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ESTROGENICITY OF THE SYNTHETIC FOOD COLORANTS TARTRAZINE, ERYTHROSIN B, AND SUDAN I IN AN ESTROGEN-RESPONSIVE HUMAN BREAST CANCER CELL LINE

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ABSTRACT—We investigated the estrogenic activity of three synthetic food colorants using two in vitro assays in estrogen receptor positive T47D breast cancer cells. Specifically, we examined whether tartrazine (FD&C Yellow No. 5), erythrosin B (FD&C Red No. 3), and sudan I in nanomolar ranges could mimic estradiol-17β (E2). Using E-screen assays, we detected the highly significant proliferative effects (PE) of tartrazine ($P = 0.0004$), erythrosin B ($P < 0.0001$), and sudan I ($P = 0.0005$). The proliferative effect of each was significantly reversed by pretreatment of cells with tamoxifen (overall $P \leq 0.001$). Further testing with luciferase reporter gene assay revealed that each compound significantly activated the estrogen response element resulting in reporter gene expression ($P = 0.0001$). This is the first report of the estrogenic effect of tartrazine. In addition, we have shown that the suspected carcinogen, sudan I, acts at least in part through activation of the estrogen receptor (ER). The results obtained for erythrosin B support previous research on the compound in HTB 133, demonstrating proliferative effects through activation of the ER.

Synthetic colorants are used to make foods more appealing to consumers, create distinctive colorations for medicines, and develop various shades in facial cosmetics. However, studies have revealed toxicological effects of many colorants. In vitro carcinogenicity of sudan I has been revealed in *Salmonella typhimurium* (Cameron et al., 1987; Zeiger et al., 1988) and mouse lymphoma L5178Y TK+/− cells (Cameron et al., 1987). In 1991, Westmoreland and Gatehouse revealed the clastogenic properties of sudan I in an in vivo rodent micronuclei test; more recent studies have suggested possible carcinogenicity in humans through the formation of DNA adducts (dose range 0.1–100 μM) (Stiborova et al., 2002). In addition, Kozuka et al. (1980) have shown that sudan I is a causative agent for pigmented contact dermatitis in humans. Currently, sudan I is a banned food colorant due to its carcinogenic properties. Erythrosin B (FD&C Red No. 3) has been shown to effect acetylcholine release at the neuromuscular junction, in vivo (Augustine and Levitan, 1983). In studies of chronically exposed male rats (28 months, 4% of diet), erythrosin B resulted in a significantly higher incidence of thyroid follicular cell adenomas and carcinomas (Food and Drug Administration, 1990). More recently, erythrosin B (dose range: 25–100 μg/mL) has been shown to stimulate proliferation in estrogen receptor (ER) positive HTB 133 cells and to increase Cdk2 activity (dose range: 3–10 μg/mL) in MCF7 cells (Dees et al., 1997). Additionally, Dees et al. (1997) suggest both erythrosin B and tartrazine (FD&C Yellow No. 5) may damage DNA as evidenced by increased p53-DNA binding in MCF7 cells treated with these compounds, though the reported effect of tartrazine was relatively low. Tartrazine has been reported to cause urticaria, asthma, and in some cases a cross-sensitivity in aspirin and NSAID-sensitive individuals (Dipalma, 1990). The mode of action of tartrazine is still under investigation, and it has been categorized as a pseudo-allergen (Dipalma, 1990).

Acceptable daily intake (ADI in mg/kg body weight) is the amount of a food additive that can be consumed daily without appreciable risk based on all available toxicological data. The ADI for a food additive is generally calculated by dividing the "no observable adverse effect level" or NOAEL in animal studies by a large factor such as 100 and is established in the United States by the Food and Drug Administration (FDA). A review of toxicological data for sudan I has rendered it a banned food additive (i.e., ADI = 0 mg/kg body weight). Erythrosin B is approved in the United States for food and ingested drugs with an ADI of 2.5 mg/kg body weight (Lipman, 1995). The gastric absorption rate of ^{131}I-erythrosin has been estimated to be approximately 1% or less in humans (unpublished study reviewed in FDA, 1990). Tartrazine is approved in the United States for food, drug, and cosmetic use at an ADI of 5 mg/kg body weight (FDA, 1985). Gastric absorption of tartrazine has not been determined in humans, but in rats the gastric absorption data appear similar to data for erythrosin B with approximately 1% or less being absorbed (Honohan et al., 1977). Tartrazine is also found as an active ingredient in aquatic herbicides such as *Aquashade*. Depending on the particular preparation of aquatic herbicide used, the Environmental Protection Agency (EPA) estimates concentrations of tartrazine to be 0.0023–0.048 mg/L in the body of water to which the preparation has been applied (EPA, 2005). Maximum exposure for terrestrial wildlife through drinking herbicide treated water is estimated at 0.0024 mg per day (0.1 mg *Aquashade* by 2.39% tartrazine in product) (EPA, 2005).

Endocrine disrupting chemicals (EDCs) mimic and disrupt the signaling cascade of endogenous hormones and, unlike classical poisons, act at low concentrations (Dickerson and Gore, 2007). Hormones are biologically effective in nanogram to microgram quantities; thus, very low concentrations of

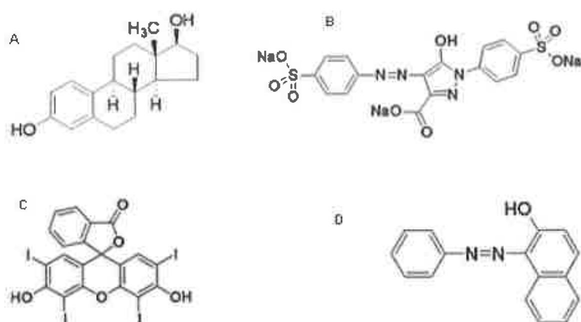

FIG. 1. Chemical homology of food colorants to Estradiol 17β: A. Estradiol 17β; B. Tartrazine; C. Erythrosin B; and D. Sudan I. The food colorants exhibit a key structural similarity to E2, a phenolic group or benzene attached to a hydrophilic group. This key structure is necessary for estrogen receptor recognition of its ligand.

compounds that mimic endogenous hormones could have biological effects. Given the ADIs and estimated environmental concentrations, low absorption rates such as 1% could still result in body burdens in the nanomolar to micromolar range for erythrosin B and tartrazine. For example, if a 25 kg child consumed 1% of the ADI for tartrazine and 1% of that amount was absorbed, estimated blood concentrations would be 0.0083 mg/L or 15.6 nM (tartrazine MW = 534.36). The same calculation for erythrosin B yields an estimate of 4.74 nM (erythrosin B MW = 879.86).

Xenoestrogens are synthetic chemicals that specifically mimic and disrupt the signaling cascade of estrogens causing reproductive abnormalities in humans and wildlife. In 1979, Gill et al. reported the reproductive disorders in children of women who had been treated with diethylstilbestrol (DES) during their pregnancy. Xenoestrogens have also been linked to increased incidence of cryptorchidism and hypospadias in men (Gill et al., 1979; Giwercman et al., 1993; Jackson, 1988), increased incidence of testicular hypoplasia (Gill et al., 1979) and malignancy (Osterlind, 1986), decrease in sperm count and quality in men (Giwercman et al., 1993), abnormalities in menopause in women (WHO, 1995), and increased incidence of prostate cancer and breast cancer (Wolff and Toniolo, 1995). Xenoestrogens have also been linked to reproductive and developmental defects in wildlife (Arai et al., 1983; Bitman et al., 1968; Falk et al., 2006; Purdom et al., 1994; Sumpter and Jobling, 1995). The adverse effects reported by others coupled with the chemical similarities among tartrazine, sudan I, erythrosin B, and estradiol-17β (E2) (Fig. 1) led us to ask whether these colorants were potential EDCs, specifically xenoestrogens, acting not at pharmacological concentrations but rather within physiological concentrations.

Estrogenicity was assessed using a cell proliferation assay (Soto et al., 1995; Matsuoka et al., 2005) and a luciferase reporter gene bioassay (Legler et al., 1999; Wilson et al., 2004) in T47D cells. The T47D cell-line (ATCC, HTB-133) was derived from a ductal carcinoma of the human breast and expresses endogenous alpha and beta ERs (Dotzlaw et al., 1996). T47D cells exhibit approximately 67.6 ± 6.2 fmol/mg cytosolic ER proteins (Watanabe et al., 1990). T47D cells are used extensively in research involving breast cancer and in vitro endocrine disruptor screening bioassays (Dees et al., 1997; Legler et al., 1999; Meerts et al., 2001; Wilson et al., 2004; Zava et al., 1997).

MATERIALS AND METHODS

Chemicals—RPMI 1640 containing glutamine, antibiotic/antimycotic solution (15240-096) (AbAm), and Dulbecco's phosphate buffered saline (DPBS), were all purchased from Invitrogen/Gibco, Gaithersburg, Maryland. Fetal bovine serum (FBS) and trypsin were purchased from Atlanta Biologicals, Lawrenceville, Georgia. Bradford reagent was obtained from Bio-Rad Laboratories, Hercules, California. Porcine insulin, EDTA, charcoal-dextran, E2 (E8875-1G), tamoxifen (T5648-1G), tartrazine (T0388-100G), erythrosin B (E9259-5G) and sudan I (103624-25G) were purchased from Sigma-Aldrich, St. Louis, Missouri. E2 and sudan I were stored as 10 mM stock solutions in 90% ethanol at −20°C. Tartrazine and erythrosin B were dissolved in sterile, nanopure water to a final concentration of 10 mM. For experiments, the chemicals were all diluted to desired-concentrations in phenol red-free RPMI 1640, and cells were never exposed to greater than 0.001% ethanol.

The *puc9.neo* plasmid vector was provided by Dr. Phillip Hartig, United States EPA, Research Triangle Park, North Carolina. The Panomics Translucent Reporter Vector (*LR0020*) was purchased from Promega, Madison, Wisconsin. This vector has an estrogen response element (ERE) upstream from the luciferase gene. Restriction enzymes, *HindIII* (10656313001, 10 U/μl) and *BamHI* (10220612001, 10 U/μl), were obtained from Roche Diagnostics, Indianapolis, Indiana. FuGENE 6 Transfection Reagent (11815091001) was purchased from Roche. Luciferase Assay System (E1500) was purchased from Promega.

Culture of T47D Cells—Estrogen-receptor positive T47D breast cancer cells (ATCC, HTB 133) were obtained from the American Type Culture Collection, Manassas, Virginia. T47D cells were cultured in Growth Medium: RPMI 1640 plus 10% v/v charcoal-stripped FBS (CS-FBS), 0.2 U/mL porcine insulin and 2% v/v AbAm. The growth medium was changed every 48 h. Cells were incubated at 37°C, 90% humidity, and 5% CO_2 in air. Upon confluence, the adherent cell layer was trypsinized from the 25 cm^2 culture flask (Falcon, 3013), washed, and resuspended in fresh medium. Trypsinization medium consisted of 0.25% trypsin plus 0.53 mM EDTA in DPBS, pH 7.2.

Cell Proliferation Assay—The cell proliferation assay was performed as described by Matsuoka et al. (2005) with modifications. Briefly, T47D cells were plated in a 24-well plate (CLS3526, Corning® Costar® cell culture plate) at an initial density of 4.0 by 10^4 cells per well in phenol red-free RPMI 1640 supplemented with 5% v/v CS-FBS, 0.2 U/mL porcine insulin and 2% AbAm. After 24 h, the medium was changed to phenol red-free RPMI 1640 supplemented with 5% CS-FBS and 2% AbAm and either control (vehicle only), E2, tartrazine, erythrosin B, or sudan I (0.001, 0.01, 0.1, 1, and 10 nM). Each treatment was performed in quadruplicate and each compound was tested at the five concentrations given. For all assays, E2 was used as a positive control at concentrations similar to its physiological concentrations (0.001 to 10 nM). For antagonist assays, the antagonist, tamoxifen (1 μM), was added 1 h before treatment application (Makela et al., 1994). The medium was changed every 48 h. Following 96 hours of treatment, cells were washed twice with phosphate buffered saline, pH 7.2 (PBS), and solubilized in 0.1 N NaOH. A Bradford protein assay (Bradford, 1976) was performed in duplicate for each sample. Bovine serum albumin

was used to generate a protein standard curve. Absorbance at 595 nm was converted into amount of total protein per well. A minimum of three independent cell proliferation assays were performed for each compound tested.

Reporter Gene Construct—The plasmid, puc9.neo, was amplified and the neomycin gene cassette (1.8 kb) was removed with BamHI restriction digestion. Simultaneously, the LR0020 vector (ERE upstream from luciferase gene) was linearized with BamHI. The vectors were incubated at 80°C for 10 min to deactivate BamHI, and all the digested products were purified to remove salt residues, buffers, and restriction endonucleases with Genopure Plasmid Midi Kit (Roche, 3143414). Ligation was performed with 3:1 molar ratio of insert to vector with 10 μL T4 ligase and 2.0 μL ligation buffer (10× buffer) in a 20 μL reaction mixture at 16°C overnight. Ligated DNA was stored at −20°C and subjected to purification with Genopure Plasmid Midi Kit (Roche, 3143414). The resulting ERE.Luc.neo expression vector was used for the transient transfections.

Luciferase Reporter Gene Assay—Transient transfection was performed as described by the Fugene 6 manufacturer's protocol. Briefly, T47D cells were plated in a 24-well plate at a density of 5.0 by 10^5 cells per well in phenol red-free growth medium 24 h prior to transfection. On the day of transfection, cells were washed twice with sterile PBS and once with serum-free and antibiotic-free growth medium. Medium was replaced with fresh serum-free and antibiotic-free growth medium. Cells were transfected with 5 μg ERE.Luc.neo plasmid vector per well at a ratio of 3:1 (Fugene6: DNA). After 6 h, the transfection medium was replaced with phenol red-free RPMI medium 1640 plus 5% v/v CS-FBS and either control (vehicle only), E2 (0.1 nM), tartrazine (0.1 nM), erythrosin B (0.01 nM), or sudan 1 (1 nM) in triplicate. Antibiotic/Antimycotic was not included here as the mixture creates background levels of luciferase activity (Wilson et al., 2004). After 48 h of incubation, treatments were removed, cells were washed twice with PBS, scraped up, and transferred to microfuge tubes. Cells were pelleted and lysed with 50 μL Promega Luciferase assay lysis buffer.

Luciferase activity was measured with a luminometer (Synergy HT1 Multi-Detection Microplate Reader) in a 96-well format at sensitivity 200; each sample was assayed in duplicate, and relative light units (RLU) were read within 1 min after the addition of substrate. Duplicate determinations for each sample were averaged. Each assay consisted of a minimum of two replicates per treatment. Each independent experiment was repeated a minimum of three times.

Statistical Analysis—Cell proliferation is reported as proliferative effect (PE). Proliferative effect was calculated as total protein of treatment divided by total protein of control (vehicle only). Data (mean ± *SEM*) were analyzed by two-way analysis of variance (ANOVA), where independent experiment number was the blocking factor and concentration was the treatment factor; $P \leq 0.05$ was considered statistically significant. A significant ANOVA was followed by post-hoc Tukey HSD (significance level of $P \leq 0.05$) to test whether there were significant differences amongst different concentrations of each treatment. In cell proliferation experiments which included tamoxifen, two-way ANOVA was conducted where independent experiment number was the blocking factor and the various combinations of test compounds was the treatment factor. A significant ANOVA ($P \leq 0.05$) was followed by post-hoc Tukey HSD (significance level of $P \leq 0.05$) to test whether there were differences amongst the various combinations of treatments.

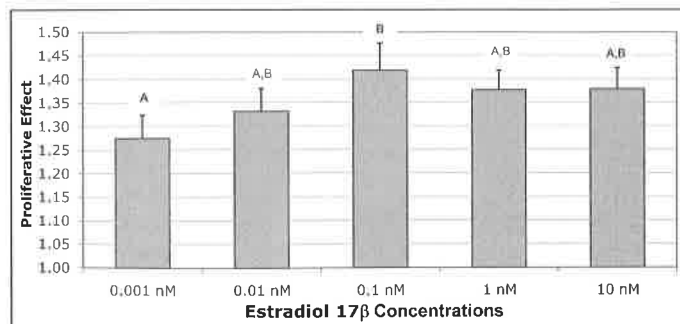

FIG. 2. Proliferative effect of E2 in T47D cells treated for 96 h (mean ± *SEM* of 9 independent experiments and four replicates per experiment). E2 significantly stimulated the proliferation of T47D cells ($P < 0.0001$). Bars with different letters are significantly different from one another in a post-hoc Tukey HSD analysis ($P = 0.05$). Proliferative effect (PE) = (total protein of treatment)/(total protein of control).

Data from luciferase reporter gene assays are reported in Relative Light Units (RLU). Relative light units were normalized in each experiment as a percent of control (vehicle only) RLU. Data (mean ± *SEM*) were analyzed by two-way ANOVA, where independent experiment number was the blocking factor and test compound was the treatment factor. A significant ANOVA ($P \leq 0.05$) was followed by comparison of means using least square means Student's *t*-test ($\alpha = 0.05$).

All statistical analyses were performed with JMP® 6 Statistical Software (SAS Institute, 2005).

RESULTS

Proliferative Effect of Tartrazine, Erythrosin B, and Sudan I in T47D Cells—Cell proliferation assays measure the proliferative effect of estrogen or xenoestrogens on estrogen-responsive cells in a hormone-stripped medium (Soto et al., 1995). The PE of increasing concentrations of E2 (positive control) and synthetic food colorants are shown in Figures 2 and 3. Data are represented as mean ± *SEM* of n independent experiments. E2 ($n = 9$) induced highly significant proliferation of T47D cells over control (vehicle only) at all concentrations ($P < 0.0001$). A post-hoc Tukey HSD indicated the PE induced by 0.1 nM was significantly different from the PE induced by 0.001 nM E2. There were no significant differences amongst the PE induced by 0.01–10 nM E2. The three food colorants tartrazine ($n = 3$ for 0.001 and 0.01 nM; $n = 4$ for 0.1, 1 and 10 nM; $P = 0.0004$), erythrosin B ($n = 3$; $P < 0.0001$), and sudan I ($n = 3$; $P = 0.0005$) at all concentrations induced significant proliferation of T47D cells over control. A post-hoc Tukey HSD indicated no significant differences amongst the various concentrations of food colorants.

Difference in Proliferative Effect of Tartrazine, Erythrosin B, and Sudan I in Presence of Antagonist Tamoxifen (1 μM) in T47D Cells—Tamoxifen competitively binds to ER in the presence of E2 or its analogues and inhibits cell proliferation in breast cancer cells (Jordan et al., 2001). The difference in PE of E2 and food colorants in the presence and absence of tamoxifen (1 μM) is shown (Fig. 4). The assay was performed

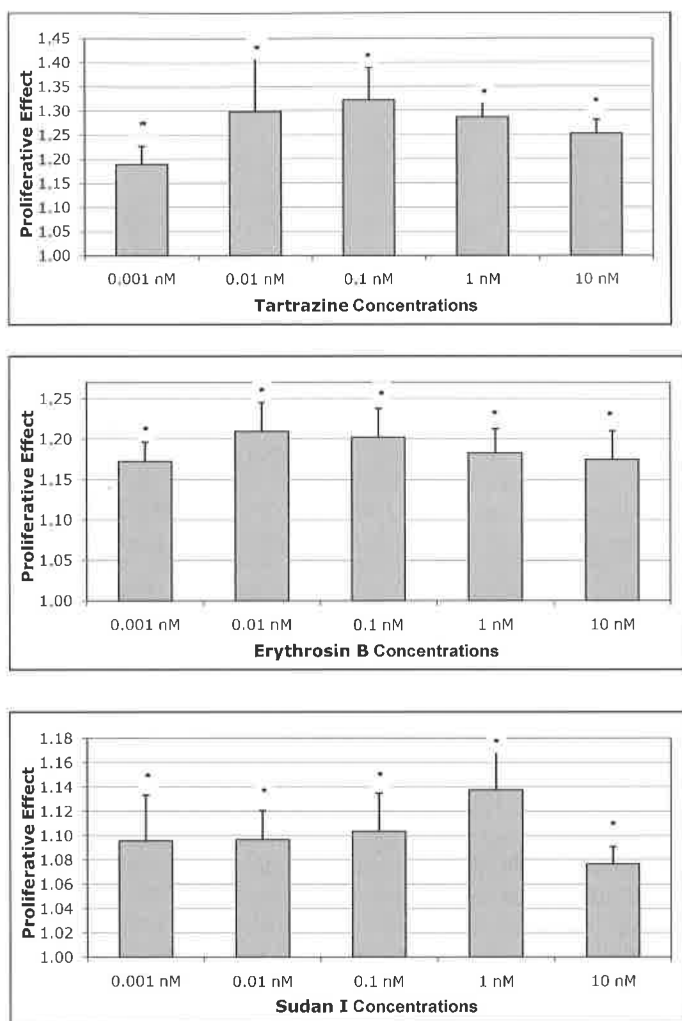

FIG. 3. Proliferative effect of Tartrazine (top), Erythrosin B (middle), and Sudan I (bottom) in T47D cells treated for 96 h (mean ± *SEM* of *n* independent experiments and four replicates per experiment). Tartrazine ($n = 3$ for 0.001 and 0.01 nM; $n = 4$ for 0.1, 1 and 10 nM); Erythrosin B ($n = 3$), and Sudan I ($n = 3$). Tartrazine ($P = 0.0004$), Erythrosin B ($P < 0.0001$), and Sudan I ($P = 0.0005$) significantly stimulated the proliferation of T47D cells at all concentrations tested. Asterisks denote a statistically significant difference compared to control (vehicle only). A post-hoc Tukey HSD indicated that there was no significant difference among the different concentrations of each colorant.

using E2 (0.1 nM), tartrazine (0.1 nM), erythrosin B (0.01 nM), and sudan I (1 nM). Tamoxifen (1 μM) significantly blocked PE of E2, tartrazine, erythrosin B, and sudan I. Two-way ANOVA for the tartrazine experiment (Fig. 4a) was significant at $P = 0.0013$, for the Sudan I experiment (Fig. 4b) at $P < 0.0001$, and for the erythrosin B experiment (Fig. 4c) at $P = 0.0003$. Post-hoc Tukey HSD ($P \leq 0.05$) indicated that in each experiment, E2-treatmeant was significantly different from Tamoxifen-only, E2+Tamoxifen, and Colorant+Tamoxifen. Also, in each experiment colorant-treatment was significantly different from Tamoxifen only, E2+Tamoxifen, and Colorant+Tamoxifen.

Activation of Estrogen-Receptor Mediated Luciferase Reporter Gene Expression by E2, Tartrazine, Erythrosin B, and Sudan I in T47D Cells, Transiently Transfected with ERE.Luc.neo—The luciferase reporter gene assay can be used to evaluate whether xenoestrogens act via the classical nuclear hormone pathway (Joyeux et al., 1997). Luciferase induction, reported as a percent of control RLU, by E2 (0.1 nM), tartrazine (0.1 nM), erythrosin B (0.01 nM), and sudan I (1 nM) in T47D cells transiently transfected with ERE.Luc.neo and treated for 48 h is shown (Fig. 5). Two-way ANOVA indicated a significant effect of treatment ($P = .0001$). A comparison of means by least square means Student's *t*-test ($\alpha = 0.05$) indicated that the effect of E2 and food colorants was significantly different from the control.

Discussion

The goal of our research was to evaluate estrogenicity of synthetic food colorants that are chemically similar to E2. We evaluated tartrazine, erythrosin B, and sudan I with cell proliferation and reporter gene assays. The food colorants exhibit a key structural similarity to E2, a phenolic group or benzene attached to a hydrophilic group (Fig. 1). This key structure is necessary for estrogen receptor recognition of its ligand (Nilsson et al., 2001). Results from both assays show that the three compounds behave as xenoestrogens in vitro.

Cell proliferation assays (E-screen) measure the proliferative effect of estrogen or xenoestrogens, on estrogen responsive cells in a hormone-stripped medium (Soto et al., 1995). The total number of viable cells after an E-screen is directly proportional to the effect of chemicals on the estrogen responsive cells (Soto et al., 1995). In our research, estrogenicity induced by E2 in T47D cells at concentrations between 0.001 nM and 10 nM (Fig. 2) was consistent with Wilson et al. (2004). As demonstrated (Fig. 3), all three investigated compounds showed significant response in the effective concentration range of 0.001 nM–10 nM, similar to the physiological concentration range of E2 and within estimated human exposure following consumption of the ADI of tartrazine and erythrosin B or wildlife exposure following drinking of tartrazine containing water. This is significant because it suggests that these compounds have the ability to interact with the estrogen signaling cascade at concentrations well below that which would be tested in classical toxicology studies. In the last several years, we have learned that exposures to environmentally relevant concentrations of EDCs at critical periods of development can have effects that are manifested well after the original exposure (Welshons et al., 1999; Howdeshell et al., 1999; Markey et al., 2001; Markey et al., 2005). This understanding has led the United States Congress in 1996 to require the EPA to begin screening chemicals for the potential of endocrine disruption (EPA, 2007).

To complement and substantiate the cell proliferation results, additional experiments were performed to study effects of the food colorants in the presence of tamoxifen. Tamoxifen is an ER antagonist in estrogen responsive breast cancer cells (Jordan et al., 2001; Zhang et al., 2005); if a compound was stimulating cell proliferation via ER binding and activation, then such binding will be competitively inhibited by tamoxifen. In our research, cell proliferation induced by E2 and the food colorants was significantly inhibited by tamoxifen (Fig. 4). These data indicate that the positive control (E2) and food colorants stimulated cell proliferation at least in part via the ER. The fact that tamoxifen-only treated cells had lower PE

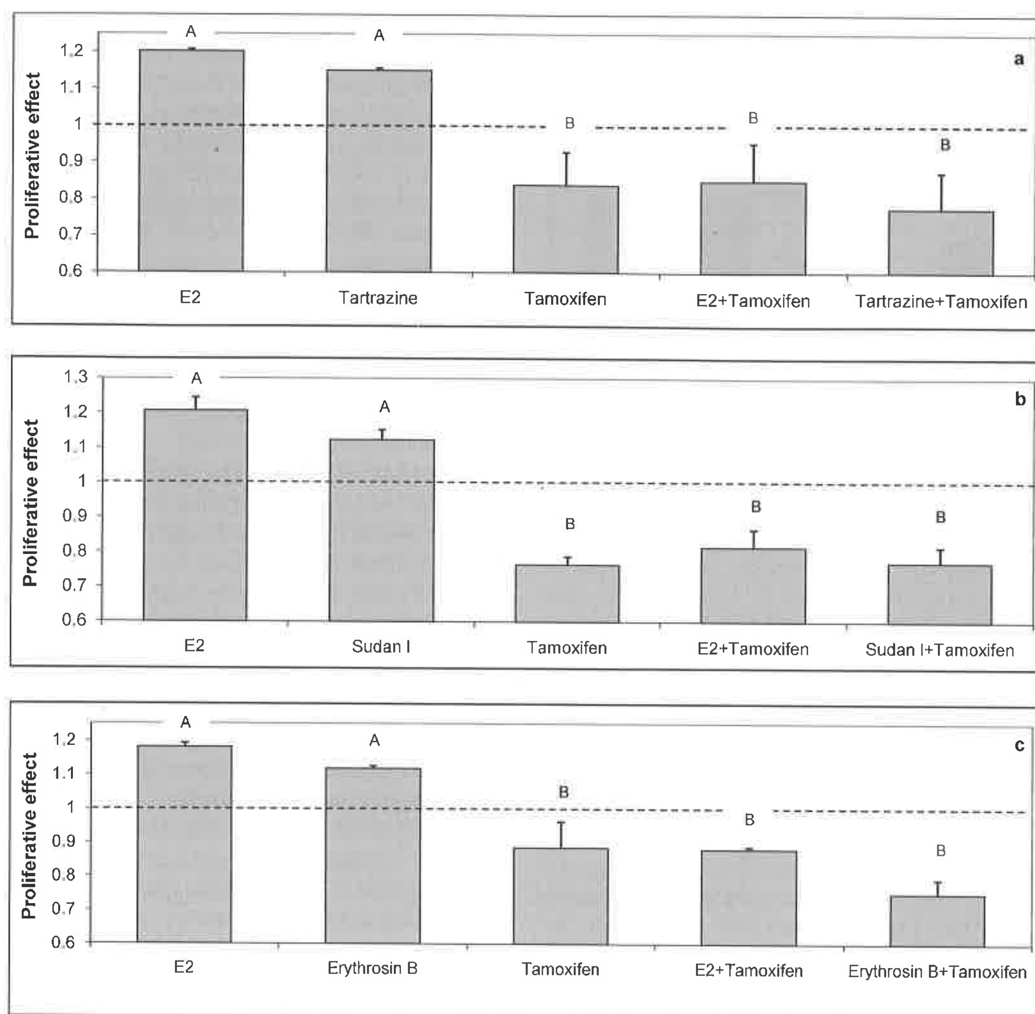

FIG. 4. Proliferative effect of 0.1 nM E2 and (4a) Tartrazine (0.1 nM), (4b) Sudan I (1 nM), and (4c) Erythrosin B (0.01 nM) in presence or absence of antagonist tamoxifen (1 μM) in T47D cells treated for 96 h (mean ± *SEM* of three independent experiments and four replicates per experiment). The dashed line represents control (vehicle only) PE. Two-way ANOVA for the Tartrazine experiment (Panel 4a) was significant at $P = 0.0013$, the Sudan I experiment (Panel 4b) at $P < 0.0001$, and for the Erythrosin B experiment (Panel 4c) at $P = 0.0003$. Post-hoc Tukey HSD ($P \leq 0.05$) indicated that in each experiment, E2-treatmeant was significantly different from Tamoxifen-only, E2+Tamoxifen, and Colorant+Tamoxifen. Also, in each experiment, colorant-treatment was significantly different from Tamoxifen only, E2+Tamoxifen, and Colorant+Tamoxifen. Within each panel, bars with different letters are significantly different from one another.

than the untreated cells indicates that there may be undefined estrogenic activity in the control medium (Bondy and Zacharewski, 1993).

The luciferase reporter gene assay was performed to demonstrate the competence of the food colorants to activate the nuclear ER. This is based on the principle of estrogens (and/or xenoestrogens) activating endogenous ER in T47D cells. The activated ER binds to the estrogen response element on the reporter vector followed by activation of the reporter gene (luciferase). On lysis of transfected cells, luciferase is released from the cell. It reacts with its substrate (luciferin) to emit light, which is proportional to the estrogenic activity (Fielden et al., 1997; Joyeux et al., 1997; Villeneuve et al., 1998). In our research, all three of the investigated compounds significantly stimulated luciferase induction over control ($P = 0.05$) and further established a nuclear ER mediated effect of the food colorants by either direct binding or indirect activation of the ER.

The data presented in this study show that all three food colorants tested—tartrazine (FD&C Yellow No. 5), erythrosin B (FD&C Red No. 3), sudan I—have estrogenic potential. These data are in agreement with a previous report by Dees et al. (1997) on the estrogenicity of erythrosin B and by Stiborova et al. (2002) on the carcinogenicity of Sudan I. To the best of our knowledge, this is the first report of the estrogenic effect of the frequently used synthetic food colorant tartrazine (FD&C Yellow No. 5). A significant aspect of our research reveals that tartrazine, erythrosin B, and sudan I are estrogenic in nanomolar concentrations and within the physiological range of E2. These concentrations are reasonably estimated to occur upon consuming and absorbing a fraction of a percent of the ADI for tartrazine and erythrosin B. In addition, we have shown that the colorants act at least in part through activation of the ER. The role of these synthetic food colorants as potential xenoestrogens correlates with the potential adverse physiological effects of food additives in human diet.

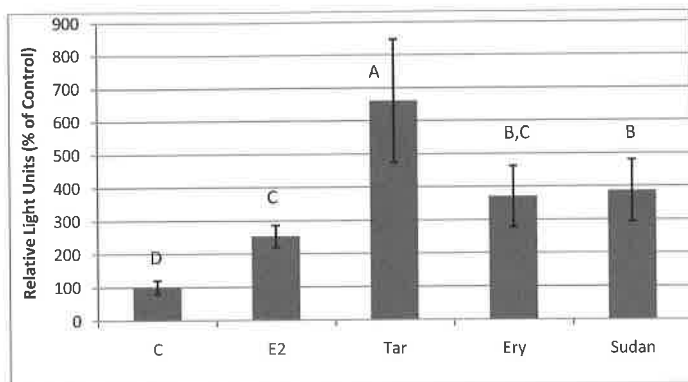

FIG. 5. Relative light units (RLU) stimulated by E2 (0.1 nM), Tartrazine (Tar, 0.1 nM), Erythrosin B (Ery, 0.01 nM), and Sudan I (1 nM) expressed as a percentage of control (C, vehicle only) RLU in T47D cells, transiently transfected with *ERE.Luc.neo*. Data represent mean ± *SEM* of 3 independent experiments. Each sample was assayed in duplicate, and each assay consisted of a minimum of two replicates per treatment. Two-way ANOVA indicated a significant effect of treatment ($P = 0.0001$). A comparison of means by Student's *t*-test ($\alpha = 0.05$) indicated E2 and food colorants were significantly different from control. Bars with different letters are significantly different from one another.

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