizing voltage of 70 eV. 1H NMR spectra were recorded at 400 MHz on Varian 400 MHz, whereas 13C NMR at 50.26 MHz and performed in CDCl₃, or in DMSO-d₆. Chemical shifts (δ) were reported in ppm downfield from internal tetramethylsilane. The abbreviations used are follows: s, singlet; d, doublet; t, triplet; m, multiplet; b, broad. Precoated silica gel plates 0.25 mm were used for analytical TLC and silica gel 35–70 μm for chromatography.

I3C (1 g) was dissolved in acetic acid (20 ml). The mixture was stirred at room temperature until the reagent disappeared (monitored by silica gel TLC). The solvent was evaporated under reduced pressure, and the crude precipitate was dissolved in ethyl ether (125 ml) and washed with brine (3×). The solvent was evaporated under reduced pressure and purified on silica gel column (elution with cyclohexane-ethyl acetate mixtures). The product was additionally purified by crystallization from methanol at ~20°C.

The product appears as a white powder of satisfactory purity, and can be stored in darkness at 0°C for several days, without any appreciable degradation.

**Spectroscopic Data I.** R: 3400, 2985, 2905, 1460, 1379, and 745 cm⁻¹. Ms: 516 [M⁺], 504, 387 (100), 245, and 130. 1H NMR: DMSO-d₆ (δ): 4.18 (m), 5.37 (m), 6.70–7.60 (m), and 10.70 (m). 13CH-NMR: CDCl₃ (δ): 21.2, 107.4, 111.0, 119.5, 120.2, 122.3, 128.8, 135.3, and 136.8.

**Cell Culture.** Three ER-positive (MCF-7, 734B, and BT474), two ER-negative (BT20 and MDA-MB-231), and an ER-negative and pRb-negative (I3C) human breast carcinoma cell line were used in the study. All of the cell lines were cultured in DMEM supplemented with 10% FCS, 2 mm L-glutamine, 1% nonessential amino acids, 50 μg/ml streptomycin, and 50 units/ml penicillin, with (MCF-7 and 734B) or without (MDA-MB-231, BT20, and BT-549) 10 μg/ml insulin.

The VERO African green monkey fibroblastoid kidney cells, the permanent ECV304 cells, and the Hep2 human epithelial cells were cultured in their appropriate growth medium supplemented with 10% FCS, 50 μg/ml streptomycin, and 50 units/ml penicillin.

All of the cell lines were grown in monolayer in a humidified atmosphere at 37°C with 5% CO₂. The experiments were performed with cells in the logarithmic phase of growth.

**[3H]Thymidine Incorporation.** Cells were seeded at a density of 30,000/well in 24-well tissue culture dishes and allowed to attach overnight. Duplicate samples of growing cells were treated for 24 h (ECV304), 48 h (Hep2, MCF-7, MDA-MB-231, 734B, and BT20), or 72 h (VERO, MCF-7, MDA-MB-231, 734B, BT20, BT474, and BT549) with increasing concentrations of tetramer or I3C TETRAMER INDUCED BREAST CANCER CELL GROWTH ARREST

**RESULTS**

Preparation and Characterization of the I3C Cyclic Tetrameric Derivative. I3C easily undergoes successive photoinduced transformations that lead to the formation of polymeric products. The photodreadful degradation is usually faster in acidic media and in the presence of an oxidizing agent (25). The formation of I3C tetramer dimer and trimer has already been reported (16, 26), but the mixture of oxidation products is really more composite. The high rate of oxidation of these molecules is because of the facile formation of a very stable benzylic-type carbocation through the loss of an alcoholic oxide and to the reactivity of this intermediate, which attacks the positions 2 or 3 of the indolic ring causing subsequent polymerization (27).

Here we report the preparation and characterization of one of the I3C polymerization products, that is the I3C cyclic tetrameric derivative (tetramer; Fig. 1).

This new molecule is mainly prepared by oxidation of the reagent with several oxidizing agents as potassium permanganate, potassium dichromate, acetic acid, phosphoric acid, hydrogen peroxide, and iodine, in the presence of solvents like ethyl ether, ethyl acetate, methanol, ethanol, and water.

Among the several ways to perform the I3C oxidation (data not shown), the reaction with iodine or acetic acid is better in terms of

**Western Blot Analysis.** CDK6, p27kip1, and pRb protein expression was assessed by Western blot. Untreated and tetramer-treated cells were lysed for 20 min on ice with 20 mM HEPES (pH 7.9), 25% glycerol, 0.4% NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂ containing 0.5% NP40, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM sodium fluoride, and 1 mM sodium orthovanadate. From the total protein extracted, 25 μg were fractionated on 10% (CDK6 and p27kip1) and 7.5% (pRb) SDS-PAGE, and then electrically transferred to nitrocellulose membranes. Blots were incubated with anti-CDK6 (1:200), anti-p27kip1 (1:500), and anti-pRb (1:1000) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C, and after horseradish peroxidase-conjugated secondary antibody. Blots were treated with enhanced chemiluminescence reagents, and all of the proteins were detected and quantitated by ChemiDoc System (Bio-Rad, Hercules, CA). Equal protein loading was confirmed by the level of actin protein present in the membrane treated with antiactin antibody 1:500 (Sigma).

**RNA Isolation, Hybridization, and Microarray Data Analysis.** Gene expression was analyzed using a commercially available nucleic acid array, the Panorama Human Apoptosis Gene Array (Sigma Genosys, St. Louis, MO). The membrane consists of 198 different human apoptosis-related genes, 8 housekeeping genes used as positive control for data normalization, 3 negative control genes, and 4 genomic DNA. Total RNA isolation was obtained from MCF-7 cells treated or not with 50 μM tetramer by TRIZol LS Reagent.

The results were normalized dividing the intensity of the signal gene by the average intensity of the signals of the 5 housekeeping genes that had a relatively constant expression level among the different samples. This procedure corrects differences in labeling, washing, and duration of exposure. To eliminate false-positive results, we reported only the genes with expression level differences ≥2.5 fold. **REFERENCES**

Preparation and Characterization of the I3C Cyclic Tetrameric Derivative. I3C easily undergoes successive photoinduced transformations that lead to the formation of polymeric products. The photodreadful degradation is usually faster in acidic media and in the presence of an oxidizing agent (25). The formation of I3C tetramer dimer and trimer has already been reported (16, 26), but the mixture of oxidation products is really more composite. The high rate of oxidation of these molecules is because of the facile formation of a very stable benzylic-type carbocation through the loss of an alcoholic oxide and to the reactivity of this intermediate, which attacks the positions 2 or 3 of the indolic ring causing subsequent polymerization (27).

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yield in cyclic tetramer (25%). The tetramer can be purified by silica gel column chromatography and crystallized from alcohol.

Effect of Tetramer on Cell Growth of Human Breast Cancer Cells. The antiproliferative activity of the I3C tetrameric derivative was initially tested on the estrogen-dependent MCF-7 breast cancer cell line. The cells were grown at subconfluency for 72 h in absence or presence of increasing concentrations of the new molecule. The effect of tetramer on the growth of MCF-7 cells was evaluated by [3H]thymidine incorporation into DNA. Results shown in Fig. 2, top, indicate that tetramer markedly reduced DNA synthesis in MCF-7 cells. The inhibition resulted dose-dependent, with an inhibition of cell proliferation >90% already at 30 μM.

To directly compare the effect of tetramer and I3C on cell proliferation, MCF-7 cells were cultured for 72 h in the presence of increasing concentrations of the two compounds. Results illustrated in Fig. 2, bottom, indicate that the I3C tetrameric derivative was about five times more effective in inhibiting DNA synthesis in MCF-7 cells than its indolic precursor. Specifically, IC50 values (that are the concentrations required to inhibit [3H]thymidine incorporation into DNA by 50%) were found to be 10.2 μM and 59 μM for tetramer and I3C, respectively. High-performance liquid chromatography evaluation of tetramer stability in the cell culture medium and in the MCF-7 cultures showed that the molecule is stable for at least 72 h and is not metabolized back to I3C (data not shown).

To test whether tetramer can suppress cell growth of human breast cancer cells independently from ER expression, we comparatively evaluated the effect of the compound on DNA synthesis of both ER-positive (MCF-7, 734B, and BT474) and ER-deficient (BT20, MDA-MB-231, and BT549) breast cancer cells. Fig. 3 indicates that tetramer given for 72 h at concentrations ranging from 5 to 50 μM caused a dose-dependent growth inhibition in all of the cell lines. To investigate whether this marked antiproliferative effects of tetramer is a common feature on mammalian cell, several cell lines were studied. For this purpose the VERO, ECV304, Hep2, and MCF-7 cells were exposed to increasing concentrations of tetramer. Results shown in Fig. 4 indicate that a strong dose-dependent inhibition of DNA synthesis was appreciable only in MCF-7 breast cancer cells. Conversely, only a modest modulation of [3H]thymidine incorporation was observed in the other cell lines.

Taken together, these results indicated that tetramer is a potent inhibitor of DNA synthesis of human breast cancer cells.

Tetramer Induces a G1 Cell Cycle Arrest on Human Breast Cancer Cells. To assess the cell cycle effects of tetramer, the four human breast cancer cell lines were exposed to different concentrations of the drug, and, at different intervals from the beginning of treatment, were stained with propidium iodide. Flow cytometry profiles of nuclear DNA content in the ER-positive cell lines MCF-7 and 734B revealed that the I3C-tetrameric derivative induced a dose-dependent accumulation of cells in the G1 compartment, which was already appreciable after 24 h of treatment. In treated MCF-7 cells, the extent of this accumulation was constant at the different time points, whereas a progressive increase in the percentage of cells blocked in the G1 phase was observed over time in treated 734B cells. The G1 phase cell accumulation was paralleled by a marked reduction in the percentage of S phase cells in both cell lines (Fig. 5, A and B). Although to a lesser extent, an accumulation of cells in the G1 compartment after treatment with tetramer was also observed in ER-negative cell lines BT20 and MDA-MB-231 cells (Fig. 5, A and B). However, when we exposed the ER-negative pRB-negative BT549 cell line to the same concentrations of I3C tetrameric derivative we failed to observe any G1 phase cell accumulation at all of the exposure times considered (Fig. 5B).
Tetramer Induces a Cytostatic Effect on Breast Cancer Cells.

To evaluate whether the reduced level of \(^{3}H\)thymidine incorporation observed in I3C tetrameric derivative-treated cells was attributable either to a cytotoxic or a cytostatic effect, we evaluated the cell viability of MCF7 cells after treatment with increasing concentrations (5–50 \(\mu M\)) of the compound. After 24, 48, and 72 h of treatment, the cells were harvested, and cell death was determined by trypan blue dye exclusion test. As shown in Fig. 6A, tetramer caused a dose-dependent inhibition of cell growth; however, it is interesting to note that the compound did not cause any significant cell killing, even at concentrations that caused >90% inhibition of cell growth (see top of Fig. 6A). Similar findings were obtained in MCF-7 cells treated with I3C (Fig. 6B). Furthermore, tetramer-induced growth suppression of MCF-7 cells was largely reversible on drug removal (data not shown). These results would suggest that tetramer blocks cell proliferation without affecting cell viability. This hypothesis is also supported by the data obtained from apoptosis studies. In fact, flow-cytometric analyses carried out in MCF-7 cells after treatment with tetramer did not evidence the presence of any significant pre-G\(_1\) apoptotic cell population (data not shown). Moreover, when the percentage of treated cells with an apoptotic nuclear morphology was determined under fluorescence microscopy after cell staining with propidium iodide or Hoechst 33342 and ethidium bromide, we found that apoptotic cells represented <1% of the overall population.

The results of these experiments indicate that tetramer does not cause any significant cell death in human breast cancer cells and suggest that the growth arrest is because of cytostatic mechanisms.

Effect of Tetramer in Cell Cycle-dependent Proteins. To investigate the molecular basis of tetramer-induced arrest of cells in the G\(_1\) phase, we focused our attention on the effect exerted by the compound on proteins involved in the G\(_1\) to S transition. MCF-7 and MDA-MB-231 cells were seeded at subconfluency and cultured for 72 h in the absence or presence of various concentrations of tetramer. Results from Western blot experiments carried out in MCF-7 and MDA MB-231 cells showed that the tetramer selectively decreased the level of CDK6 and increased the level of the CDK inhibitor p27\(^{kip1}\) in both cell lines, although to a different extent. As shown in Fig. 7, A and D, in MCF-7 cells tetramer reduced the baseline level of CDK6 to 50% when used at a concentration of 20 \(\mu M\) and to 25% when used at a concentration of 50 \(\mu M\). In MDA-MB-231 cells similar levels of CDK6 inhibition were obtained with 40 and 100 \(\mu M\) of tetramer, respectively (Fig. 7, A and E).

In MCF7 cells treated with tetramer the level of p27\(^{kip1}\) protein increased in a dose-dependent manner up to 180% of the baseline (Fig. 7, B and D), whereas in MDA-MB-231 cells the level of p27\(^{kip1}\) increased >20 fold when tetramer was used at 50 \(\mu M\) and >30 fold if tetramer was used at 100 \(\mu M\). However, the effect of tetramer on cell cycle proteins was already markedly pronounced after 22 h of treatment. At this time, indeed, treatment of MCF-7 cells with 50 \(\mu M\) tetramer reduced the baseline level of CDK6 to 30%, increased of p27\(^{kip1}\) protein up to 170%, and decreased the hyperphosphorylatate Rb to 27%. Interestingly, the intracellular level of CDK4, the other kinase that associates with cyclin D in the G\(_1\) phase of the cell cycle, was not altered by treatment (data not shown). This result demonstrates the specificity of tetramer action on the intracellular level of CDK6 and p27. Overall, this experiment indicates that the changes in intracellular levels of CDK6 and p27 may be involved in the origin of growth inhibition and G\(_1\) cell cycle arrest of both ER-positive and ER-deficient human breast cancer cells.

Tetramer-dependent Modification of CDK6, p27\(^{kip1}\), and pRb.

One of the most important substrates of the G\(_1\)-CDKs is the pRb. To determine whether the changes in the intracellular levels of
CDK6 and p27kip1 induced by tetramer on MCF-7 and MDA-MB-231 cells are associated with specific changes in the pattern of RB phosphorylation, we examined the level of phosphorylated pRb in these cells after treatment with tetramer by Western blot analysis using phosphorylation-sensitive Rb-specific antibody. As shown in Fig. 8, tetramer treatment induced a significant decrease in the amount of pRB and ppRb; at concentration of 50 μM in MCF-7 cells and 100 μM in MDA-MB-231 cells, the expression of ppRb became nearly undetectable.

These results suggest that in MCF-7 and MDA-MB-231 cells...
treated with tetramer, the observed decrease in CDK6 and increase in p27kip1 levels are associated with very low levels of endogenous ppRb and cell cycle arrest.

**MCF7 Treated with Tetramer Showed Up-Regulated Expression of Apoptosis-related Genes.** To investigate whether tetramer treatment induced specific changes in the expression of apoptosis-related genes, we studied the patterns of gene expression using a cDNA macroarray containing 198 genes involved in apoptosis and cell cycle, and prepared 32P-labeled cDNA from MCF-7 cells untreated or treated for 36 h with 50 μM tetramer. In the treated cells the expression of several apoptosis-related genes was up-regulated (data not shown). Among genes of which the products are known to have antiapoptotic functions, we found overexpression of cyclophilin c (4.6-fold), Bid (2.5-fold), p75 (low affinity nerve growth factor receptor, 2.7-fold increase), prostate apoptosis response 4 (2.5-fold increase), Fas, (7.7-fold increase), and DR5 (or TRAIL-R2, 4.9-fold increase). Moreover, consistent with Western blot results, we observed that treatment of MCF7 cells with tetramer also increased the expression of the p27kip1 gene (2.7-fold).

**DISCUSSION**

Breast cancer is the most common neoplasm in women and is increasing in both developing and developed countries (4, 28). A considerable fraction of patients is resistant, intrinsically or after prolonged treatment, to conventional hormonal or chemotherapeutic agents, and surgery has been the treatment of choice in the majority of cases. Epidemiological studies have evidenced that the consumption of vegetables may have chemopreventive activity against various kinds of cancer (2). In particular, I3C, a natural component of Brassica vegetables, has been shown to arrest the growth of human cancer cells in vitro and possess anticarcinogenic activity in vivo (6–8, 10–12).

In this study we have synthesized a stable I3C derivative (a tetramer) and investigated its activity on human breast cancer cell lines. Our results show that tetramer markedly reduced, in a dose-dependent manner, DNA synthesis in breast cancer cells and induced accumulation of cells in the G1 phase of the cell cycle. We have demonstrated that tetramer interferes with the cell cycle-related proteins. In particular, this new molecule selectively decreases the level of CDK6, increases the level of the CDK inhibitor p27kip1, and decreases the amount of endogenous ppRb. As a consequence, the hypophosphorylated form of Rb binds and inactivates the E2F transcription factors, thus arresting the cell cycle. These results indicate that, at least for the parameters investigated, tetramer inhibits the proliferation of breast cancer cells through molecular mechanisms that are similar to those described for I3C (6, 7), although experiments carried out in 734B (ER-positive) and BT20 (ER-negative) breast cancer cell lines indicated that tetramer is more effective than I3C in modulating the expression of proteins involved in G1 to S transition (data not shown). Furthermore, the finding that the ppRb-negative BT-549 cell line was highly sensitive to the tetramer even in the absence of any G1 phase cell accumulation would suggest that CDK6 is not the sole relevant molecular target for the tetramer activity, and that additional molecular mechanisms (to be additionally investigated) may be involved in sustaining the antiproliferative effect of this compound. Tetramer also has two potential advantages over I3C. Tetramer resulted a very stable compound, whereas the chemical instability of I3C (16, 29) may present a limitation in the development of this compound. Moreover, it is not yet clear whether the biological activity observed is because of I3C or some of its polymeric products formed in acidic environments such as in the digestive tract (6, 12, 17–19).

Second, tetramer resulted more effectively (about five times more) than I3C in inhibiting the growth of MCF-7 cells (Fig. 2B). These findings are very important and suggest that tetramer could overcome the problem of the high dose of I3C required to suppress the growth of breast cancers (30). Previous studies reported that I3C (6) and its linear trimeric product, the 2-indol-3-ylmethyl)-3,3'-diindolylmethane (17)
inhibited the growth of breast cancer cells independently of ER presence. In this study, using various breast cancer cell lines, we found that tetramer inhibited cell proliferation and induced a G1 block in cell cycle of both estrogen-dependent and -independent breast cancer cell lines. Moreover, as already reported above for ER−/H11001 cells, tetramer resulted more effectively (2-fold) than I3C in ER−/H11002 cells as well (data not shown).

Currently, the antiestrogen tamoxifen is the most widely used agent in tumors expressing ER receptors (28, 31). Unfortunately, only ~50% of the patients with ER+ tumors respond to this therapy (30) and, after prolonged treatment, the development of tamoxifen-resistance occurs in a fraction of patients (32). Thus, the combination of tetramer and tamoxifen could represent a potentially new therapy for human breast cancer expressing ERs. Experiments in progress in our laboratory support this hypothesis because we found that combinations of tamoxifen and tetramer are more effective in suppressing the growth of ER+ breast cancer cells than either agent alone.

A very interesting finding provided by this study is the preferential activity of tetramer on the breast cancer cell types. Treatment with a tetramer concentration that induced 50% growth inhibition in MCF-7 cells did not result in a detectable growth inhibition in others mammalian cell lines (Fig. 3). Because we used cell lines with a high replicative rate, this result may be of great value for the selective therapeutic index of tetramer.

Tetramer was found to exert a good antiproliferative activity, although it failed to cause any significant loss of cell viability or

Fig. 7. Effects of the I3C tetrameric derivative on expression of G1 cell cycle proteins of MCF-7 and MDA-MB-231 breast cancer cells. MCF-7 (left) and MDA-MB-231 (right) cells were plated at a density of 600,000 cells onto 60-mm tissue culture dishes and, after attachment, treated with the tetramer for 72 h. Total cell extracts were separated by SDS/PAGE on a 10% polyacrylamide gel and analyzed for (A) CDK6, (B) p27kip1 and (C) actin proteins by Western blot. The relative level of CDK6 (A) and p27kip1 (B) proteins in (D) MCF-7 and (E) MDA-MB-231 cells treated with different concentrations of the tetramer was quantitated by ChemiDoc System, normalized to actin, and expressed as a percentage of untreated value (which was set at 100%) or as a ratio versus the untreated value (E; p27kip1). The values are the means of five different experiments that are in agreement within 10%.

Fig. 8. The I3C tetrameric derivative-induced modifications of G1-related proteins reduce the level of endogenous phosphorylation of Rb protein in MCF-7 and MDA-MB-231 cells. MCF-7 (top) and MDA-MB-231 (bottom) cells were treated with the tetramer at the indicated doses for 72 h. Total cell extracts were separated by SDS/PAGE on 7.5% polyacrylamide gel and analyzed by Western blot using a phosphorylation-sensitive Rb-specific antibody. ppRb exhibits a characteristic mobility shift when compared with the hypophosphorylated (pRb) alone. The levels of pRb and ppRb were quantitated by ChemiDoc System, normalized to actin, and expressed as a percentage of the untreated value, which was set at 100%, in MCF-7 (■) and MDA-MB-231 (□) cells. The values are the mean of three different experiments; bars, ±SD.
apoptosis induction in MCF-7 cells, at least during the observation period used in this study. Therefore, the tetramer-induced growth arrest of MCF-7 cells in the G1 phase of cell cycle seems to be because of a cytostatic mechanism. Similar results have been reported for I3C (our data; Refs. 6, 7) and for 2-(indol-3-ylmethyl)-3,3-diindolylmethane, an I3C derivative (17). Ge et al. (19) reported that I3C was able to induce apoptosis in MCF-7 cells. This result is at variance with the data reported in this paper and may be because of the use of different MCF-7 clones in the different laboratories.

The apoptotic process requires the activation and action of a set of cysteine proteases, named caspase, that act in a coordinated sequence once appropriate stimuli have been received (33, 34). Of these proteases, caspase 3 plays a key role in apoptotic cell death induced by many death signals (35), including chemotherapeutics (36). This protease, once activated by apical caspases (caspase 8 and caspase 9), cleave cellular death substrates (37). MCF-7 cells were found to be deficient in caspase 3 as a consequence of a mutation in the CASP-3 gene (38, 39). It was reported that deficiency in caspase 3 may contribute to drug resistance in breast cancer and that the reconstitution of caspase 3 sensitizes MCF-7 cells to drug-induced apoptosis (40). Consequently, the deficiency in caspase 3 could explain, at least in part, why tetramer failed to induce apoptosis in MCF-7 cells. However, this hypothesis needs to be additionally investigated, because the role of caspase 3 in the apoptotic pathway still remains controversial. In fact, it was also reported that a derivative of I3C, 3,3′-diindolylmethane, induced apoptosis in various human cancer cell lines, including MCF-7 (12). Furthermore, others studies reported that caspase 3 was not necessary for apoptosis induced by vitamin D compounds (41), tumor necrosis factor α (42), staurosporine (42), tributyrin (43), or, as reported by Xue et al. (44), caspase 3 may accelerate apoptosis in MCF-7 cells induced by photodynamic therapy, but is not important in the critical lethal event. Again, alternative apoptotic pathways independent of caspase 3 in MCF-7 have also been suggested (45).

In the apoptotic pathway there is a delicate balance between anti- and proapoptotic stimuli. In our studies we observed that tetramer-treated MCF-7 cells showed alterations in the expression of some antiapoptotic (DAD1, PIN, and MDM2) and proapoptotic (cytochrome c, Bid, p75, Par 4, Fas, MDM2, and DR5) genes. Among proapoptotic genes, Fas and DR5 resulted as highly overexpressed. Fas (which is 7.7-fold higher in treated cells, when associated with its ligand, Fas-L, induces a rapid proteolytic cascade resulting in apoptotic death (46, 47). Fas induced apoptosis by the activation of caspase 8, which lead to a series of downstream events including activation of effector caspases, cleavage of several caspase substrates, and induced BID dependent-mitochondria damage (48). It has been shown that some chemotherapeutic agents increase Fas expression, whereas the loss of Fas expression is involved in the development of resistance to chemotherapy (49). DR5 (also called TRAIL-R2), another death receptor (for TRAIL), showed a 4.9-fold increase in tetramer-treated cells. Interaction of TRAIL with DR5 induces apoptosis in tumor cells via the activation of caspase 8 (50).

Such evidence would suggest that, although tetramer induced an accumulation of MCF-7 cells in the G1 phase without evidence of apoptosis, tetramer-treated cells, which display a marked overexpression of some dead receptors, may activate the apoptotic pathway after interaction with specific ligands. The overexpression of several death receptors in MCF-7 cells treated with tetramer may be very relevant in vivo. Indeed, other than to arrest the proliferation of breast cancer cells in the patient, we speculate that tetramer could induce the overexpression of Fas and DR5 in the same cells that could be recognized and killed by competent TCD4+ and TCD8+ circulating cells.

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A New Indole-3-Carbinol Tetrameric Derivative Inhibits Cyclin-dependent Kinase 6 Expression, and Induces G₁ Cell Cycle Arrest in Both Estrogen-dependent and Estrogen-independent Breast Cancer Cell Lines

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