Biochemical Pharmacology, Vol. 51, pp. 1069–1076, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00060-3

Indole-3-carbinol and Diindolylmethane as Aryl Hydrocarbon (Ah) Receptor Agonists and Antagonists in T47D Human Breast Cancer Cells

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ABSTRACT. Indole-3-carbinol (I3C) is a major component of *Brassica* vegetables, and diindolylmethane (DIM) is the major acid-catalyzed condensation product derived from I3C. Both compounds competitively bind to the aryl hydrocarbon (Ah) receptor with relatively low affinity. In Ah-responsive T47D human breast cancer cells, I3C and DIM did not induce significantly CYP1A1-dependent ethoxyresorufin O-deethylase (EROD) activity or CYP1A1 mRNA levels at concentrations as high as 125 or 31 μM, respectively. A 1 nM concentration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induced EROD activity in these cells, and cotreatment with TCDD plus different concentrations of I3C (1–125 μM) or DIM (1–31 μM) resulted in a > 90% decrease in the induced response at the highest concentration of I3C or DIM. I3C or DIM also partially inhibited (< 50%) induction of CYP1A1 mRNA levels by TCDD and reporter gene activity, using an Ah-responsive plasmid construct in transient transfection assays. In T47D cells cotreated with 5 nM [^{3}H]TCDD alone or in combination with 250 μM I3C or 31 μM DIM, there was a 37 and 73% decrease, respectively, in formation of the nuclear Ah receptor. The more effective inhibition of induced EROD activity by I3C and DIM was due to *in vitro* inhibition of enzyme activity. Thus, both I3C and DIM are partial Ah receptor antagonists in the T47D human breast cancer cell line. BIOCHEM PHARMACOL 51;8:1069–1076, 1996.

KEY WORDS. indole-3-carbinol; diindolylmethane; T47D cells; Ah receptor antagonist

Several studies have reported that laboratory animals fed a diet containing cruciferous vegetables are protected from the development of various spontaneous and carcinogen-induced tumors [1–3]. For example, there is a decreased incidence and growth of DMBA§-induced mammary tumors in female Sprague–Dawley rats maintained on a diet containing brussels sprouts [2]. I3C is a major secondary metabolite found in cruciferous vegetables, and this compound also exhibits a broad spectrum of anticarcinogenic activities [4–9]. I3C inhibits several carcinogen-induced tumors at various sites in rodent models [5–9] and also decreases the development of spontaneous mammary and endometrial tumors in female C3H/OuJ mice [4] and Donryu rats [7], respectively. I3C induces phase I and phase II drug-metabolizing enzymes in both laboratory animals and humans, and these include several CYP isoforms and their dependent enzyme activities: glutathione S-transferase, glucuronosyl transferase, NAD(P)H:quinone oxidoreductase, and epoxide hydrolase [10–18]. In studies with carcinogens such as benzo[*a*]pyrene and aflatoxin B_1, the induction of these enzyme activities correlates with altered metabolism and decreased levels of carcinogen-DNA and other adducts [19, 20]. Several studies have also demonstrated that I3C undergoes acid-catalyzed self-condensation to give several products including DIM and ICZ [17, 21, 22]. These I3C-derived compounds bind with variable affinities to the Ah receptor and thereby constitute one of the major classes of endogenous or natural compounds that bind to this receptor [15, 17, 23]. For example, competitive Ah receptor binding studies, using TCDD as the radioligand, give relative binding affinities of 1.0, 3.7×10^{-2}, 7.8×10^{-5}, and 2.6×10^{-7} for TCDD, ICZ, DIM, and I3C, respectively [17]. Similar results were also reported by Jellinck and coworkers [15]. Many of the induction responses observed in animals treated with I3C are consistent with the activity of this compound and related heteropolynuclear aromatic hydrocarbons as Ah receptor agonists, which typically induce CYP1A1- and CYP1A2-dependent glutathione S-transferase, UDP-glucuronosyl transferase and NAD(P)H:quinone oxidoreductase activities [24–27]. Previous

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§*Abbreviations:* Ah, aryl hydrocarbon; CAT, chloramphenicol acetyltransferase; DIM, diindolylmethane; DMBA, 7,12-dimethylbenzanthracene; EROD, ethoxyresorufin O-deethylase; I3C, indole-3-carbinol; ICZ, indolo[3,2-*b*]carbazole; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, polychlorinated dibenzofurans; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TCDF, 2,3,7,8-tetrachlorodibenzofuran.

Received 15 September 1995; accepted 6 December 1995.

studies have also demonstrated that TCDD and related compounds exhibit antiestrogenic activity in mammalian cells in culture [28–34] and inhibit mammary tumor growth or formation in rodent models [29, 35, 36] and possibly in humans [37]. Similarly, I3C and ICZ also exhibit antiestrogenic activity in the MCF-7 human breast cancer cell line [38, 39], and inhibition of both carcinogen-induced and spontaneous mammary tumors and endometrial cancer in rodents may also be Ah receptor mediated [2, 4, 7]. I3C induces CYP1A2-dependent estradiol-2-hydroxylase activity in rodents and humans, and this metabolic pathway may also be associated with decreased risk from estrogen-dependent tumors [4, 40].

It has been shown previously that Ah receptor agonists with moderate to weak binding affinity for the receptor may also exhibit partial antagonist activity [41–46]. This study reports the Ah receptor agonist and partial antagonist activities of I3C and DIM in the T47D human breast cancer cell line. T47D cells treated with TCDD form the nuclear Ah receptor complex, and the induction of CYP1A1-dependent EROD activity is among the highest observed in human or rodent cancer cell lines. Results of studies reported in this paper demonstrated that both I3C and DIM exhibit Ah receptor antagonist activity in T47D human breast cancer cells.

MATERIALS AND METHODS

Cells, Chemicals, and Biochemicals

T47D human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). DIM was prepared as previously described [14, 17, 21]. I3C was purchased from the Sigma Chemical Co. (St. Louis, MO). Solutions of I3C and DIM were stored carefully in the dark due to their photolability. [^{3}H]TCDD (37 Ci/mmol) was prepared by chlorination of [1,6-$^3H_2$]dibenzo-*p*-dioxin and purified by high pressure liquid chromatography to greater than 95% purity. Unlabeled TCDD and TCDF are prepared routinely in this laboratory (> 98% pure by gas chromatographic analysis). All other chemicals and biochemicals used in these studies were the highest quality available from commercial sources.

Cell Growth and Formation of Nuclear Receptor Complexes in T47D Cells Treated with [^{3}H]TCDD

Cells were grown as monolayer cultures in α-Eagle's Minimum Essential Medium supplemented with 2.2 g/L sodium bicarbonate, 5% fetal bovine serum, and 10 mL antibiotic-antimycotic solution (Sigma). Cells were maintained in 150-cm^2 culture flasks in an air:carbon dioxide (95:5) atmosphere at 37°. After reaching confluence, the cultures were trypsinized and washed once with used culture medium. The washed cells were resuspended in this medium in 25-cm^2 flasks at a cell concentration of 3×10^6 cells/mL (final volume 10 mL). The cells were incubated with 5 nM [^{3}H]TCDD in the presence or absence of 31 μM DIM and 250 μM I3C in DMSO (0.1% final concentration). Nuclear extract baselines were obtained by cotreatment of the cells with [^{3}H]TCDD plus a 200-fold excess of TCDF. The flasks were incubated by gentle shaking for 2 hr at 37°. After incubation, the suspended cells were decanted into 50-mL centrifuge tubes and centrifuged at 400 *g*. This and all subsequent procedures were performed at 4°.

Isolation and Analysis of Nuclear Extracts

Harvested cells were washed twice in 20 mL of HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1.0 mM dithiothreitol; pH 7.6). The washed cell pellet was resuspended in 3 mL of HED buffer (HEDG without glycerol) and incubated for 10 min. After incubation, the cells were pelleted and resuspended with an additional 1.5 mL of HEGD buffer and homogenized using a tight Teflon pestle/drill apparatus. The homogenate was transferred to a centrifuge tube in 20 mL of HEGD buffer and centrifuged at 1500 *g* for 10 min. The pellet was then resuspended in 3 mL of HEGD buffer containing 0.5 M potassium chloride (pH 8.5) and allowed to stand at 4° for 1 hr. Nuclei prepared by this method were found to be intact and were greater than 90% free of extranuclear cellular contamination, as determined by microscopic examination and trypan-blue staining. Aliquots (200 μL) of the samples were layered onto linear sucrose gradients (5–25%) prepared in HED buffer containing 0.4 M potassium chloride. Gradients were centrifuged at 4° for 2.5 hr at 404,000 *g*. After centrifugation, 30 fractions were collected from each gradient, and the radioactivity of each fraction was determined by liquid scintillation counting. ^{14}C-Labeled BSA (4.4S) and catalase (11.3S) were used as the markers for determining sedimentation values.

EROD Activity

EROD activity was assayed as described [47] with some modifications. Trypsinized cells were plated into 25-cm^2 tissue culture flasks (10^5 cells/mL), allowed to attain 60% confluency, and treated with 1 nM TCDD, 1–31 mM DIM, 1–125 mM I3C, and combinations of TCDD plus DIM or I3C for 24 hr. Cells were harvested by manual scraping from the plate, centrifuged at 400 *g* for 5 min at 4°, and resuspended in 100 μL of Tris–sucrose buffer (38 mM Tris–HCl, 0.2 M sucrose; pH 8.0). Aliquots (50 μL) of the cells were incubated with 1.15 mL cofactor solution (1 mg BSA, 0.7 mg NADH, 0.7 mg NADPH, 1.5 mg $MgSO_4$ in 0.1 M HEPES buffer; pH 7.5) in a 37° water bath for 2 min. The reaction was started by adding 50 μL ethoxyresorufin (1 mg ethoxyresorufin/40 mL methanol). After incubation for 15 min, the reaction was stopped by adding 2.5 mL methanol. Samples were centrifuged for 10 min at 1500 *g*. The supernatant was used for fluorescence measurement at an excitation wavelength of 550 nm and an emission wavelength of 595 nm.

Northern Blot Analysis

Cells were plated into 100 mm Petri dishes, and when 60% confluent, the cells were treated with 1 nM TCDD, DIM, and I3C alone or their combinations for 24 hr. After trypsinization, cells were pelleted by centrifugation at 500 *g* for 5 min at 4°. To the cell pellet, 700 μL of a solution containing 4 M guanidinium thiocyanate, 10% sarcosyl, 3 M sodium acetate (pH 5.2), and 0.1% 2-mercaptoethanol was added. The cell pellet was resuspended immediately with a 1-mL syringe and a 22-gauge needle. To the cell homogenate, 0.5 mL of water-saturated phenol was added and mixed thoroughly. A solution of chloroform:isoamyl alcohol (24:1) was added, vortexed, and then allowed to incubate at 4° for 15 min. Samples were then centrifuged at 10,000 *g* for 15 min, and 600 μL of the upper aqueous phases was extracted carefully. After addition of 1 vol. of isopropyl alcohol, the RNA was allowed to precipitate overnight at −20°. After precipitation, the samples were centrifuged at 10,000 *g* for 15 min, and the RNA was washed with cold ethanol once and 70% ethanol once. Samples were evaporated and redissolved in 15–20 μL of deionized formamide. The murine CYP1A1 cDNA probe was obtained from the American Type Culture Collection. The plasmid pGMB1.1 was a gift from Dr. Don Cleveland (Johns Hopkins University) and carries the mouse β-tubulin cDNA cloned into the *Eco* RI site of pGMB1.1. Digestion of the plasmid yielded a 1.3-kb fragment that was used to detect β-tubulin mRNA.

The RNA (10 μg) was mixed with sample buffer, electrophoresed on a denaturing agarose gel (1.2%), and transferred to a nylon membrane as previously described [41]. The membrane was then exposed to UV light for 5 min to cross-link RNA to the membrane and baked at 80° for 2 hr. The membrane was then prehybridized in a solution containing 0.1% BSA, 0.1% Ficoll, 0.1% polyvinyl pyrrolidine, 10% dextran sulfate, 1% SDS and 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA) for 18–24 hr at 65°. Probes were ^{32}P-labeled using a Boehringer-Mannheim kit. The membrane was hybridized for approximately 24 hr in the prehybridization solution with the addition of 10^6 cpm/mL ^{32}P-labeled CYP1A1 cDNA probe. After hybridization, the membrane was washed twice at 20° in 1 × SSC, 1% SDS, and again twice for 45 min at 65°. After two additional rinses in 0.1 × SSC, 1% SDS at 20°, the membrane was sealed in a plastic bag, quantitated on a Betagen Betascope 603 blot analyzer imaging system, and visualized by autoradiography. The membrane was stripped by washing twice in stripping buffer (0.1 × SSC, 0.5% SDS) at 100° and rehybridized. The CYP1A1 mRNA was standardized relative to β-tubulin mRNA [41].

Transient Transfection Assays

The plasmid pRNH11c contains the regulatory human CYP1A1 region from the *Taq* I site at −1142 to the *Bcl* I site at +2434 fused to the bacterial CAT reporter gene [48]. Cells were seeded in 100-mm Petri dishes and grown as described above in the proliferation assays until 70% confluent. Five micrograms of the pRNH11c plasmid and 20 μg polybrene/mL were incubated for 6 hr; cells were then shocked using 15% glycerol. After 18 hr, cells were treated with 1 nM TCDD, 31 μM DIM, and 125 μM I3C for 30 hr. Cells were then washed with PBS and scraped from the plates. Cell lysates were prepared in 0.16 mL of 0.25 M Tris-HCl, pH 7.5, by three freeze–thaw-sonication cycles (3 min/each cycle) to ensure maximum levels of CAT activity. Cell lysates were incubated at 56° for 7 min to remove endogenous deacetylase activity. CAT activity was determined using 0.2 mCi *d*-threo-[*dichloroacetyl*-1-^{14}C]chloramphenicol and 4 mM acetyl-CoA as substrates. The protein concentrations were determined using BSA as a standard. Following TLC, acetylated products were visualized and quantitated using a Betascope 603 blot analyzer. CAT activity was calculated as the percentage of that observed in cells treated with DMSO alone, and results are expressed as means ± SD. The experiments were carried out at least in triplicate.

Isolation of Microsomes from T47D Cells

After treatment with 1 nM TCDD for 24 hr, T47D cells were homogenized in HEGD buffer using a Teflon pestle/drill apparatus. The homogenates were centrifuged at 10,000 *g* for 20 min. The resulting supernatant was centrifuged further at 105,000 *g* for 30 min. The microsomal pellet was resuspended in 100 μL Tris–sucrose buffer (38 mM Tris–HCl, 0.2 M sucrose; pH 8.0) and stored at −80°. Test compounds were incubated with TCDD-induced microsomes, BSA, NADH, and NADPH at 3° for 2, 10, or 20 min, and EROD activity was determined fluorimetrically [47].

Statistical Analysis

All the experiments were carried out in triplicate, and the results are expressed as means ± SD. Statistical significance was determined by performing ANOVA using Student's *t*-test.

RESULTS

The results in Fig. 1 summarize the concentration-dependent induction of EROD activity by I3C and DIM in T47D cells. At concentrations of I3C and DIM as high as 31 and 125 μM, respectively, no significant induction was observed for either compound. In contrast, EROD activity in cells treated with 1 nM TCDD was 300–400 pmol/min/mg. Cotreatment of T47D cells with 1 nM TCDD and I3C or DIM gave a concentration-dependent decrease in induced EROD activity. I3C significantly inhibited TCDD-induced EROD activity at a concentration of 31 μM, and this activity was decreased to less than 10% of the maximal response at the highest concentration of I3C (125 μM). Sim-

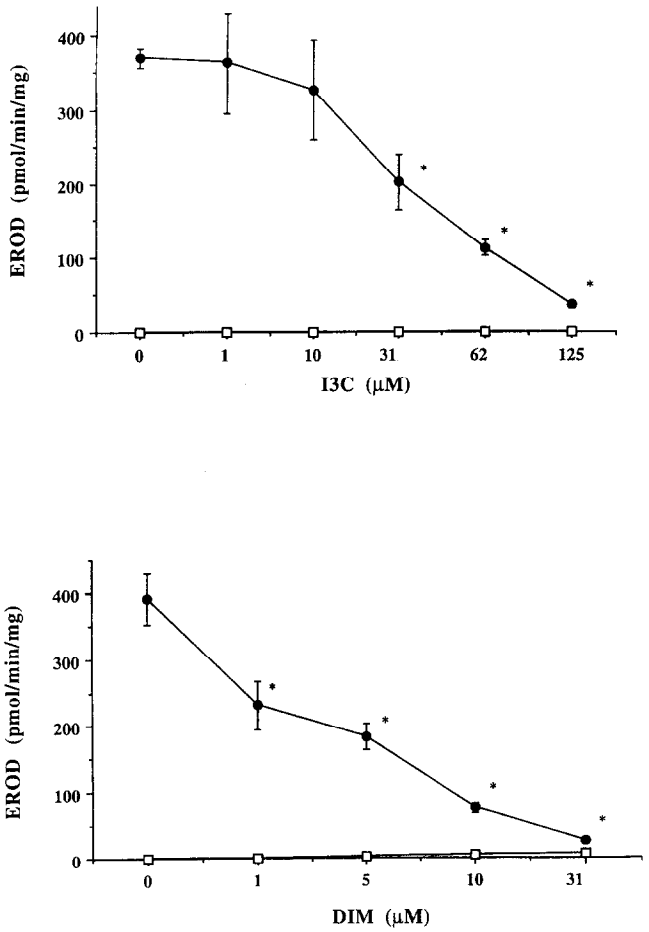

FIG. 1. Inhibition of TCDD-induced EROD activity in T47D cells by I3C (top) and DIM. T47D cells were treated with 1 nM TCDD, different concentrations of I3C (□, top) or DIM (□, bottom) alone or cotreated with 1 nM TCDD plus different concentrations of I3C (●, top) or DIM (●, bottom) for 24 hr. Cells were isolated, and EROD activity was determined as described in Materials and Methods. Results are expressed as means ± SD for 3 separate determinations for each data point. I3C and DIM significantly inhibited ($P < 0.05$) induced EROD activity at concentrations as low as 31 and 1 μM, respectively.

ilar results were also observed for DIM in which 1 μM DIM significantly decreased induced EROD activity (Fig. 1, bottom). The results in Fig. 2 summarize the effects of 1 nM TCDD, 1 and 31 μM DIM, 10 and 125 μM I3C, and TCDD plus DIM or I3C on CYP1A1 mRNA levels in T47D cells. DIM alone caused a small but marked increase (62%) in CYP1A1 mRNA levels at the highest concentration (31 μM), whereas I3C did not induce CYP1A1 mRNA levels significantly. In T47D cells cotreated with 1 nM TCDD and 1 or 31 μM DIM, there was a 53% decrease in TCDD-induced CYP1A1 mRNA levels. Similarly, in T47D cells cotreated with 1 nM TCDD and 10 or 125 μM I3C, there was a 58% decrease in TCDD-induced CYP1A1 mRNA levels only at the 125 μM concentration of I3C. The results in Fig. 3 summarize the effects of 1 nM TCDD, 31 μM DIM, 125 μM I3C, and TCDD plus DIM or I3C on CAT activity in T47D cells transiently transfected with the pRNH11c plasmid. CAT activity was induced 6- to 10-fold by TCDD (in two separate experiments), whereas 125 μM I3C and 31 μM DIM alone caused a 3.4- and 2.7-fold increase in activity, respectively. In cells cotreated with TCDD plus DIM or I3C, there was a significant decrease in CAT activity compared with results obtained with TCDD alone.

The results in Fig. 4 illustrate the velocity sedimentation analysis of nuclear extracts from T47D cells treated with 5 nM [^{3}H]TCDD alone and in the presence of 250 μM I3C, 31 μM DIM or 1 μM TCDF. A higher concentration of [^{3}H]TCDD was used in this experiment to ensure that sufficient specifically bound nuclear extract could be obtained for sucrose density gradient centrifugation. Preliminary studies showed that with the higher concentration of [^{3}H]TCDD, cotreatment with 125 μM unlabeled I3C resulted in only minimal decreases in accumulation of the radiolabeled nuclear Ah receptor; however, cotreatment with 250 μM I3C resulted in a 37% decrease in the [^{3}H]TCDD nuclear Ah receptor complex. In cells cotreated with 5 nM [^{3}H]TCDD plus 31 μM DIM, there was a 73% decrease in the radiolabeled nuclear Ah receptor complex.

The *in vitro* effects of I3C on CYP1A1-dependent activ-

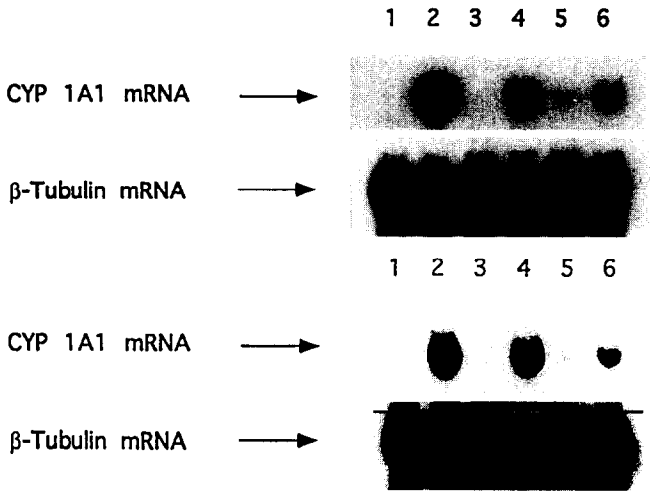

FIG. 2. Induction of CYP1A1 mRNA levels by TCDD, I3C, DIM, and TCDD plus I3C (top) or DIM (bottom) in T47D cells. T47D cells were treated with DMSO, 1 nM TCDD, 10 μM I3C, 10 μM I3C plus 1 nM TCDD, 125 μM I3C, and 125 μM I3C plus 1 nM TCDD (top, lanes 1 through 6, respectively) for 24 hr. Cells were treated with DMSO, 1 nM TCDD, 1 μM DIM, 1 μM DIM plus 1 nM TCDD, 31 μM DIM, 31 μM DIM plus 1 nM TCDD (bottom, lanes 1 through 6, respectively) for 24 hr. Cells were harvested, and mRNA was isolated and analyzed by northern blot analysis as described in Materials and Methods. CYP1A1 mRNA levels were standardized relative to β-tubulin mRNA, and the values (means ± SD for 3 determinations) were: (top) 1.00 ± 0.15, 4.07 ± 0.75, 0.94 ± 0.08, 3.22 ± 0.49, 1.18 ± 0.10, and 1.96 ± 0.01 (lanes 1 through 6, respectively); (bottom) 1.00 ± 0.19, 6.27 ± 0.86, 1.35 ± 0.09, 5.30 ± 0.29; 1.62 ± 0.15, and 2.94 ± 0.35 (lanes 1 through 6, respectively). Quantitation of induced bands was determined using a Betagen Betascope 603 blot analyzer.

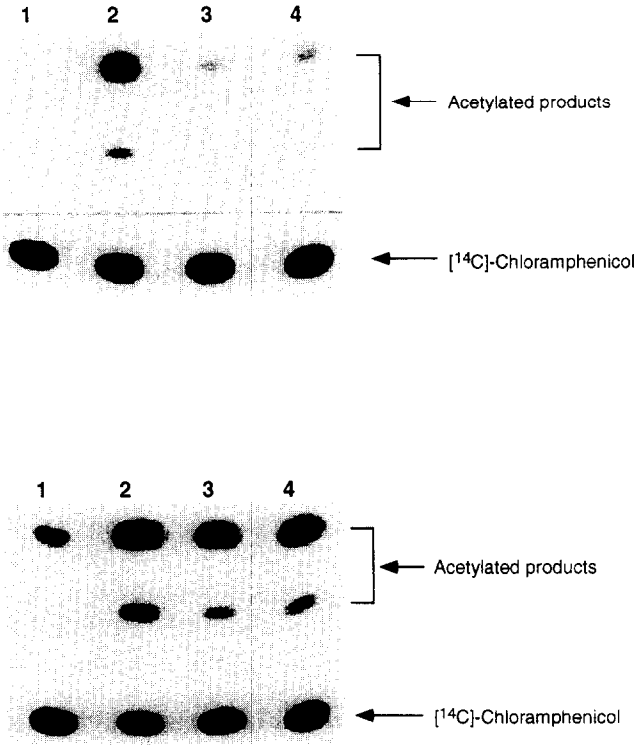

FIG. 3. Induction of CAT activity in T47D cells by TCDD, I3C, DIM, and their combinations. T47D cells were transiently transfected with pRNH11c and treated with the various chemical combinations for 24 hr; CAT activity was determined as described in Materials and Methods. In two separate experiments, CAT activity was induced 6- to 10-fold by 1 nM TCDD (lane 2) compared with activity in cells treated with DMSO (lane 1). The relative intensities of the acetylated products in cells with 1 nM TCDD, 125 μM I3C, and TCDD plus I3C (lanes 2 through 4, respectively, top) were: 100 ± 4, 34 ± 3, and 67 ± 8%. The relative intensities of acetylated products in cells treated with 1 nM TCDD, 31 μM DIM, and TCDD plus DIM (lanes 2 through 4, respectively, bottom) were: 100 ± 25, 42 ± 16, and 59 ± 20%. I3C (top) and DIM (bottom) alone significantly induced CAT activity and also significantly inhibited the TCDD-induced response ($P < 0.005$). The relative intensities (means ± SD for 3 separate determinations) of the acetylated products were determined using a Betagen Betascope 603 blot analyzer.

ity (Fig. 5) were determined by incubating different concentrations of I3C (10, 63, and 125 μM) or DIM (1, 10, or 31 μM) with microsomes from T47D cells treated with 1 nM TCDD for 2, 10, or 20 min. The results showed that both I3C and DIM caused a concentration- and time-dependent decrease in EROD activity using a complete microsomal enzyme incubation system and that DIM was the more potent inhibitor. In the absence of the reduced nucleotide cofactors, comparable inhibitory responses were observed (data not shown).

DISCUSSION

MCDF and related 6-alkyl-1,3,8-trichlorodibenzofurans have been characterized previously as weak Ah receptor agonists for the induction of CYP1A1 and for several toxic responses including immunotoxicity, porphyria, and fetal cleft palate formation in mice [44–46]. Moreover, in cells or in mice cotreated with an effective (or toxic) dose of TCDD plus MCDF, there was a significant inhibition of these same TCDD-induced responses. Surprisingly, MCDF did not inhibit TCDD-induced antiestrogenic responses in the rat uterus or in human breast cancer cell lines but exhibited Ah receptor agonist activity for this response [49–51]. The combination of low toxicity but high antiestrogenic activity suggests that MCDF and related compounds may be useful clinically as antiestrogens [51]. Previous studies have shown that I3C exhibits several properties similar to those described for MCDF; I3C binds with low affinity to the Ah receptor and at high doses or concentrations, I3C induces CYP1A1/1A2-dependent activity [11–17]. For example, 500 μM I3C induces immunoreactive CYP1A1 protein in MCF-7 breast cancer cells [52], whereas minimal induction responses are observed at lower concentrations. Similar results were observed in this study using T47D cells, since 125 μM I3C induced only a minimal increase in CYP1A1 mRNA levels (Fig. 2). It was reported previously that lower concentrations of I3C also inhibit several estrogen (E_2)-induced responses including cell proliferation and nuclear estrogen receptor binding [38, 39]. This profile of responses in MCF-7 cells, namely antiestrogenic activity and minimal induction of CYP1A1-dependent EROD activity, resembled those previously reported for MCDF, and therefore the major objective of this study was to determine if I3C or its major dimerization product, DIM [52], also exhibited Ah receptor antagonist activity.

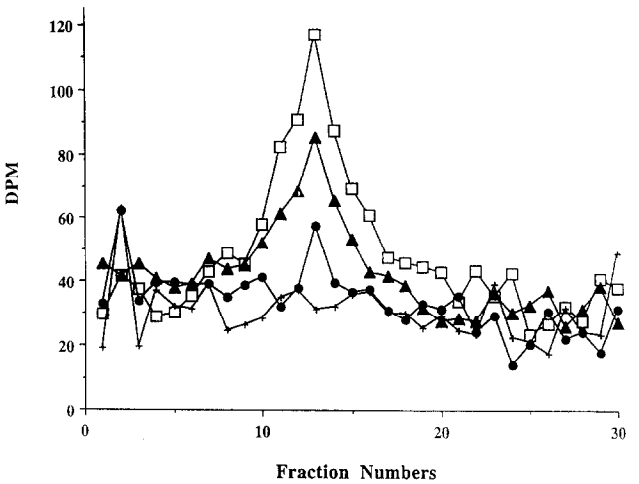

FIG. 4. Velocity sedimentation analysis of nuclear extracts from T47D cells treated with [^{3}H]TCDD plus I3C or DIM. Cells in suspension were treated with 5 nM [^{3}H]TCDD alone (□) or in combination with 250 μM I3C (▲), 31 μM DIM (●) or 1 μM TCDF (+) for 2 hr; nuclear extracts were isolated and analyzed on sucrose density gradients as described in Materials and Methods. Relative nuclear Ah receptor levels in cells treated with [^{3}H]TCDD alone, [^{3}H]TCDD plus I3C, and [^{3}H]TCDD plus DIM were 100 ± 17, 63 ± 13, and 27 ± 12%, respectively. Representative gradients are shown.

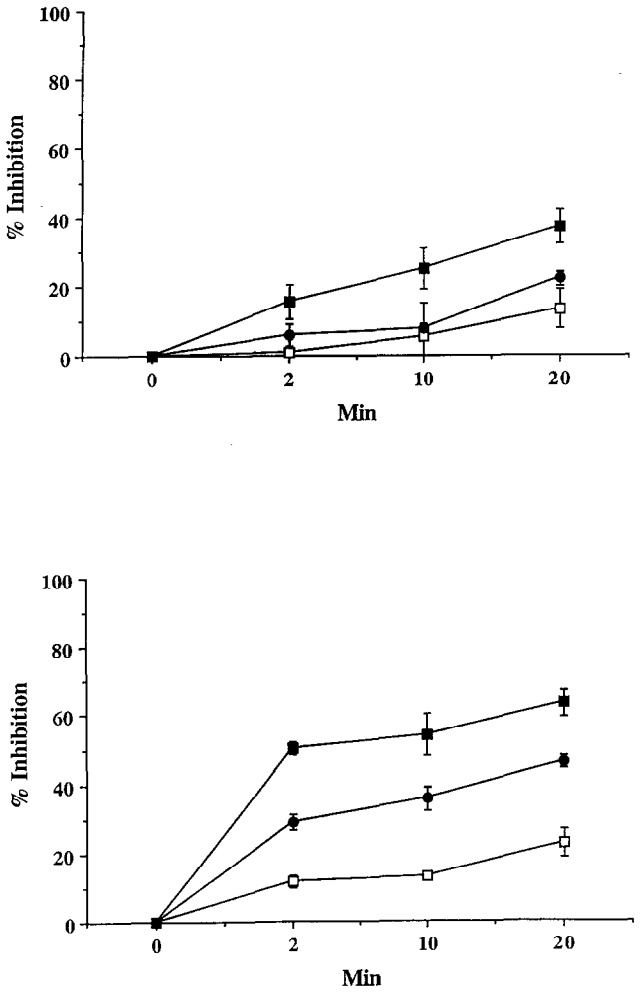

FIG. 5. *In vitro* inhibition of EROD activity by I3C (top) or DIM (bottom). Microsomes from T47D cells treated with 1 nM TCDD for 24 hr were incubated with 10 (□), 63 (●), or 125 (■) μM I3C (top) and 1 (□), 10 (●), or 31 (■) μM DIM (bottom) for 2, 10, or 20 min, and EROD activity was determined fluorimetrically as described in Materials and Methods. The results are expressed as means ± SD for each data point (3 separate determinations). Significant inhibition ($P < 0.05$) was observed for all concentrations of I3C and DIM after incubation with microsomes from T47D cells, and similar inhibitory effects were observed using induced rat hepatic microsomes (data not shown). The induced EROD activity was 437 pmol/min/mg.

T47D human breast cancer cells were utilized in this study because of the reported high inducibility of CYP1A1-dependent EROD activity [53].

The results in Figs. 1 and 2 confirmed that both I3C and DIM are relatively weak Ah receptor agonists since at concentrations as high as 125 and 31 μM, respectively, no induction of EROD activity was observed. I3C (125 μM) caused a small but insignificant increase in CYP1A1 mRNA levels in T47D cells, whereas 31 μM DIM markedly induced mRNA levels (Fig. 2); similar results were observed in transient transfection assays using pRNH11c, an Ah-responsive plasmid that contains a dioxin-responsive element enhancer sequence (Fig. 3). These data are consistent with results of previous studies showing that DIM is more potent than I3C as an Ah receptor agonist; however, both compounds are much less active than either ICZ or TCDD [15, 17].

In T47D cells treated with 1 nM TCDD, there was a significant induction of EROD activity; however, in cells cotreated with TCDD plus I3C or DIM, there was a concentration-dependent decrease in the induced response and, at the highest concentrations of these compounds (125 and 31 μM, respectively), < 10% of induced activity was observed. I3C and DIM also markedly inhibited TCDD-induced CYP1A1 mRNA levels (Fig. 2) and CAT activity (Fig. 3) in cells transiently transfected with pRNH11c. The inhibitory effects of I3C and DIM on TCDD-induced responses in T47D cells also correlated with the decreased accumulation of the radiolabeled nuclear Ah receptor complex in cells cotreated with I3C or DIM (Fig. 4). These results are similar to those previously observed for other Ah receptor antagonists, such as α-naphthoflavone and MCDF, which also decrease formation of the radiolabeled nuclear Ah receptor complex in cells cotreated with [^{3}H]TCDD plus the antagonists [41–45].

The results of this study demonstrate that both I3C and DIM are weak Ah receptor agonists that also exhibit partial Ah receptor antagonist activity for the induction of *CYP1A1* gene expression by TCDD. However, there was a disparity between the > 90% inhibition of TCDD-induced EROD activity (Fig. 1) and the < 50% inhibition of CYP1A1 mRNA levels (Fig. 2). Jellinck and coworkers [15] noted that several Ah receptor agonists which induce CYP1A1/1A2 *in vivo* inhibit the induced microsomal enzyme activity *in vitro*. The results in Fig. 5 demonstrate that I3C and DIM caused a concentration- and time-dependent decrease in EROD activity using microsomes from T47D cells treated with 1 nM TCDD for 24 hr. Thus, the *in vitro* activity of DIM and I3C as inhibitors of CYP1A1-dependent EROD activity is consistent with the >90% inhibition of this induced response in cells cotreated with TCDD plus I3C or DIM (Fig. 1).

I3C and related hetero-polynuclear aromatic hydrocarbons in vegetables represent a class of endogenous Ah receptor agonists ("natural dioxins") that are consumed in the diet along with industrial and combustion-derived halogenated aromatic compounds, such as TCDD, which are also Ah receptor agonists (exodioxins). Levels of PCDDs and PCDFs in the human diet vary from 1000 to 2000 pg/day or 80 to 120 pg/day of toxic (or TCDD) equivalents. Moreover, in the general population, average background levels of TCDD equivalents are estimated to be 58 ng/kg serum lipid [54]. For individuals who consume an average of 25 g of *Brassica* vegetables each day, the intake levels of I3C are in excess of 700,000,000 pg/day [17]. Since I3C, DIM, and related compounds exhibit both Ah receptor agonist and partial antagonist activities, risk assessment of low level dietary exposure to exodioxins should also take into account the possible inhibitory or additive effects of dietary compounds that also bind to the Ah receptor. The inter-

actions (additive or inhibitory) between I3C and related condensation products with exodioxins will depend on their relative serum levels; however, at present, the values of I3C-derived compounds are unknown. Moreover, due to rapid metabolism of I3C and related indoles, serum levels of these compounds will be highly variable, and this may also influence interactions with TCDD and related exodioxins. Current ongoing studies in this laboratory are investigating the relative potencies of TCDD, I3C, DIM, and other "natural dioxins" and their interactions for several other Ah receptor-mediated responses.

The financial assistance of the National Institutes of Health (ES04917) and the Texas Agriculture Experiment Station is gratefully acknowledged. S. Safe is a Sid Kyle Professor of Toxicology.

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