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Cytostatic and Antiestrogenic Effects of 2-(Indol-3-ylmethyl)-3,3′-diindolylmethane, a Major *In Vivo* Product of Dietary Indole-3-carbinol

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ABSTRACT. Under acidic conditions, indole-3-carbinol (I3C) is converted to a series of oligomeric products thought to be responsible for the biological effects of dietary I3C. Chromatographic separation of the crude acid mixture of I3C, guided by cell proliferation assay in human MCF-7 cells, resulted in the isolation of 2-(indol-3-ylmethyl)-3,3′-diindolylmethane (LTr-1) as a major antiproliferative component. LTr-1 inhibited the growth of both estrogen-dependent (MCF-7) and -independent (MDA-MB-231) breast cancer cells by approximately 60% at a non-lethal concentration of 25 μM. LTr-1 had no apparent effect on the proliferation of MCF-7 cells in the absence of estrogen. LTr-1 was a weak ligand for the estrogen receptor (ER) (IC_{50} 70 μM) and efficiently inhibited the estradiol (E_2)-induced binding of the ER to its cognate DNA responsive element. The antagonist effects of LTr-1 also were exhibited in assays of endogenous pS2 gene expression and in cells transiently transfected with an estrogen-responsive reporter construct (pERE-vit-CAT). LTr-1 activated both binding of the aryl hydrocarbon (Ah) receptor to its cognate DNA responsive element and expression of the Ah receptor-responsive gene *CYP1A1*. LTr-1 was a competitive inhibitor of CYP1A1-dependent ethoxyresorufin-*O*-deethylase (EROD) activity. In summary, these results demonstrated that LTr-1, a major *in vivo* product of I3C, could inhibit the proliferation of both estrogen-dependent and -independent breast tumor cells and that LTr-1 is an antagonist of estrogen receptor function and a weak agonist of Ah receptor function. BIOCHEM PHARMACOL **58**;5:825–834, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. indole-3-carbinol; 2-(indol-3-ylmethyl)-3; 3′-diindolylmethane; antiestrogenic; breast tumor cells; cytostatic; HPLC

I3C§ is a product of enzymatic hydrolysis of the indolylmethyl glucosinolate glucobrassicin, present in common vegetables of the *Brassica* genus, including cabbage, kale, rutabaga, cauliflower, turnips, broccoli, kohlrabi, mustard, collard, and Brussels sprouts, and is a promising cancer preventive agent (see structure in Fig. 1). Oral administration of I3C to rodents prior to treatment with a carcinogen reduces tumorigenesis in the stomach, liver, lung, and oral cavity by a mechanism that is thought to include induction of phase I and phase II xenobiotic metabolism and increased clearance of the carcinogen [1–5]. When administered at high doses following treatment with a carcinogen, I3C is reported to promote tumorigenesis in the thyroid gland, colon, pancreas, and liver [6–8]. Although the mechanism for these adverse effects of I3C is yet to be determined, one concern is that they might be associated with activation of the Ah receptor. Persistent activation of this receptor is thought to be responsible for the adverse effects of the potent environmental toxin TCDD [9].

The most pronounced and consistently protective effects of I3C have been reported against tumorigenesis in estrogen-responsive tissues. In one of the earliest studies of the protective effects of non-nutritive food components, I3C was shown to reduce by 75% the frequency of dimethylbenzanthracene-induced mammary tumors in rodents [1]. A recent extension of these studies found an even greater protective effect of I3C, i.e. 95% reduction in tumor multiplicity, when the indole was administered prior to and following treatment with the carcinogen [10]. I3C treatment also produced a dramatic decrease (65%) in mammary tumor multiplicity induced by the direct-acting carcinogen methylnitrosourea [10]. Furthermore, I3C is reported to inhibit spontaneous formation of mammary and endometrial tumors in rodents [11, 12]. The protective effects of

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§ *Abbreviations*: I3C, indole-3-carbinol; LTr-1, 2-(indol-3-ylmethyl)-3,3′-diindolylmethane; ER, estrogen receptor; E_2, 17β-estradiol; Ah, aryl hydrocarbon; EROD, ethoxyresorufin-*O*-deethylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxine; ICZ, indolo[3,2-*b*]carbazole; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; SSC, 0.15 M sodium chloride + 0.015 M sodium citrate; THF, tetrahydrofuran; DIM, 3,3′-diindolylmethane; RXM, acid reaction mixture; and ERE, estrogen responsive element.

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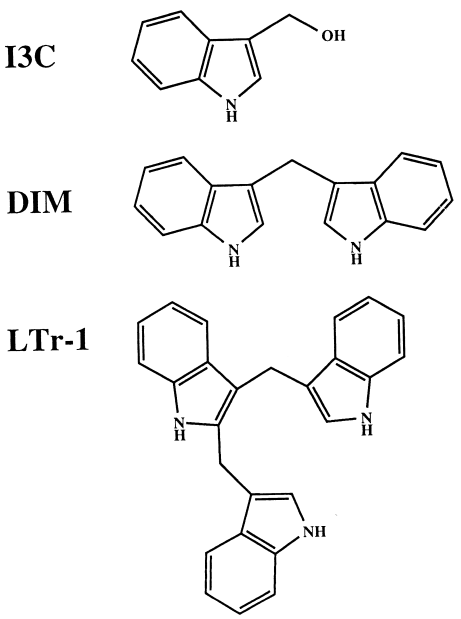

FIG. 1. Structures of I3C, DIM, and LTr-1.

I3C in these studies have been attributed to alterations in E_2 metabolism and/or modifications of ER function.

An important consideration in studies of the biological effects of I3C is its chemical instability. I3C, being a vinylogous hemiaminal, is dehydrated readily in acidic and basic solutions and is converted rapidly to a mixture of oligomeric products [13]. These products, which are produced readily in gastric acid following ingestion, are thought to be responsible for the biological effects of orally administered I3C [14]. We have shown previously that a minor product, ICZ, is a potent activator of Ah receptor pathways and exhibits antiestrogenic activities [15, 16]. DIM, a major product of I3C, is a weak ligand for the Ah receptor, inhibits CYP1A1 enzyme activity, and can act as an antagonist of E_2-mediated tumor cell growth and gene activation [17, 18].

As part of our continuing efforts to understand the mechanisms of action of I3C, we have begun to identify the components of the RXM of I3C that are responsible for the cancer protective effects of I3C and the antiproliferative activity of RXM in cultured breast tumor cells. We report here the effects in tumor cells of the linear trimeric product LTr-1, a second major component of RXM. We show that this novel compound can inhibit proliferation of both estrogen-dependent and -independent cultured breast tumor cells and that it is an antagonist of ER function with little agonist activity. We show further that LTr-1 is a weak agonist of Ah receptor function.

MATERIALS AND METHODS

Materials

DMEM, Opti-MEM, and Lipofectamine were supplied by Gibco/BRL. Phenol red-free DMEM, FBS, calf serum, tamoxifen, and E_2 were supplied by the Sigma Chemical Co. [γ-^{32}P]ATP and [^{3}H]acetyl-CoA were supplied by New England Nuclear. All other reagents were of the highest grade available.

Preparation of RXM

The procedure reported by Grose and Bjeldanes [13] was followed for the preparation of RXM. Briefly, I3C (100 mg, Aldrich Chemical Co.) was suspended in 1 M HCl (100 mL) at room temperature for 15 min. The acid suspension was neutralized with aqueous ammonia to pH 7.0, and the precipitate was filtered and dried under vacuum to give RXM as a reddish powder. LTr-1 is stable under the neutral aqueous conditions of the cell proliferation assay.

Fractionation of RXM

RXM (200 mg) was dissolved in THF (1 mL) and purified by silica gel vacuum liquid chromatography. Mixtures of hexane/THF with increasing polarity were used as the mobile phase. HPLC purification of bioactive components of the crude fractions was performed using a Shimadzu HPLC system (SCL-10A; Shimadzu Scientific Instruments, Inc.) equipped with a C_{18} bonded-phase semi-preparative column (Ultrasphere-ODS, 10 $\times$ 250 mm, 5 μm; Beckman) and UV/VIS detector (SPD-10AV; Shimadzu Scientific Instruments, Inc.). The peaks were monitored at 280 nm. For isocratic elution, we used a mixture of acetonitrile: water (60:40) at a flow rate of 2 mL/min. Crude RXM fractions were resuspended in THF before injection into HPLC. The electron impact mass spectrometry analyses of HPLC fractions of interest were obtained by the Mass Spectrometry Facility of the College of Chemistry, University of California at Berkeley.

Cell Culture

The human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 and the murine hepatoma cell line Hepa-1c1c-7, obtained from the American Type Culture Collection (ATCC), were grown as adherent monolayers in DMEM (Gibco, Life Science Technology), supplemented to 4.0 g/L of glucose and 3.7 g/L of sodium bicarbonate in a humidified incubator at 37° and 5% CO_2, and passaged at approximately 80% confluence. Cultures of human cells were used in subsequent experiments for fewer than 25 passages.

Cell Proliferation

Before the beginning of the treatments, cells were depleted of estrogen for 7–10 days in medium composed of DMEM base without phenol-red (Sigma), with 4 g/L of glucose, 3.7 g/L of sodium bicarbonate, and 5% calf serum twice stripped in dextran-coated charcoal and microfiltered, supplemented with non-essential amino acids (Gibco), 2 mM

glutamine, and 10 ng/mL of insulin. During the depletion period, medium was changed every other day. Treatments were administered by the addition of 1 μL of 1000x stock solutions in DMSO per mL of medium. Once the treatment period started, medium was changed daily to counter possible loss of readily metabolized compounds. Cells were harvested by trypsinization and counted in a Coulter particle counter.

Estrogen Receptor Binding Assay

Rat uterine cytosol was prepared as described previously [19]. Briefly, 2.5 g of uterine tissue from five Sprague–Dawley rats (12 weeks old) was excised and placed on ice. The fresh tissue was homogenized with 30 mL of ice-cold TEDG buffer (10 mM Tris, pH 7.4, 1.5 mM EDTA, 1 mM DTT, 10% glycerol) using a Polytron at medium speed for 1 min on ice. The homogenate was centrifuged at 1000 *g* for 10 min at 4°. The supernatant solution was transferred to ultracentrifuge tubes and centrifuged at 100,000 *g* for 90 min at 4°. The supernatant solution was divided into 1.0-mL aliquots, quickly frozen in a dry-ice/ethanol bath, and stored at −80°. Protein concentration of the uterine cytosol was measured by the Bradford assay using bovine serum albumin as the standard. For each competitive binding assay, 5 μL of 20 nM [^{3}H]E_2 in 50% ethanol, 10 mM Tris, pH 7.5, 10% glycerol, 1 mg/mL of BSA, and 1 mM DTT was placed in a 1.5-mL microcentrifuge tube. Competitive ligands were added as 1.0 μL of 100x stock solutions in DMSO. After mixing, 95 μL of uterine cytosol was added, and the tubes were vortexed and incubated at room temperature for 2–3 hr. Proteins were precipitated by the addition of 100 μL of 50% hydroxylapatite slurry equilibrated in TE (50 mM Tris, pH 7.4, 1 mM EDTA) and incubated on ice for 15 min with vortexing every 5 min to resuspend hydroxylapatite. The pellet was washed with 1.0 mL of ice-cold wash buffer (40 mM Tris, pH 7.4, 100 mM KCl), and centrifuged for 5 min at 10,000 *g* at 4°. The supernatant was aspirated carefully, and the pellet was washed two more times with 1.0 mL of wash buffer. The final pellet was resuspended in 200 μL of ethanol and transferred to a scintillation vial. The tube was washed with another 200-μL portion of ethanol, which then was added to the same counting vial. A negative control contained no uterine cytosol. Non-specific binding was determined using 100-fold (0.1 μM) excess unlabeled E_2. Relative binding affinities were calculated using the concentration of competitor needed to reduce [^{3}H]E_2 binding by 50% as compared with the concentration of unlabeled E_2 needed to achieve the same result.

Reporter Plasmids and Expression Vectors

The ER responsive CAT reporter plasmid pERE-vit-CAT [20] was a gift from D. J. Shapiro (University of Illinois). pERE-vit-CAT contains the 5′-flanking and promoter region (−596 to +21) of the *Xenopus* vitellogenin-B1 gene, including two imperfect endogenous EREs (at −302 and −334) and one consensus exogenous ERE inserted at position −359. The transfection efficiency control vector pCMVβ constitutively expressing β-galactosidase was obtained from ATCC.

Transient Transfections with Reporters

Transfections were done by the lipofection method using Lipofectamine (Gibco BRL). Cells were grown in 10% FBS-DMEM until 80% confluent and transferred to 6.0-cm Petri plates 24 hr before transfection. The plates were seeded with the appropriate number of cells to be 50–60% confluent at the time of transfection. For each 6-cm plate, 8 μL of Lipofectamine was diluted with 92 μL of Opti-MEM serum-free medium (Gibco). Plasmid DNA (0.1 to 1.0 μg) was diluted in 100 μL of serum-free medium. Lipid and plasmid dilutions were combined, mixed gently, and incubated at room temperature for 30–45 min. Meanwhile, the plates were washed with 4 mL of serum-free medium, and 2 mL of serum-free medium was added to each plate. A 200-μL portion of the lipid/DNA suspension was added to each plate and mixed gently. The plates were returned to the incubator for 5–6 hr, and 2 mL of medium containing 10% calf serum was added. The next day, the plates were treated with fresh stripped medium without phenol red (5% DCC-FBS), and the 48-hr treatments were started by the addition of 1 μL of 1000x stock solutions in DMSO per mL of medium. The transfection efficiency determined with the constitutive galactosidase expression plasmid pCMVβ in duplicate sets of plates was unaffected by the treatments.

Chloramphenicol Acetyl Transferase (CAT) Assay

The CAT assay was done using a modification of the phase extraction assay described by Seed and Sheen [21]. At the end of the 48-hr treatment period, the transfected cells were harvested by scraping with a rubber policeman, transferred with the medium to a conical 15-mL tube, centrifuged at 600 *g* for 2 min, resuspended in 1 mL of cold PBS, transferred to Eppendorf tubes, centrifuged at 600 *g* for 2 min, and washed in PBS a second time. Cell pellets were resuspended in 200 μL of 0.1 M Tris, pH 8.0, and lysed by 3 cycles of freeze/thaw treatment (alternating 5 min in a dry-ice/alcohol bath and 5 min in a 37° bath). Cell lysates were incubated at 65° for 15 min to inactivate acylases and centrifuged at 14,000 *g* for 8 min. A 165-μL aliquot of the cytosol was transferred to a 7-mL scintillation vial, and a 20-μL aliquot was reserved for determination of protein concentration by the Bradford assay. The substrate mixture (85 μL) was added to the scintillation vial for final concentrations of 100 mM Tris–HCl, pH 8.0, 250 nmol chloramphenicol, and 1 μCi [^{3}H]acetyl-CoA (200 mCi/mmol) in a total volume of 250 μL and mixed thoroughly. The organic scintillation fluid (4 mL) was added slowly, and the vials were incubated at 37° for 1–2 hr or until sufficient counts were obtained.

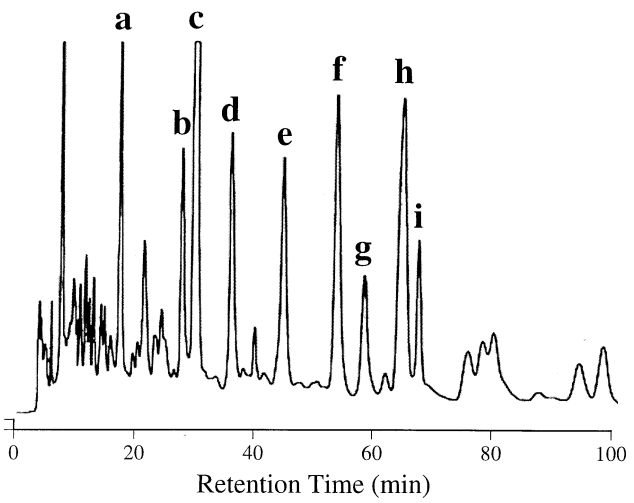

FIG. 2. HPLC chromatogram of fraction B under the conditions described in Materials and Methods. Major peaks were labeled and collected individually.

RNA Extraction and Northern Blot Analysis

Cells were lysed by addition of Tri-reagent (Molecular Research Center, Inc.), and chloroform was used for phase separation. After centrifugation, the water-soluble upper phase was collected, and total RNA was precipitated with isopropanol, washed with 75% ethanol, and dissolved in diethyl pyrocarbonate-treated water. Total RNA was electrophoresed on a 1.2% agarose gel containing 3% formaldehyde, using MOPS as the running buffer. The gel then was washed gently with 10x SSC and blotted with a Zeta nylon membrane (Bio-Rad) overnight. The RNA was fixed to the membrane by UV cross-linking. The hybridization probes were labeled with [^{32}P]CTP using random primers and the pS2-cDNA and GADPH-cDNA plasmids provided by ATCC as the template. Hybridization and quantitation of results were done as described previously [14]. Specific pS2 mRNA levels were normalized using GADPH as a standard.

Nuclear Extracts

Three near confluent (80–90%) cultures of MCF-7 cells in 100-mm Petri dishes were used for each treatment. LTr-1, E_2, and tamoxifen were added as 1 μL of a 1000x stock solution in DMSO per mL of medium. After 2 hr of incubation at 37°, the plates were placed on ice and washed twice with 5 mL of hypotonic buffer (10 mM HEPES, pH 7.5) and incubated with 2 mL of the same buffer for 15 min. Cells were harvested in 1 mL of MDH buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES, pH 7.5) with a rubber scraper, homogenized with a loose-fitting Teflon pestle, and centrifuged at 1000 *g* for 4 min at 4°. The pellets were washed twice with 3 mL of MDHK buffer (3 mM $MgCl_2$, 1 mM DTT, 0.1 M KCl, 25 mM HEPES, pH 7.5), resuspended in 1 mL of MDHK, and centrifuged at 600 *g* for 4 min at 4° in a microcentrifuge. The pellets were resuspended in 100 μL of HDK buffer (25 mM HEPES, pH 7.5, 1 mM DTT, 0.4 M KCl), incubated for 20 min on ice with mixing every 5 min, and centrifuged at 14,000 *g* for 4 min at 4°. Glycerol was

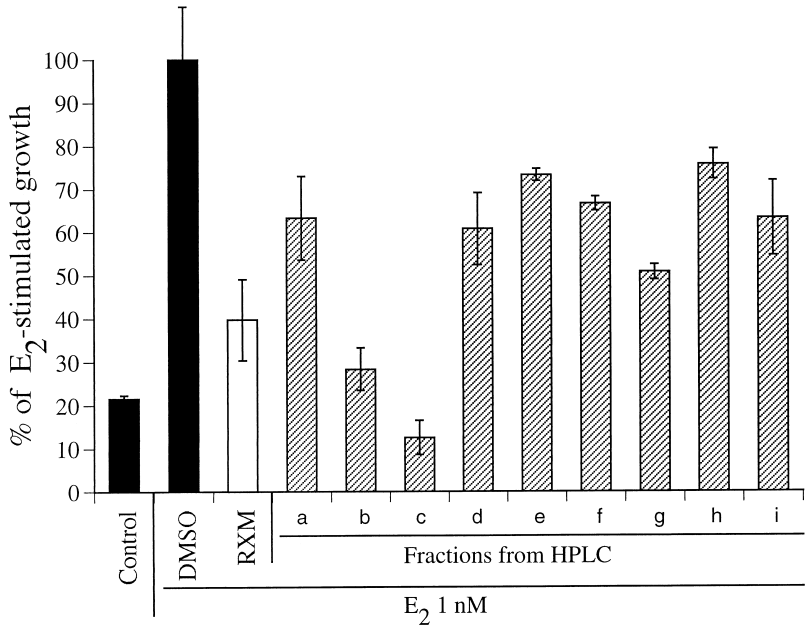

FIG. 3. Antiproliferative effect of subfractions Ba–Bi on MCF-7 cells. Estrogen-depleted MCF-7 cells were plated at a density of 4 × 10^4 cells per well in 24-well plates and treated as indicated with RXM and HPLC fractions at a concentration of 50 μM I3C equivalent. Duplicate aliquots of cells from individual wells were counted after 5 days. The results are shown as the average and SD from three identical wells. Growth was reduced significantly following treatment with RXM and all subfractions (a–i) as determined by Dunnett's test with a procedurewise error rate of < 0.05.

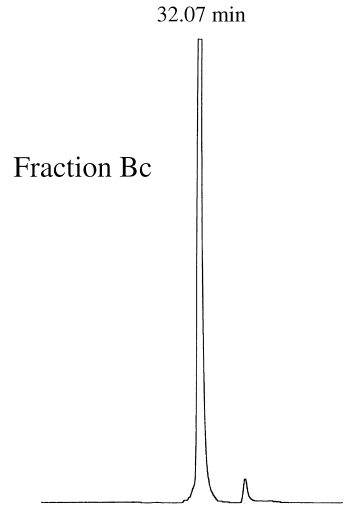

FIG. 4. HPLC analysis of subfraction Bc under the conditions described in Materials and Methods.

added to the supernatants to a concentration of 10%, and aliquots of the nuclear extracts were stored at −80°.

Gel Mobility Shift Assay

The following two sets of complementary oligonucleotides: 5′-GATCCCAGGTCACAGTGACCTGAGCTAAAAT-3′ and 5′-GATCATTTTAGCTCAGGTCACTGTGACC-TGG-3′ containing the palindromic consensus ERE motif (underlined), and 5′-GATCTGGCTCTTCTCACGCA-ACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGA-AGAGCCA-3′ containing the consensus DRE motif (underlined), were annealed and 5′-end-labeled with [γ-^{32}P]ATP using T4 nucleotide kinase. The resulting labeled double-stranded DNA probes were purified on a Sephadex G50 spin-column, precipitated in ethanol, dissolved in TE buffer, and diluted in 25 mM HEPES, 1 mM DTT, 10% glycerol, 1 mM EDTA to contain approximately 25,000 cpm of ^{32}P/μL. Nuclear extracts (7 μg of proteins) were mixed with 90 ng poly-dIdC, 25 mM HEPES, 1 mM DTT, 10% glycerol, 1 mM EDTA, 160 mM KCl in a total volume of 21 μL. For antibody supershift experiments, 0.5 μg of monoclonal mouse-IgG anti-human-ER (Santa Cruz Biotechnology) was added to the incubation mixture. After incubation for 15–20 min at room temperature, 4 μL (100,000 cpm) of end-labeled ^{32}P probe was added and incubated for another 15 min at room temperature. After the addition of 2.8 μL of 10x Ficoll loading buffer (0.25% bromophenol blue, 25% Ficoll type 400), 22-μL aliquots were loaded onto a pre-run, non-denaturing 4.0% polyacrylamide gel in TAE (67 mM Tris, 33 mM sodium acetate, 10 mM EDTA, pH 8.0) at 120 V for 2 hr. The gel then was dried and autoradiographed.

EROD Assay

Enzymatic activity associated primarily with cytochrome P4501A1 was measured by the EROD assay as described

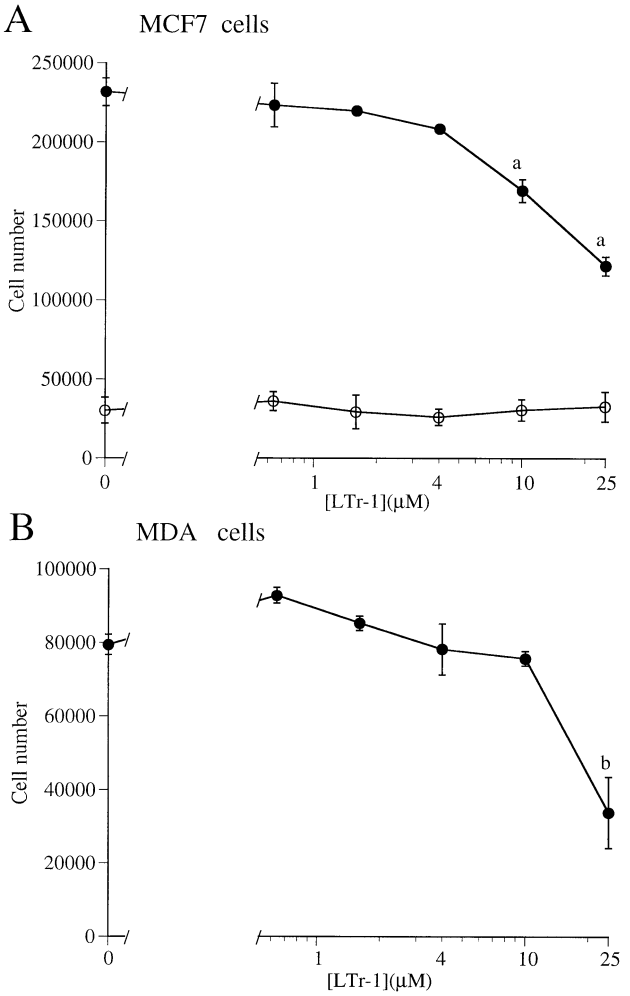

FIG. 5. Effect of LTr-1 on proliferation of breast cancer cells. Panel A: estrogen-depleted MCF-7 cells were plated at a density of 10^5 cells per well in 6-well plates and treated with LTr-1 at the concentrations indicated, in the presence (●) or absence (○) of E_2 (1 nM). Panel B: MDA-MB-231 cells were plated at the same density in complete medium and treated with LTr-1 at the concentrations indicated. Duplicate aliquots of cells from individual wells were counted after 5 days. Data from three identical wells were averaged. The statistical differences between groups were determined using ANOVA and Tukey's Studentized range test. The results are expressed as means ± SD for at least three replicate determinations for each experiment. Key: (a) significantly different ($P < 0.05$) from E_2 induced, and (b) significantly different ($P < 0.05$) from the DMSO control.

previously [22]. Briefly, after the 18- to 24-hr treatment, cells were trypsinized, and 5 mL of PBS was added to the cells. The reaction was done at room temperature, and the cells and the reaction solutions were incubated first at 37°. An aliquot of the cell suspension was counted to obtain the cell number, and 1.5 mL of the cell suspension was placed into a fluorometer cuvette, followed by the addition of 0.5 mL of 2.5 mM ethoxyresorufin (Sigma). The reaction mixture was mixed by inversion of the cuvette, and the linear fluorescence produced was measured at the excitation wavelength of 510 nm and the emission wavelength of 586 nm with a 20-nm slit width using a Perkin-Elmer

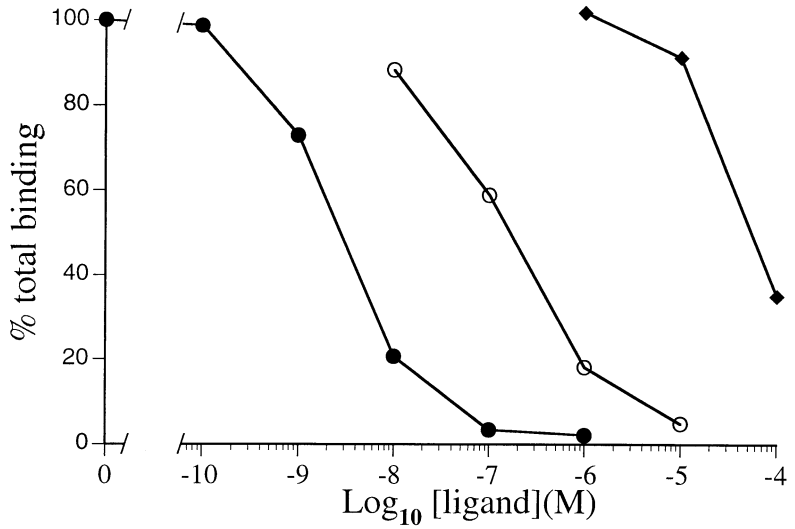

FIG. 6. Competitive binding to the ER. The binding of [^{3}H]E_2 (1 nM) to the ER from rat uterine cytosol was measured in the presence of the unlabeled competitors, E_2 (●), tamoxifen (○), and LTr-1 (♦), at the concentrations indicated and reported as the percentage of binding in the absence of competitors. Results are presented as the averages of two independent determinations. Relative binding affinities were calculated using the concentration of competitor needed to reduce [^{3}H]E_2 binding by 50% as compared with the concentration of unlabeled E_2 needed to achieve the same result.

650–10S spectrofluorometer. Chart speed was recorded for time determination, and a standard curve was obtained using resorufin (Sigma) added to the heated control cells. The enzyme activity was then presented as picomoles of resorufin produced per minute per million cells.

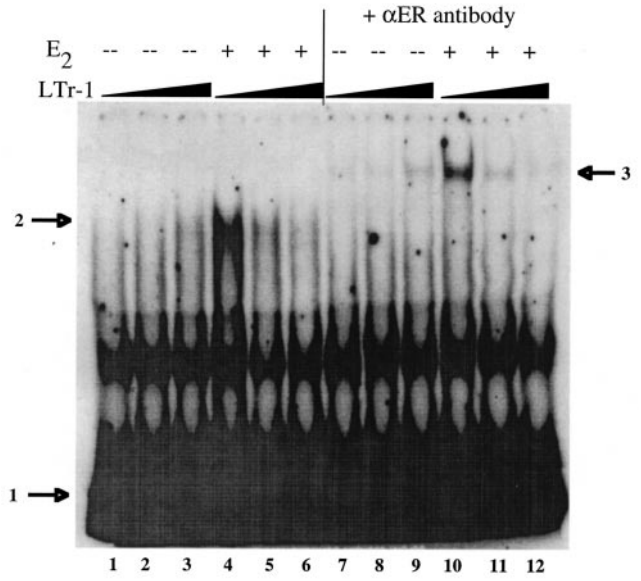

FIG. 7. Binding of nuclear proteins to the ERE. Gel mobility shift analysis of nuclear extracts from estrogen-depleted MCF-7 cells treated for 2 hr with DMSO (lanes 1, 4, 7, 10) or with LTr-1 (1.0 and 10.0 μM in lanes 2, 5, 8, 11 and 3, 6, 9, 12, respectively) and E_2 (1 nM) (lanes 4–6 and 10–12). A monoclonal antibody specific for the human ER also was added to the incubation mixture for lanes 7–12. Arrows indicate the locations of the free labeled probe (arrow #1), the ligand-responsive shifted band (arrow #2), and the antibody-supershifted band (arrow #3).

RESULTS

RXM Fractionation

Silica gel vacuum liquid chromatography was used for the initial crude fractionation of RXM. Five fractions were collected, 100% hexane (A), hexane:THF, 2:1 (B), hexane:THF, 1:1 (C), hexane:THF, 1:2 (D), and 100% THF (E), with gradually increasing mobile phase polarity. At a concentration of 50 μM (I3C equivalent) based on weight of residual material after evaporation of solvent, RXM and all five fractions inhibited MCF-7 cell proliferation in the presence of 1 nM estrogen. Fraction B, the most active fraction, was purified further on a reverse-phase semi-preparative HPLC column using the conditions described in Materials and Methods. The chromatogram shown in Fig. 2 contained a predominant peak and many minor peaks. Of the nine major fractions collected (from Ba to Bi), fraction Bc exhibited the strongest toxic and antiproliferative activities against MCF-7 cells (Fig. 3). In several experiments, crude RXM inhibited cell proliferation by about 70% at the highest non-lethal concentrations (Fig. 3).

HPLC analysis of fraction Bc (Fig. 4) indicated that it contained a compound with the retention time of LTr-1, identified previously as a major component of RXM [23, 24]. Mass spectrometric analysis confirmed this structural assignment.

Cell Proliferation Studies of LTr-1

Results of tumor cell growth experiments showed that both the estrogen-induced proliferation of MCF-7 cells and the estrogen-independent proliferation of the MDA-MB-231 breast tumor cell line were inhibited by LTr-1 by up to 60% in a concentration-dependent manner (Fig. 5). The de-

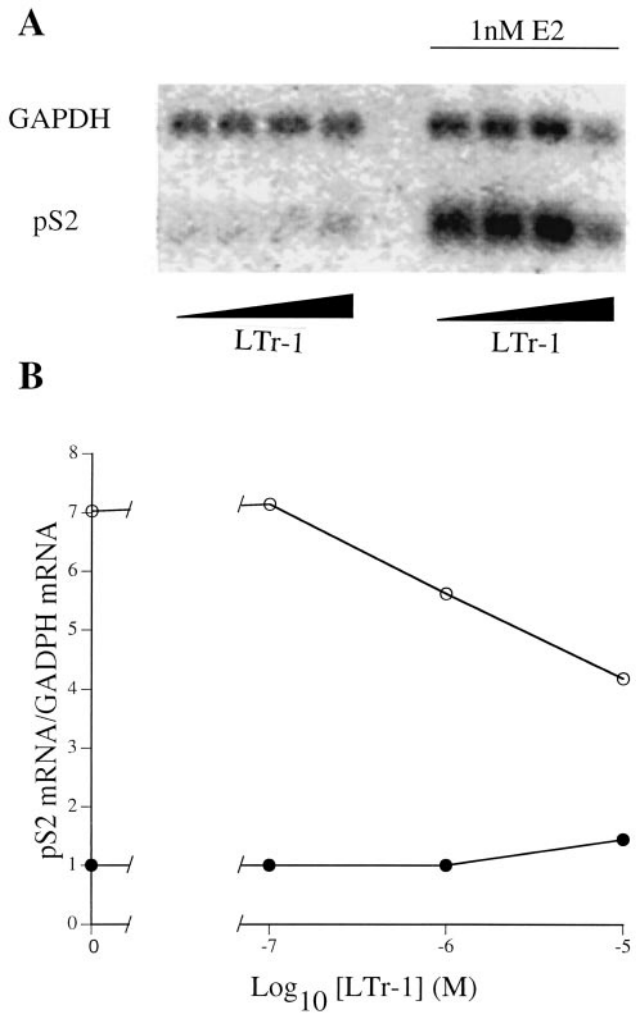

FIG. 8. Effect of LTr-1 on pS2 mRNA expression. Estrogen-depleted MCF-7 cells were treated for 48 hr with LTr-1 at concentrations ranging from 0.1 to 10.0 μM, with (○) or without (●) E_2 (1 nM). pS2 mRNA levels were measured by northern blot analysis (A) and normalized using GADPH mRNA as an internal standard (B). Results are presented as fold induction over the DMSO control (averages of two independent determinations).

creased cell counts apparently were not due to a general toxicity of the trimer, since we saw no evidence of cell killing over the 5-day treatment period. Interestingly, LTr-1 exhibited no apparent effect on proliferation of MCF-7 cells in the absence of E_2.

Effects of LTr-1 on Estrogen Receptor Binding and Function

Because LTr-1 inhibited the E_2-induced proliferation of MCF-7 cells, we examined the effects of this indole derivative on components of the ER signal transduction pathway. The relative binding affinity of LTr-1 for the ER, as measured by a competitive binding assay, indicated an IC_{50} of approximately 70 μM compared with 200 and 3.0 nM measured for tamoxifen and E_2, respectively (Fig. 6). Thus, LTr-1 exhibits a weak affinity for the ER.

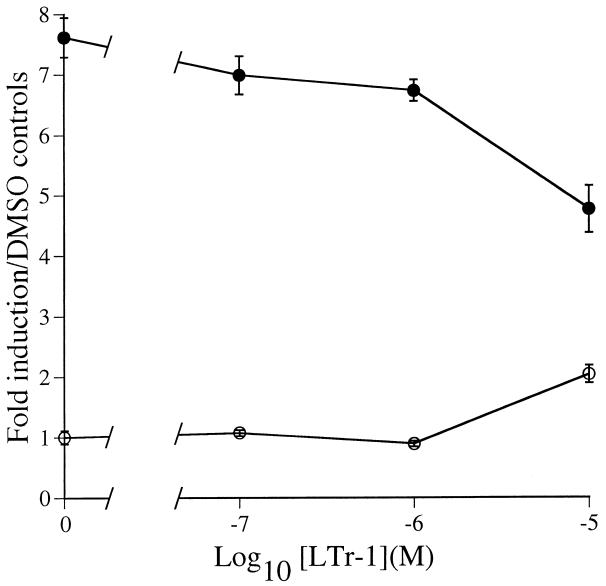

FIG. 9. Effect of LTr-1 on CAT expression from the ERE-vit-CAT reporter gene. MCF-7 cells were transiently transfected with the pERE-vit-CAT reporter plasmid and treated for 48 hr with LTr-1 at the concentrations indicated, in the presence (●) or absence (○) of E_2 (1 nM). CAT activity in cytosol preparations from individual plates was normalized for protein concentration. Results are presented as fold induction over the DMSO control (mean ± range of two independent determinations).

We next examined by gel mobility shift assay the effect of LTr-1 on the binding activity of ER to its cognate DNA motif. LTr-1 exhibited a strong concentration-dependent inhibitory effect on the E_2-induced binding of ER to a consensus ERE with nearly complete loss of the shifted band at 10 μM LTr-1 (Fig. 7). In the absence of E_2, however, LTr-1 exhibited weak agonist activity on ERE binding to DNA.

To determine whether LTr-1 can affect transcription of estrogen-responsive genes, we examined its effects on expression of the endogenous pS2 gene, often used as a marker of estrogen-responsive breast tumors, and on the pERE-vit-CAT reporter construct transiently transfected into MCF-7 cells. The pERE-vit-CAT construct contains the promoter and 5′-flanking region of the *Xenopus* vitellogenin gene upstream of the *CAT* structural gene. The results of northern blot analysis indicated that E_2-induced transcription of pS2 was inhibited in a concentration-dependent manner (approximately 50% at 10 μM) by LTr-1 (Fig. 8). In the absence of E_2, LTr-1 did not induce significant transcription of pS2. A similar inhibitory effect of LTr-1 was seen on E_2-induced expression of the pERE-vit-CAT reporter construct (Fig. 9). In this case, however, LTr-1 exhibited a weak activation of this reporter in the absence of E_2. Thus, LTr-1 could suppress activation of E_2-responsive genes at concentrations that inhibited breast tumor cell proliferation.

Effects of LTr-1 on Ah Receptor Signaling

We reported previously that LTr-1 has an appreciable affinity for the Ah receptor [23]. Since persistent activation of the Ah receptor is thought to be responsible for the toxic effects of certain ligands, including TCDD, we examined further the effects of LTr-1 on this pathway.

The effects of LTr-1 on binding of the Ah receptor to its cognate DNA motif, DRE, as determined by gel mobility shift assay, are represented in Fig. 10. At a concentration of 1 μM, LTr-1 promoted detectable binding of the Ah receptor to the DRE. At a concentration of 10 μM, binding was as strong as the positive control, ICZ, indicating that LTr-1 could efficiently transform the Ah receptor to a DNA binding form.

As shown in Fig. 11, LTr-1 was a weak inducer of Ah receptor responsive CYP1A1 activity in the concentration range of 1–10 μM, with a maximum induction of approximately 20% of the EROD activity induced by ICZ. We found also that LTr-1 was an inhibitor of induced CYP1A1 activity. As shown in Fig. 12, LTr-1 suppressed EROD activity with an ic_{50} of about 1.0 μM. The result of the double-reciprocal plot analysis indicates that LTr-1 was a competitive inhibitor of EROD activity (K_i = 0.913 mM) (data not shown). Taken together, these results indicate that LTr-1 is a weak but efficient agonist of Ah receptor function and an inhibitor of the Ah receptor-induced CYP1A1 enzyme activity.

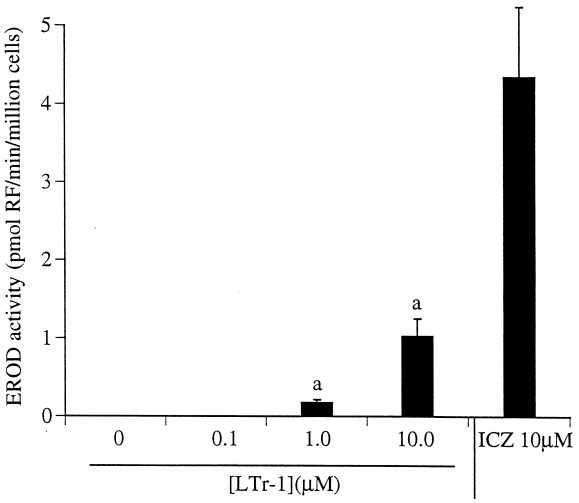

FIG. 11. Induction of expression of cytochrome P4501A1 by LTr-1. MCF-7 cells grown in 10% FBS-DMEM were treated with LTr-1 or ICZ at the concentrations indicated for 24 hr. Expression of P4501A1 was measured by the EROD assay and normalized for cell number in individual plates. RF = resorufin. The statistical differences between groups were determined using ANOVA and Tukey's Studentized range test. The results are expressed as means ± SD for at least three replicate determinations for each experiment. Key: (a) significantly different ($P < 0.05$) from the ICZ control.

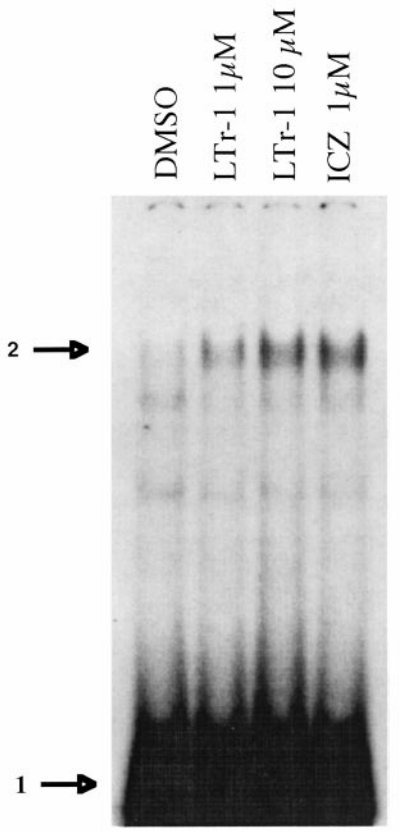

FIG. 10. Binding of nuclear proteins to the DRE. Gel mobility shift analysis of nuclear extracts from Hepa-1c1c-7 cells treated for 2 hr with DMSO, LTr-1, or ICZ. Arrows indicate the locations of the free labeled probe (arrow #1) and the ligand-responsive shifted band (arrow #2).

DISCUSSION

Our results show that LTr-1 can inhibit ER function and activate Ah receptor function. In this respect, LTr-1 appears to produce effects similar to those of potent Ah receptor ligands including TCDD and ICZ. However, whereas LTr-1 can inhibit the estrogen-dependent proliferation of MCF-7 cells and the estrogen-independent proliferation of MDA-MB-231 cells, this indole produced little effect on the estrogen-independent proliferation of MCF-7 cells. These antiproliferative effects of LTr-1 are in contrast to those of TCDD, which strongly inhibits the estrogen-induced growth of MCF-7 cells but, in the same concentration range, has little effect on the proliferation of MDA-MB-231 cells [22]. Thus, the antiproliferative effects of LTr-1 in the two cell lines appear to require processes in addition to, or other than, those activated by TCDD. Other possible explanations for these effects include differences in the metabolism of LTr-1 in the cell lines such that a more generally cytostatic metabolite accumulates in the MDA-MB-231 cells and not in the MCF-7 cells.

The biological effects of LTr-1 differ in several respects from those of DIM, which we will report elsewhere.* Whereas the antiproliferative effects of these substances on

* Riby JE, Chang GHF, Firestone GL and Bjeldanes LF, manuscript submitted for publication.

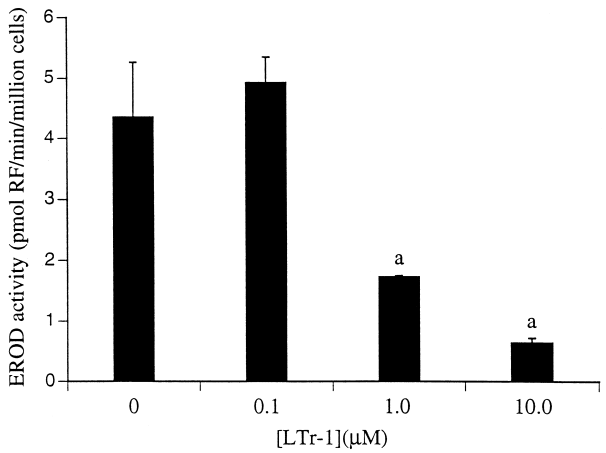

FIG. 12. Inhibition of EROD activity. MCF-7 cells that were 70–80% confluent were treated with 1 μM ICZ for 24 hr. LTr-1 was added to the cell suspension in the fluorometer cuvette to obtain final concentrations of 0.1, 1, and 10 μM, respectively, and incubated at room temperature for 5 min. Finally, 0.5 mL of 2.5 mM ethoxyresorufin was added to the cuvette, and the EROD activity was measured as described above. RF = resorufin. The statistical differences between groups were determined using ANOVA and Tukey's Studentized range test. The results are expressed as means ± SD for at least three replicate determinations for each experiment. Key: (a) significantly reduced ($P < 0.05$) from the DMSO control.

the E_2-dependent proliferation of MCF-7 cells and the E_2-independent proliferation of MDA cells were similar, LTr-1 exhibited little effect on the MCF-7 cells in the absence of E_2. In contrast, DIM showed a marked induction of MCF-7 cell proliferation under E_2-depleted conditions. Consistent with these growth effects, LTr-1 was primarily an antagonist in our assays of ER function, whereas DIM was consistently an agonist of this activity. Similar to DIM, LTr-1 was a weak agonist of Ah receptor function. Thus, in contrast to DIM, LTr-1 is a consistent inhibitor of breast tumor cell proliferation and shows little evidence that it might function as an agonist of ER-mediated transcriptional events. In this respect, the activities of LTr-1 in cultured cells are similar to the effects we observed for RXM. However, determination of whether LTr-1, DIM, or other RXM products can account for all or part of the effects of I3C administered orally requires further *in vivo* and *in vitro* studies of the individual and combinatorial effects of these substances.

Previous studies have established that LTr-1 and DIM are major *in vivo* products of orally administered I3C. Estimates of LTr-1 produced in the stomachs of rodents within several hours of oral administration of I3C range in yield from about 0.2 to 2.0% [23, 24]. These products appear to be absorbed to a similar extent from the gastrointestinal tract, since the relative concentrations of the two compounds found in the liver are similar to those found initially in the stomach. It is interesting to note that the concentrations of the two compounds found in the livers of treated rodents (up to 13 μM) [13] are within the range in which these substances are active in our cell proliferation and ER-dependent gene activation assays.

Efforts to estimate likely exposure to LTr-1 following ingestion of *Brassica* vegetables are difficult, since no direct measurements of glucobrassicin conversion to LTr-1 are available. Nevertheless, since it is well established that LTr-1 is a major product of I3C oligomerization *in vitro* and *in vivo*, in some cases exceeding DIM in yield, it is reasonable to estimate LTr-1 exposures based on estimates of DIM exposures. Thus, based on the published levels of glucobrassicin in fresh Brussels sprouts of about 1.4 g/kg and assuming a conversion of glucobrassicin of about 5%, the level of LTr-1 produced from a 100-g portion of vegetable is about 5–10 mg. On the assumption that absorption from the gastrointestinal tract is nearly complete, this would provide blood concentrations for an average person of as high as 8 μM. Thus, following ingestion of a 100-g portion of cruciferous vegetables, an average person may attain a blood concentration of LTr-1 of within an order of magnitude of the effective levels determined in this study.

Thus, these studies establish LTr-1 as a novel inhibitor of breast tumor cell proliferation that can affect both estrogen-dependent and -independent cellular pathways in cultured cells in a range of concentrations similar to concentrations found *in vivo* following ingestion of naturally occurring I3C. Further studies of the antiproliferative efficacy and modes of action of this compound are in progress.

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