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Effects of Indole Oligomers Induced from Indole-3-carbinol on the Growth of MCF-7 Breast Cancer Cells

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Abstract

Inhibitory effect of indole oligomers induced from indole-3-carbinol (I3C) on the growth of breast cancer cells was studied. We generated the reaction mixtures (RXM) at ambient temperature by treating a stirred aqueous solution of I3C (typically 0.25 ml at a concentration of 12 μmol/ml) with hydrochloric acid (typically 28 μl of a 1 mmol/ml solution). RXM was fractionated by the column chromatography. The fractions with similar UV-pattern were further fractionated by HPLC and 3,3'-diindoylmethane (DIM) and other indole oligomers were identified. I3C, RXM, and its derived indole compounds were added to MCF-7 cells and cultured in the presence of 10^{-7}M estradiol for 7 days. The growth-inhibitory effect of I3C and DIM on the growth of MCF-7 cell was very strong. The synthetic DIM also revealed antiproliferative effect on MCF-7 cell. The fractions containing high DIM content (77%), were most effective in inhibiting MCF-7 cell growth induced by estradiol. With these results, we suggest that I3C and DIM might have anticarcinogenic effect on the breast cancer.

Key words : I3C, RXM, DIM, indole oligomer, anticarcinogen, cancer

INTRODUCTION

Vegetables of the *Brassica* genus contain an 3-indolylmethyl glucosinolate, commonly known as glucobrassicin. Levels of thioglucosides are reported to be as high as 1100 μg/g in some cultivars (1), while Korean cabbage and radish contained average 125 μg/g, 160 μg/g, respectively (2). 3-Indolylmethyl glucosinolate was shown to be the predominant indole glucosinolate in cabbage, broccoli, Brussels sprouts, Korean cabbage, and radish (3,4). Virtanen (5) demonstrated that, following rupture of the cells of plant material, 3-indolylmethyl glucosinolate is hydrolyzed by the endogenous enzyme myrosinase (thioglucoside glucohydrolase, EC3.2.3.1) to a number of compounds including indole-3-carbinol (I3C) and thiocyanate ion. I3C and thiocyanate ion have been shown to be the major product of the autolysis of 3- indolylmethyl glucosinolate, but due to the instability of I3C in aqueous solution the condensation product 3,3'-diindoylmethane (DIM) and/or the oxidation product 3-indolecarbonaldehyde tend to predominate (5,6). Increased consumption of raw vegetables and cabbage has been associated with a decreased risk of rectal and bladder cancers. The inhibition of dimethylbenzanthracene-induced mammary carcinoma in rats by I3C (3) and inhibition of aflatoxin B_1-induced hepatic oncogenesis in rats fed with a 25% freeze-dried cabbage diet were reported (4). In the studies with laboratory animals, indole derivatives, specifically I3C, DIM, and 3-indole acetonitrile, have been shown to inhibit forestomach neoplasia and mammary tumor formation and to increase the activity of key enzymes involved in cellular detoxification systems (7). I3C is a potent inducer of cytochrome P-4501A1 (CYP1A1)-dependent monooxygenase and can modulate the potency of carcinogens. When administered before a carcinogen, I3C can act as an inhibitor of cancer initiation, most likely by altering carcinogen metabolism (7). If admininistered after a carcinogen, I3C can act as a promoter of carcinogenesis (8). I3C also modifies estrogen metabolism in humans and is under study as a protective agent against mammary cancer (9). These dual cancer-modulating activities are generally not seen in substances of small molecular weight such as I3C but is a characteristic of certain polycyclic aromatic substances (10). An explanation for

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this unusual activity of I3C arises from the observation that I3C is highly sensitive to comparatively mild acidic conditions (11) and under these conditions I3C is converted to biologically active substances (12). Bjeldanes et al. (13) reported prevously that among these substances there is indole [3,2-b]carbazole which, in contrast with I3C, is a potent inducer of CYP1A1 without contact with acid and has a high affinity for Ah receptor protein. The Ah receptor is a control factor for the synthesis of CYP1A1 and binds with high affinity to TCDD (14).

In this study we investigated the major oligomeric products of I3C produced in aqueous acid and the effects of reaction conditions on the composition of mixture. Our purpose was to gain insight into the chemical processes by which I3C is converted into biologically active substances under aqueous acidic conditions mimicking those found in gastric juice. Also we studied the effect of I3C fractions separated by HPLC on MCF-7 cell proliferation.

MATERIALS AND METHODS

Chemicals

We purchased I3C from Aldrich Chemical Co. (Milwaukee, WI, USA) as the hydrate and purified it by recrystallization from toluene. MCF-7 human breast cancer cells were obtained from Grace Chang (5 generations), University of California at Berkeley (USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serium were from Gibco (Grand Island, NY, USA). 17-β estradiol (E_2) were purchased from Sigma Chem. Co. (St. Louis, MO, USA).

Preparation of the acid reaction mixture (RXM)

Using the procedure reported by Leete and Marion (11), we generated the reaction mixtures at ambient temperature by treating a stirred aqueous solution of I3C (typically 0.25 ml at a concentration of 12 μmol/ml) with hydrochloric acid (typically 28 μl of 1 mmol/ml solution). The solution was stirred for 10 min and neutralized with aqueous ammonia (typically 115 μl of 0.25 mmol/ml solution). For the HPLC analysis of the reaction products, we then added tetrahydrofuran (THF) (typically 607 μl to give a final sample volume of 1 ml), other organic solvents and reagents, and injected portions of the solution. For the preparative-scale reactions (typically starting with 400 mg of I3C in 400 ml of water), we filtered the neutralized mixture, washed the precipitate with water, and then air-dried the product while keeping it protected from ambient light.

Synthesis of the diindol-3-ylmethane (DIM)

Using the method reported by Leete and Marion (11). 1.0 g I3C was refluxed with 100 ml 10% sodium hydroxide for one hour. The solution was cooled, neutralized with conc. HCl (pH 7.0), and filtered. The filtered substance was crystallized from benzene to yield glistening plates of DIM, with m.p. 164°C, yield 37%.

Isolation of active compounds

Using the same extraction scheme mentioned above, larger scale reaction mixtures (typically 400 mg or more) were prepared for preparative chromatography. The concentrated CH_2Cl_2 extract of indole compounds was applied to a short column of silica gel (0.06~0.2 mm particle size, 2.5×10 cm) and then eluted with 5% 2-propanol in hexane. The major components were eluted after about 20 fractions of 20 ml each in the preparative HPLC. Fractions with compounds in common were pooled, evaporated, dissolved in DMSO, and submitted for growth inhibition assay.

Cytotoxicity of compounds and indole fractions

To study antiestrogen effects of RXM, I3C, and DIM their growth inhibitory activities were determined in estrogen-responsive MCF-7 cells in the presence of estradiol. Growth inhibitory responses were determined for a range of doses of each sample and estrogen with a single high dose of each sample. MCF-7 cells were seeded into 24-well plastic tissue-culture plates in phenol red-free medium consisting of Dulbecco's Eagles's medium supplemented with 10% fetal bovine serum and containing penicillin, streptomycin, insulin, L-glutamine, and non-essential amino acids, and incubated in a humidified atmosphere chamber containing 5% CO_2 at 37°C. The culture was re-fed at 48 h with 4 ml growth medium containing experimental fractions with or without 1 nM estrogen dissolved in DMSO (≤0.1%, v/v). On 7th day the culture was washed, trypsinized, and aliquots were counted with a Coulter counter (ZM, USA).

HPLC methods

For all of the HPLC analyses a C-18 column (Beckman

Ultrasphere-ODS, 0.46 [I.D] × 25 cm) was used. The mobile phase was 60% (v/v) acetonitrile in 31 mM ammonia dihydrogenphosphate adjusted to pH 6.7 with aqueous ammonia. The injection volumes were 10 μl and the flow rate was 1 ml/min. The detector was a Perkin-Elmer model LS-4 fluorescence spectrometer set for excitation at 335 nm and emission at 415 nm.

RESULTS AND DISCUSSION

Yields and characterization of oligomers

The complexity of the mixture of products found in the RXM is shown in the HPLC chromatogram of Fig. 1 where the peaks were detected using UV absorption at 280 nm. By directly measuring the molar yield for peak A compound (identifeid as DIM) and the other compounds of the RXM, we estimated the corrected molar yields for each compound. We identified peak B as the symmetrical cyclic trimer 5,6,11,12,17,18-hexahydrocyclononal[1,2-b:4,5-b':7,8-b"]triindole (Ctr) using HPLC and MS analyses. Peak C was identified as the linear trimer [2-(indole-3-ylmethyl)-indol-3-yl]indol-3-ylmethane (Ltr1) on the basis of report by Leete and Marion (11). The rest of compounds were 3,3-bis(indol-3-ylmethyl)indolenine (Ltr2), a cyclic tetramer (CTet), and a linear tetramer (LTet), respectively. Formerly these three compounds were produced from indole glucosinolate of cruciferous vegetables, Korean cabbage and radish, in gastric juice. The relative concentrations were DIM 15.1%, Ctr 12.3%, and Ltr 25.0%. The fractions (#1~20) from column were collected for further experiment of anticarcinogenesis. We divided the fractions as follows: #1~6, #7~10, #11~13, #14~16, #17~18, and #19~20. The fractions of the peaks were pooled, concentrated by nitrogen gas, and subjected to HPLC analysis. The relative concentration of DIM in each fraction was 77.2% for #11~13, 64.4% for #14~16, 54.6% for #7~10, 47.2% for #1~6, 24.0% for #17~18, and 10.9% for #19~20, respectively(Table 1).

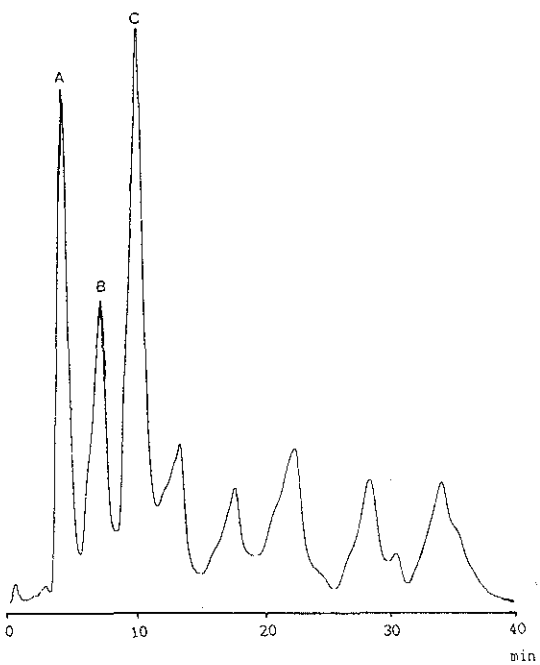

Fig. 1. HPLC chromatography of RXM.
7 μl sample injected onto a 0.46 × 25 cm C-18 column. The sample was eluted using 60% acetonitrile in water and detected at 280nm.
A: DIM, B: CTr, C: LTr1

I3C, DIM, and indole-3-acetonitrile, three indoles occuring in edible cruciferous vegetable, had been studied for their effects on 7,12-dimethylbenz(a)anthracene- induced mammary tumor formation in female Sprague- Dawley rats and on benzo(a)pyrene-induced neplasia of the forestomach in female ICR/Ha mice. When given by p.o. intubation 20 hr prior to 7,12-dimethylbenz(a)anthracene administration, I3C and DIM had an inhibitory effect on mammary tumor formation, but indole-3-acetonitrile was inactive (7). I3C inhibited nitrosamine 4-(methylnitrosamino)-1(3-pyridyl)-1-butanone-induced lung neoplasia in A/J mice. It was suggested that the basis of this inhibition is the decrease in O^6-methylguanine formation in A/J lung caused by I3C pretreatment (15). I3C was reported to be unstable in acidic environment of the stomach and yield an excess of products among which DIM, Ctr, 2,3-bisskatyindole and 5,11-dihydroindolo [3,2-b]carbazole (ICZ) were the most important (13). ICZ appeared to be responsible for the enzyme-inducing effects of I3C. But its binding affinity to aromatic hydrocarbon responsiveness-receptor was only a factor of 3.7×10^{-2} lower than that of highly toxic contaminant and cancer

Table 1. The relative concentration of indole oligomers in the fractions and original RXM

		Oligomer compounds (%)					
		DIM	Ctr	Ltr1	Ltr2	CTet	LTet
RXM		15.1	12.3	25.0	10.2	6.9	10.8
fraction	#1~6	47.2	17.3	14.5	12.1	-	-
	#7~10	54.6	15.5	13.6	9.6	-	-
	#11~13	77.2	2.7	7.0	6.4	-	-
	#14~16	64.4	2.3	10.8	14.9	-	-
	#17~18	24.0	4.1	28.1	27.7	-	-
	#19~20	10.9	64.8	14.7	6.0	-	-

promotor 2,3,7,8-tetrachlorodibenzo-p-dioxin (13). Also ascorbigen, rather than I3C, was the most important indole derivative for the enzyme-inducing effects of dietary cruciferous vegetables (16). Grose and Bjeldanes (17) compared the identities of the major oligomeric products of I3C produced under conditions approximating those found in gastric juice with the reported identities of products of 3-substituted indoles produced under enzymatic and other nonenzymatic conditions. After 10 min treatment in aqueous HCl solution, I3C was converted into 18% yield to a mixture of acetonitrile-solution products, the major component of which was DIM (5.9%) as determined by HPLC.

Effects of different concentrations of estradiol on the growth of MCF-7 cells

Fig. 2 shows the effects of different concentrations of estradiol on MCF-7 cell growth. Optimal growth was achieved at 10^{-7} M estradiol. Estradiol increased both the growth rate and final saturation density of the cells in a dose-dependent manner, with maximal stimulation occuring between 10^{-10} and 10^{-8} M estradiol. A major loss of estradiol response was found if the cells were grown for 7 to 14 days in the absence of estradiol (18).

Biological effects of indole derivative

To study effect of indole compounds on human breast cancer cells (MCF-7), the growth rate of MCF-7 cells in the presence of HPLC fractions (6 pooled divisions) was determined. As shown in Fig. 3, the number of cells was 1.5×10^5 for fraction #11-13, 1.8×10^5 for #14-16, 1.9×10^5 for #7-10, 2.2×10^5 for #1-6, 3.3×10^5 for #17-18, and 6.5×10^5 for #19-20, respectively. The plate containing RXM and DIM appeared to contain 6.2×10^5 cells and 1.2×10^5 cells, respectively. I3C was unstable in acidic condition, as RXM formed diverse indole oligomer compounds. We guessed that the oligomer of I3C had activity as the inhibitor of cancer initiation and promotion. We identified anticarcinogenesis effects at different DIM concentrations. Extremely high contents of DIM (#11-13, 77.2%) suppressed cell number to 1.5×10^5, whereas the plate containg the lowest contents of DIM (#19-20, 10.9%) had 6.5×10^5cells. RXM-containing plate had 6.2×10^5 cells, and one containing synthetic DIM was 1.2×10^5 cells. We suggested that DIM could act to inhibit the growth of mammary cancer cell, but other oligomer compounds were inactive. More study about the effects of biological activity of RXM and its oligomer compounds need to be performed *in vivo*. Dietary indoles in cruciferous vegetables were reported to induce cytochrome p-450 enzymes and prevented tumor in various animal models. Because estradiol metabolism was also cytochrome p-450 mediated and linked to the breast cancer risk, indoles might reduce estrogen-responsive tumors in humans. These results indicated that I3C strongly influenced estradiol metabolism in humans and provided a new chemopreventive approach to estrogen-dependent diseases (9).

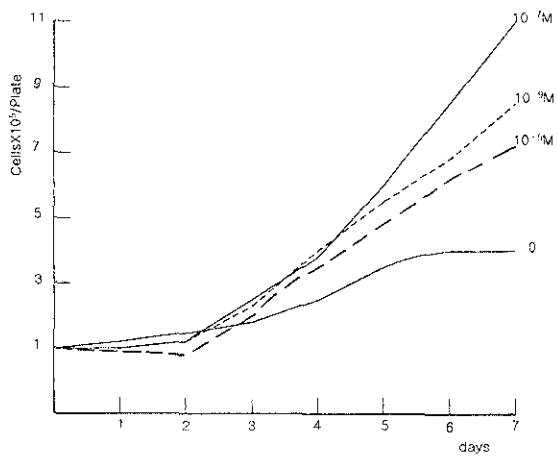

Fig. 2. Effects of different concentrations of estradiol on the growth of MCF-7 cells in 10% FBS DMEM.
Cells were seeded at a density of 1.0×10^5 cells/plate. On days 1,2,3,4,5,6,7 cells were harvested by brief trypsinization, and viable cells were counted by Coulter counter.

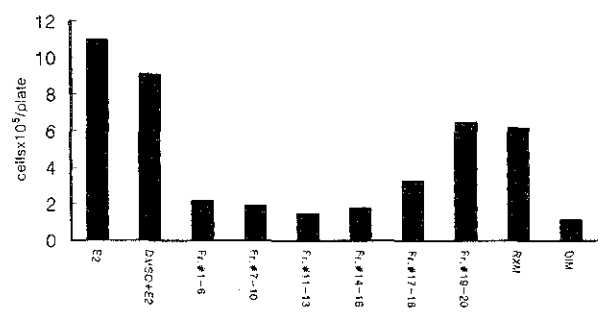

Fig. 3. The growth inhibition of MCF-7 breast cancer cells by RXM and its fraction for 7 days.
Cells were adjusted to a density of 1.0×10^5 cells/plate, and plated into 60×15 mm culture dishes containing as follows: total volume was 4 ml depleted medium, E2 (added 40 μl 10^{-7} M E2), DMSO +E2 (added 4 μl DMSO+40 μl 10^{-7} M E2), fractions, RXM, and DIM (added 40 μl 10^{-7} M E2+4 μl 10^{-1} M each fraction sample)

Effect of antiestrogen I3C on the proliferation of MCF-7 cells

Effects of 10^{-7} M E_2 and 10^{-1} M I3C on cell growth were shown in Fig. 4. Physiological concentrations of E_2 clearly stimulated cell division while the antiestrogen I3C was strongly inhibitory. I3C-treated cells decreased in the cell number for 2 days. The cell wall of the MCF-7 cells severely got pressed out of shape (data not shown) as observed by electron microscopy. After 3 days the cell wall recovered the natural shape by nature. We suggested that I3C can act as antiestrogen-like substance. Lippman had established or characterized six lines of human breast cancer maintained in long-term tissue culture for 1 year and had examined these lines for estrogen responsiveness. One of these cell lines, MCF-7, shows marked stimulation of macromolecular synthesis and cell division with physiological concentration of estradiol. Some antiestrogens were strongly inhibitory, and at concentrations greater than 3×10^{-7} M killed cells (19). Since melatonin, the major hormone of the pineal gland, had been shown to inhibit the growth of mammary tumors in animal models of human breast cancer, Hill and Blask (21) examined the hypothesis that this indoleamine had the potential to inhibit breast cancer growth by directly inhibiting cell proliferation as exemplified by the growth of the estrogen-responsive human breast cancer cell line MCF-7 in culture (20). Inhibition of activator protein-1 activity by anti-estrogen was unlikely to be explained by the presence of residual estrogens in MCF-7 cells. OH-tamoxifen was efficient in inhibiting estrogen-responsive element-mediated activity. I3C was clearly the most potent inducer of estradiol 2-hydroxylation, whereas indole-3-acetonitrile, another abundent indole in cruciferous vegetables, was only half as effective (9). Dietary indoles can also enhance estradiol metabolism. With these results, we suggest that I3C influences estradiol metabolism in humans and may provide a chemopreventive approach to estrogen-dependent diseases.

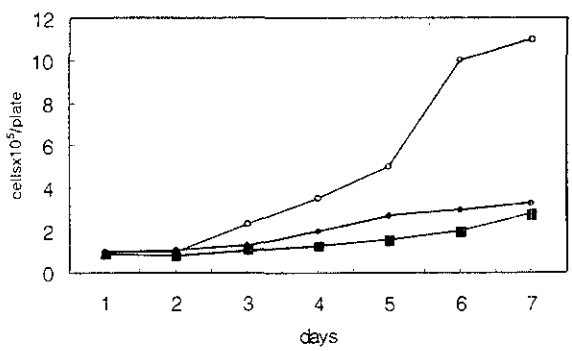

Fig. 4. The effects of antiestrogen I3C on MCF-7 cell proliferation.
Cells were seeded and harvested as described in Fig. 2.
—○— E2, —■— E2+I3C, —◆— NO E2

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