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

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Estrogenicity of the Synthetic Food Colorants, Tartrazine, Erythrosin B and Sudan I in an Estrogen-responsive Human Breast Cell Line

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Payel Datta

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I am submitting herewith a thesis written by Payel Datta entitled "Estrogenicity of the Synthetic Food Colorants, Tartrazine, Erythrosin B and Sudan I in an Estrogen-responsive Human Breast Cell Line" . I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biological Sciences.

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Chapter 1. Introduction

Additives such as synthetic colorants are integral parts of food, cosmetics and pharmaceutical products. Colorants have been used to make foods more appealing to consumers, to create distinctive colorations for medicines, and to develop various shades in facial cosmetics. However, recent studies have revealed toxicological effects of many colorants. *In vitro* carcinogenicity of sudan I has been revealed in *Salmonella typhimurium* mutagenicity tests with S-9 activation (Cameron *et al.* 1987; Zeiger *et al.* 1988) and in mouse lymphoma L5178Y TK+/- cells with S-9 activation (Cameron *et al.* 1987). In 1991, Westmoreland and Gatehouse revealed the clastogenic properties of sudan I in an *in vivo* rodent micronuclei test; recent studies have suggested possible carcinogenicity in humans through the formation of DNA adducts (dose range 0.1–100 μM) (Stilborova *et al.* 2002). In addition, Kozuka *et al.* (1988) have shown that sudan I is a causative agent for pigmented contact dermatitis in humans. Currently, sudan I is banned in many countries 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 1980; Lafferman and Silbergeld 1979). More recently, erythrosin B (dose range of 25 μg/ml to 100 μg/ml) has been shown to stimulate proliferation in estrogen receptor (ER) positive HTB 133 cells and to increase Cdk2 activity (dose range of 3 μg/ml to 10 μg/ml) in MCF7 cells (Dees *et al.* 1997). Additionally, Dees *et al.* (1997) suggest both erythrosin B and tartrazine (FD& C Yellow 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).

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.* 1992), 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 insidiousness of endocrine disrupting chemicals (EDCs), such as xenoestrogens, is that, unlike classical poisons, they act at low concentrations. The adverse effects that have been reported by others, coupled with the chemical similarities amongst tartrazine, sudan I, erythrosin B and estradiol-17β (E2) (Figure 1) led us to ask whether these colorants were potential EDCs, specifically xenoestrogens, acting not at toxicological concentrations but rather within physiological concentrations.

In the studies reported here, estrogenicity was assessed using the cell proliferation assay (Soto *et al.* 1995; Matsuoka *et al.* 2005) and estrogen-receptor-mediated-chemically activated luciferase reporter gene expression (ER-CALUX) bioassay (Leglar *et al.* 1999; Wilson *et al.* 2004) in the T47D cell-line, in presence or absence of tamoxifen. Tamoxifen is an antagonist in breast cancer cells and binds to and inactivates the ER. The T47D cell-line (ATCC, HTB-133) was derived from a ductal carcinoma of the human breast and expresses endogenous alpha and beta ERs (Dotslaw *et al.* 1996). T47D cells exhibit approximately 67.6±6.2 fmol/mg cytosolic ER proteins (Watanabe *et al.* 1990). T47D is used extensively in research involving breast cancer and *in vitro*

endocrine disruptor screening bioassays (Dees *et al.* 1997; Leglar *et al.* 1999; Meerts *et al.* 2001; Wilson *et al.* 2004; Zava *et al.* 1997).

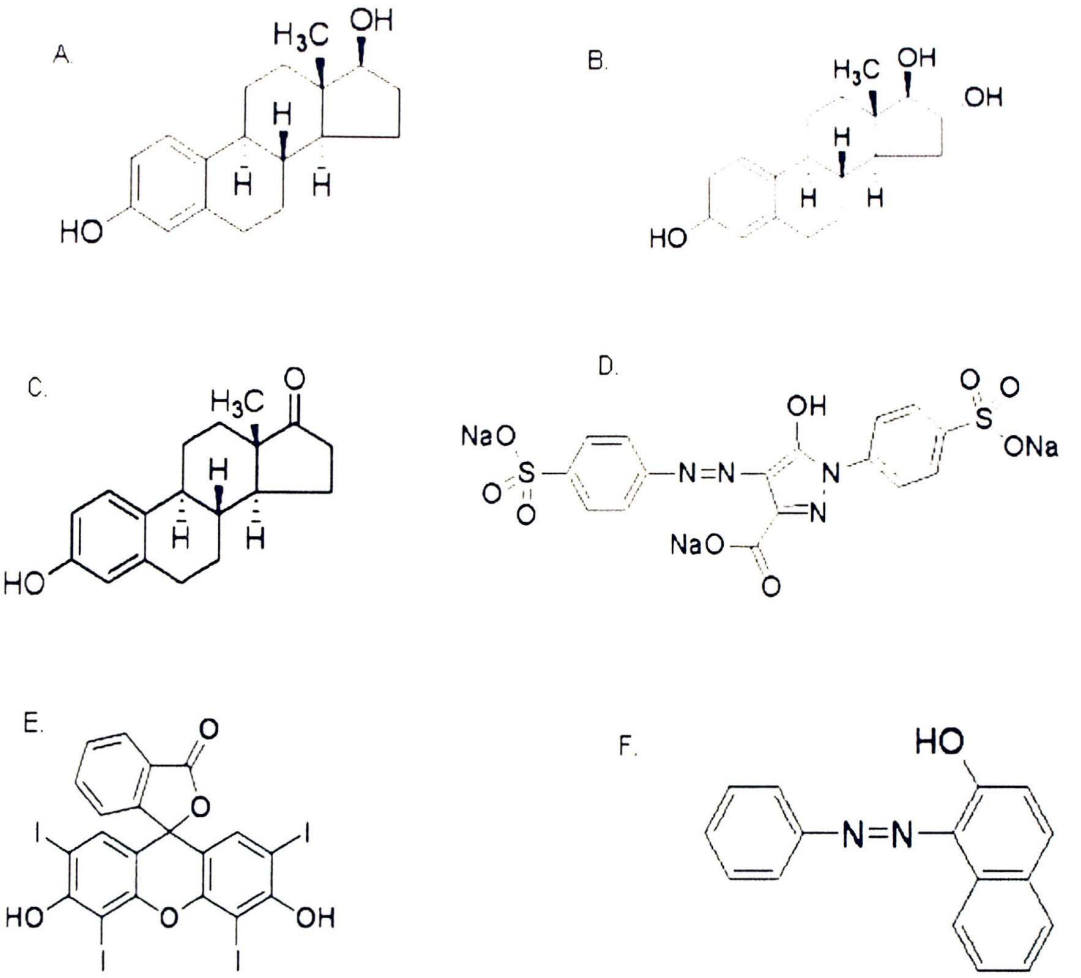

Figure 1. Chemical homology of (A) Estradiol 17β; (B) Estriol; (C) Estrone; (D) Tartrazine; (E) Erythrosin B and (F) Sudan I. The food colorants exhibit a key structural similarity to E2, a phenolic group or benzene attached to a hydrophilic group. This key structure is necessary for estrogen receptor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

Chapter 2. Literature Review

2.1 Estrogen

Estrogens are steroid hormones that are produced daily in milligram quantities in reproductive females (Table 1) and in lesser amount in males. All steroid hormones are derived from cholesterol (Figure 2). Cholesterol esters are carried by low-density lipoproteins (LDL) in the blood stream. Each LDL molecule contains apoB100 which specifically recognizes LDL receptors on the steroidogenic cells (Nelson and Cox, 2004). The binding of LDL to its receptor initiates receptor mediated endocytosis. The endosome eventually fuses with the lysosome and cholesterol esters are hydrolyzed to cholesterol and fatty acids. This phenomenon was first elucidated by Brown and Goldstein (Goldstein *et al.* 1985). Cholesterol is transported into the inner mitochondrial membrane, where cytochrome p450 cleaves the side chain on C-17 carbon atom of cholesterol and oxidizes the adjacent carbons to form pregnenolone (Nelson and Cox, 2004). Pregnenolone is further oxidized to progesterone, which is oxidized to androstenedione. Androstenediene is converted to testesterone or aromatized to form estrone (E1). Testesterone further is aromatized by the action of aromatase monooxygenase to form estradiol (E2). E2 may further be oxidised to form estriol (E3). E1, E2 and E3 are primarily synthesized in the ovaries. Additionally, E2 and E3 are produced in adrenal glands. Aromatase activity has also been detected in muscle (Matsumine *et al.* 1986), fat (Miller *et al.* 1991), nervous tissue (Naftoline *et al.* 1975), brain (Naftolin 1994) and the Leydig cells of the testes (Brodie *et al.* 1993). During pregnancy, E1, E2 and E3 are produced in the placenta (Siiteri *et al.* 1966). The synthesis of estrogen by various steroidogenic tissues depends upon the occurrence and amount of the biosynthetic enzymes present. After synthesis, estrogens are secreted into the blood stream, where they reversibly bind to sex-hormone–binding globulin (SHBG), and transported to target tissues (Sheehan and Young, 1979).

Estrogens are primarily responsible for regulating female reproductive functions, for example, oocyte maturation (Gruber *et al.* 2002). They also regulate the menstrual cycle in conjunction with progesterone and pituitary gonadotropins (Gruber *et al.* 2002). They are also responsible for maturation and function of secondary sex organs (e.g. breast development); estrogens stimulate the growth and differentiation of ductal epithelium (Porter 1974; Sodergyist *et al.* 1993). Estrogens also exert a wide variety of actions on the central nervous system (McEwen et al. 1999). For example, estrogens cause a surge of gonadotropin secretion in women; this results in sexual differentiation in the brain (Naftolin 1994). These steroids also exert important physiological actions on the cardiovascular system by increasing the formation and release of nitric oxide resulting in short term vasodilation (Kim *et al.* 1999). Estrogens affect mineral homeostasis in bone by inhibiting osteoclast differentiation and decreasing bone loss (Christianse *et al.* 1981).

Table 1. Production rates and serum concentrations of estrogens in the menstrual cycle in normal Women*

Phase	Estradiol 17β		Estrone		Estriol	
	Serum Concentration	Daily Production	Serum Concentration	Daily Production	Serum Concentration	Daily Production
	pg/ml	mg	pg/ml	mg	pg/ml	mg
Follicular	40-200	60-150	30-100	50-100	3-11	6-23
Preovulatory	250-500	200-400	50-200	200-350	-	-
Luteal	100-150	150-300	50-115	120-250	6-16	12-30
Premenstrual	40-50	50-70	15-40	30-60	-	-
Post menstrual	<20	5-12	15-80	30-80	3-11	5-22

* Adapted from Gruber *et al.* 2002

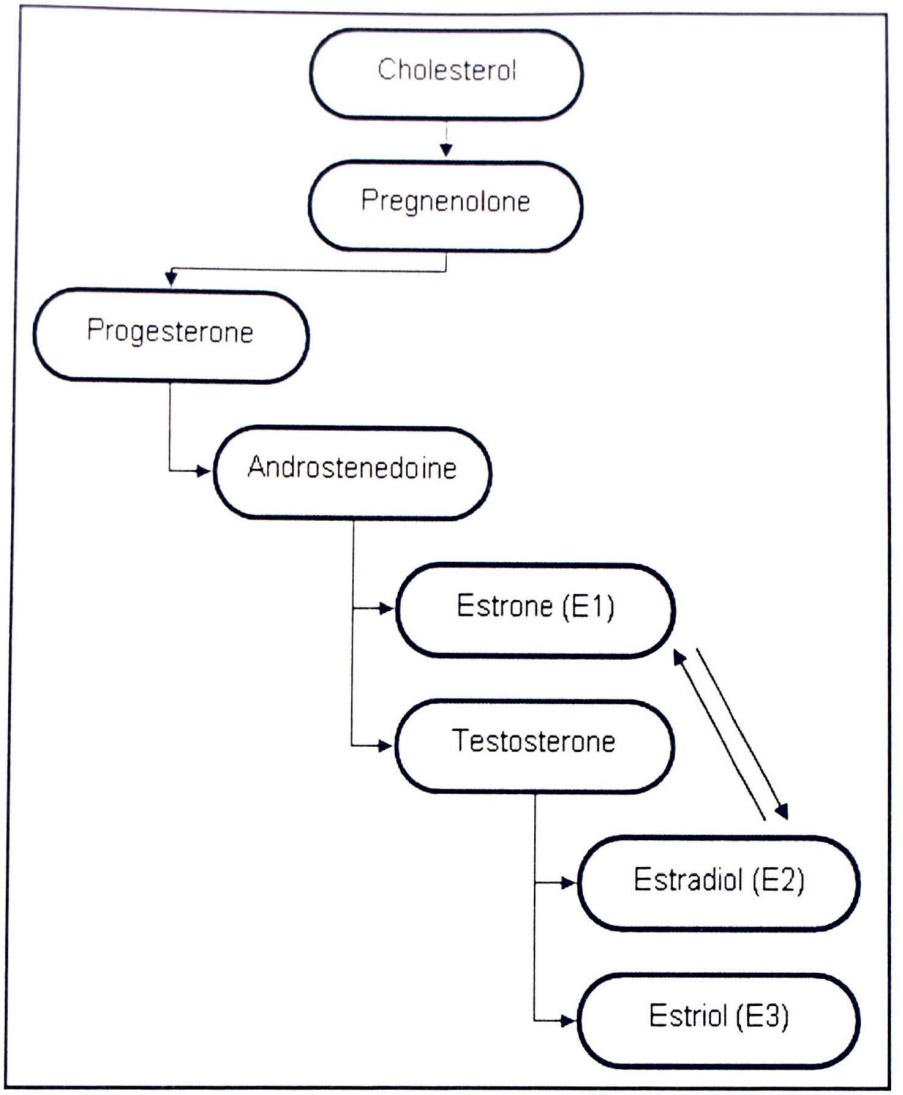

Figure 2. Biosynthetic pathway of Estrogens. The three prevalent forms of human estrogen are *estrone*, *estradiol*, and *estriol*. Because of their respective position in the biosynthetic pathway, estrone is referred as E1, estradiol as E2, and estriol as E3. E1, E2 and E3 are primarily synthesized in the ovaries. Additionally, E2 and E3 are produced in adrenal glands. Aromatase activity has also been detected in muscle (Matsumine *et al.* 1986) fat (Miller *et al.* 1991) nervous tissue (Naftoline *et al.* 1975), brain (Naftolin 1994) and the Leydig cells of the testes (Brodie *et al.* 1993). During pregnancy, E1, E2 and E3 are produced in the placenta (Siiteri *et al.* 1966). The synthesis of estrogen by various steroidogenic tissues depends upon the occurrence and amount of the biosynthetic enzymes present. After synthesis, estrogens are secreted into the blood stream, where they reversibly bind to sex-hormone–binding globulin (SHBG), and transported to target tissues.

2.2 Estrogen receptors

Estrogen exerts its effects mainly through binding and activation of the estrogen receptor (ER). The most widely studied ER is the nuclear ER (Nadal *et al.* 2001). Nuclear ERs are members of the steroid/thyroid hormone superfamily of receptors. These proteins are ligand-activated gene regulatory proteins. There are two isoforms of nuclear ER, ERα and ERβ. As illustrated in Figure 3, the receptor protein includes an A/B domain at the amino terminus that includes an ligand-independent activation function (AF1) which is responsible for binding other regulatory proteins and for target gene activation, a C domain responsible for DNA binding, and the D/E/F domain that includes the ligand binding site as well as an additional domain responsible for associating with coactivators (Nilsson *et al.* 2001). ERα shares significant homology with ERβ in the DNA binding domain (96% amino acid) and ligand binding domain (58% amino acid), but differs greatly in the AF1 domain (Nilsson *et al.* 2001). This difference in the AF1 domain is thought to explain at least in part the different effects the two receptor types have in different tissues. Furthermore, while the ligand binding domains of ERα and ERβ share homologies, there is evidence that the two isoforms have differing affinities for some ligands and subtly different conformational changes in response to ligand binding (Horwitz, 1999; Kuiper *et al.* 1997). Subtle differences in the induced conformation can alter the ability of the ER to recruit coregulators (activators or repressors) of transcription. ERα receptors have higher affinity for E2 than ERβ (Kuiper *et al.* 1997). Genistein, a phytoestrogen, binds ERβ with a higher affinity than ERα and when bound triggers antagonist-type conformational changes in the ERβ (Nilsson *et al.* 2001). The two isoforms also have distinct responses to the antagonist tamoxifen, raloxifene, and ICI-164,384(Horwitz, 1999). These compounds are partial E2 agonist with ERα and pure antagonists with ERβ (Horwitz, 1999).

ERα and ERβ can dimerize to form homodimers or heterodimers (Nilsson *et al.* 2001). The order of DNA binding affinity is: ERα homodimer>ERα-ERβ heterodimer>ERβ homodimers (Nadal *et al.* 2001). As reviewed by Gruber *et al.*

(2002) ERα and ERβ are differentially distributed in various organs. For example, ERα has been shown to be predominant in the endometrial, breast cancer cells and ovarian stroma; ERβ is present in relatively greater quantities in granulosa cells and non-classical target tissues, including bone marrow, bone, brain and prostate gland (Enmark *et al.* 1997). This differential distribution and the varied responses to the same ligands are thought to be partially responsible for the different effects of estrogen analogues on different target tissues (Horwitz, 1999).

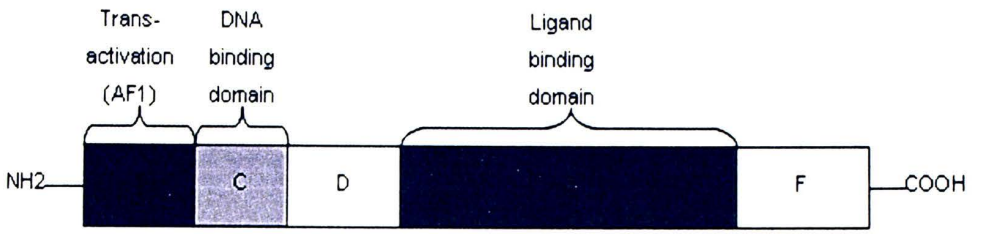

Figure 3. The nuclear estrogen receptor (alpha and beta isoforms). ERα shares significant homology with ERβ in DNA binding domain (96% amino acid) and ligand binding domain (58% amino acid), but differs greatly in the trans-activation region.

Estrogen analogues that have these differential effects are called selective estrogen receptor modulators (SERMs). For example, tamoxifen (an antiestrogen drug) has been shown to inhibit proliferation of breast cancer cells by competitively binding to and inactivating the ER (Jordan *et al.* 2001; Zhang *et al.* 2005); but tamoxifen has been shown to stimulate uterine endometrial cell proliferation by activating the ER (Zhang *et al.* 2005). It has been postulated that the duality of tamoxifen action is due to: 1) different responses of the two receptor isoforms, 2) different tissue distribution of the two isoforms, and 3) the presence of an AP-1 element in the promoter region of some E2 responsive genes. The AP-1 element is a binding site for gene regulatory proteins and can interact with the ER. When the AP-1 element occurs instead of the ERE, tamoxifen acts as an agonist; whereas, tamoxifen acts as an antagonist in the presence of the ERE. (Horwitz, 1999).

As discussed in more detail below E2 has also been shown to bind at the plasma membrane in some cells. These binding sites are responsible for rapid, non-genomic mechanisms of E2 action and are called membrane ERs. Membrane ERs may or may not be structurally similar to the nuclear ER (Falkenstein *et al.* 2000).

2.3 The molecular mechanism of action of estrogens

As reviewed by Gruber *et al.* (2002) and Nadal *et al.* (2001) estrogens act via the classical genomic pathway and alternative non-genomic pathways involving binding of estrogens at the plasma membrane (Figure 4).

Genomic Pathway of estrogen receptors (Classical model of ER action)

The most well described estrogen-mediated cellular action is the genomic pathway (Nadal *et al.* 2001). Upon diffusing in the cell, E2 (or its analogue) binds to the hormone-binding-domain of ER. Binding of E2 to ER causes conformational changes in the receptor and subsequently releases cytoplasmic chaperones, e.g. 90kDa heat shock protein (HSP90) from the ER (Kuiper *et al.* 1997; Smith *et al.* 1993). The activated ER undergoes dimerization and activation (Petterson *et al.* 1997). The cytosolic protein caveolin-1 stimulates the nuclear translocation process by directly interacting with the activated ER-complex (Schlegel *et al.* 1999). The dimer-complex binds to the estrogen-response-element (ERE) in a zinc-finger-DNA-motif (Nelson and Cox, 2000). The ERE is a palindrome-segment of DNA (13 base-pairs) and is situated in the target gene's promoter region (Nelson and Cox, 2000). Along with the nuclear activated receptor, several coactivators (e.g. steroid receptor coactivators) interact with the ER and the ERE, recruiting histone modifying enzymes and maximizing the ligand-dependant-transactivation of the target gene (Nelson and Cox, 2000). Estrogens have also been shown to regulate ERE independent genes by binding to another promoter element known as the API modulating the activity of AP1-transcription factors (Webb *et al.* 1995).

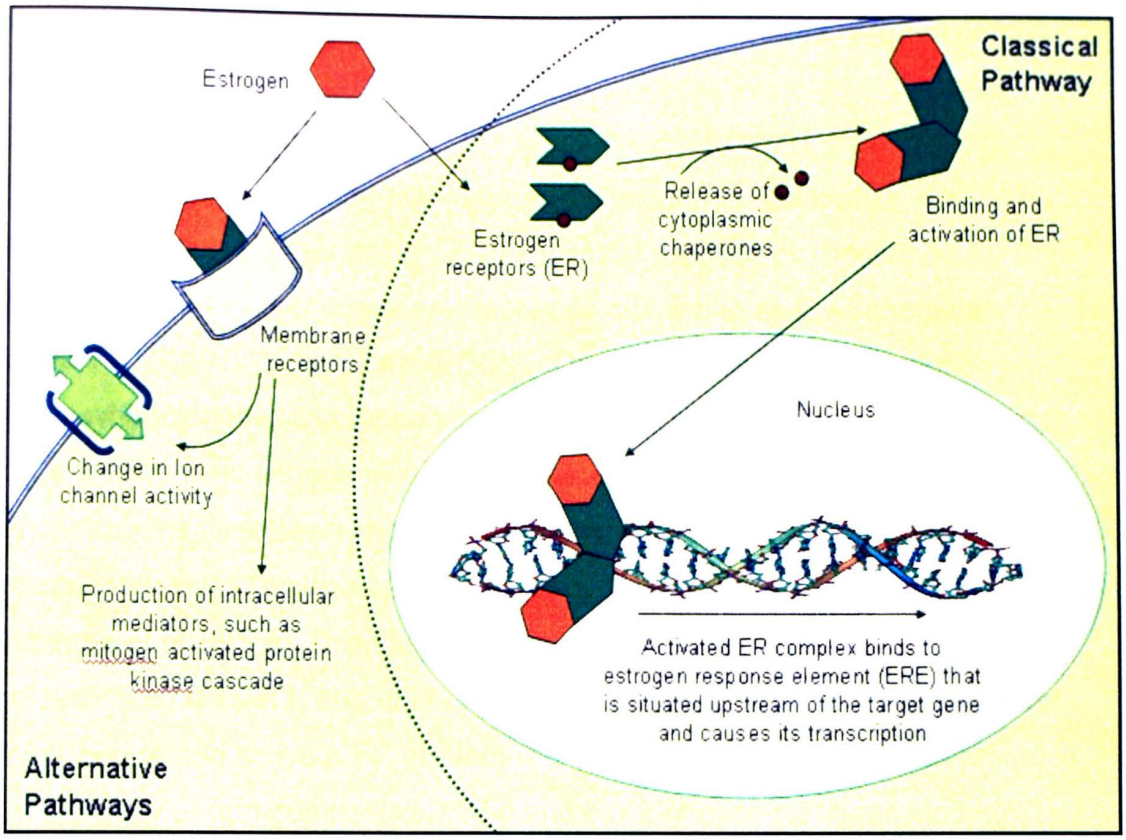

Figure 4. Different mechanisms of action of estrogen. First, classically known nuclear receptors act via binding to the estrogen response element of target genes. Secondly, membrane bound estrogen receptors activate signaling cascades of various kinases or intracellular-estrogen receptors activate signaling cascades in cytoplasm.

Alternative molecular pathways of estrogen action

In the early nineties studies revealed membrane associated estrogen receptors which mediate more rapid and presumably nongenomic effects (Aronica *et al.* 1991;Collins and Webb 1999; Ignar-Trowbridge *et al.* 1993; Kim *et al.* 1999; Newton *et al.* 1994; Smith *et al.* 1993; Watson *et al.* 1999; Weigel *et al.* 1996). The membrane associated ERs are located in cell membrane invaginations called caveolae (Gruber *et al.* 2002). When activated, these ERs can change ion channel activity and thus cell excitability (Kim *et al.* 1999; Nadal *et al.* 1998) or stimulate production of intracellular mediators, such as, mitogen-

activated protein kinase (MAPK) cascades (Kato *et al.* 1995). Some of these intracellular mediators may go on to effect transcriptional rates of target genes.

2.4 Xenoestrogens

There are natural and anthropogenic compounds which affect the endocrine system by interacting with the signally cascade of endocrine hormones. These compounds are frequently referred to as Environmental Endocrine Disrupting chemicals (EDCs). One type of endocrine disruption occurs when xenobiotic compounds mimic steroid hormone action. Xenobiotic EDCs which mimic endogenous estrogen are called xenoestrogens. Most xenoestrogens have been shown to be chemically similar to estrogen at a molecular level and possess phenolic and hydroxyl moieties (Fang *et al.* 2001; Nishihara *et al.* 2000). This chemical structure is the ligand for specific binding and activating of ERs (Fang *et al.* 2001; Nishihara *et al.* 2000). Xenoestrogens which bind to and activate ER are referred to as agonists. Xenoestrogens which competitively or non-competitively bind and block or alter the ligand binding domain of ER are referred as antagonists. Xenoestrogens in females have been linked to reproductive defects in their offspring (Gill *et al.* 1979). They have also been shown to increase breast cancer pathogenesis (Wolff and Toniolo 1995), abnormalities in menopause and menstrual cycle (http://www.who.int/ipcs/publications /new_issues/endocrine_disruptors/en/ index.html). Xenoestrogens have also been linked to increased incidence of cryptorchidism and hypospadias in men (Gill *et al.* 1979; Giwercman *et al.* 1993; Jackson 1988). These compounds have also been shown to increase incidence of testicular hypoplasia (Gill *et. al.* 1979), decreases in sperm count in men (Giwercman *et al.* 1992) and increased incidence of prostate cancer and breast cancer (Wolff and Toniolo 1995). Xenoestrogens have 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). Examples of ubiquitous xenoestrogens are 4-methylbenzylidine camphor (from

sunscreen lotion), erythrosin B (FD& C Red 3), bisphenol-A (plasticizer), methoxychlor (insecticide) and DDE (insecticide-metabolites). These compounds have been shown to be xenoestrogenic (Durando *et al.* 2007; Fry and Toone, 1981; Murray *et al.* 2006; Rogan *et al.* 1987; Timwell *et al.* 2002).

Recently, the Environmental Protection Agency (EPA) has developed guidelines for the systematic screening of potential xenoestrogens (http://www.epa.gov/oscpmont/sap/meetings/1998/may/edstac/appenk.pdf). The screening for xenoestrogenicity has been grouped into Tier I and Tier II assays. Tier I screening assays are performed to test a chemical's potential to interact with endocrine system. Tier II screening assays are performed to determine if the compound interacts with whole physiological systems and pose a risk for altering life cycles. These studies focus on tier I screening assays.

Our research focused on whether our test food colorants directly interacted with the estrogen receptors *in vitro*. Two types of Tier I screening assays were performed, the cell proliferation assay (E screen) and the reporter gene assay (estrogen-receptor mediated chemically activated luciferase expression reporter gene assay, ER-CALUX). The principle of the assays is shown in Figure 5. The cell proliferation assay (E-screen) measures 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). The number of viable cells can be measured by counting the total nuclei in a coulter counter apparatus (Soto *et al.* 1995), total protein count (Matsuoka *et al.* 2004; Zava *et al.* 1997) or total DNA (Chang *et al.* 2001; Zava *et al.* 1997). Data from other researchers (Soto *et al.* 1995) suggest that the proliferative effect of estrogen can be detected after 24 to 48 hours of hormone treatment. Additional cell proliferation experiments were performed to study the effects of the food colorants in the presence of tamoxifen. Tamoxifen, as described earlier, is an antagonist in estrogen responsive breast cancer cells; if a compound was stimulating cell proliferation via ER binding and activation, then

such binding will be competitively inhibited by tamoxifen. ER-CALUX assay is based on the principle of estrogens (and/or xenoestrogens) binding to and activating endogenous ER, present in the T47D cells (http://www.epa.gov/ oscpmont/sap/ meetings/1998/may/edstac/appenk.pdf). The activated ER binds ERE 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.

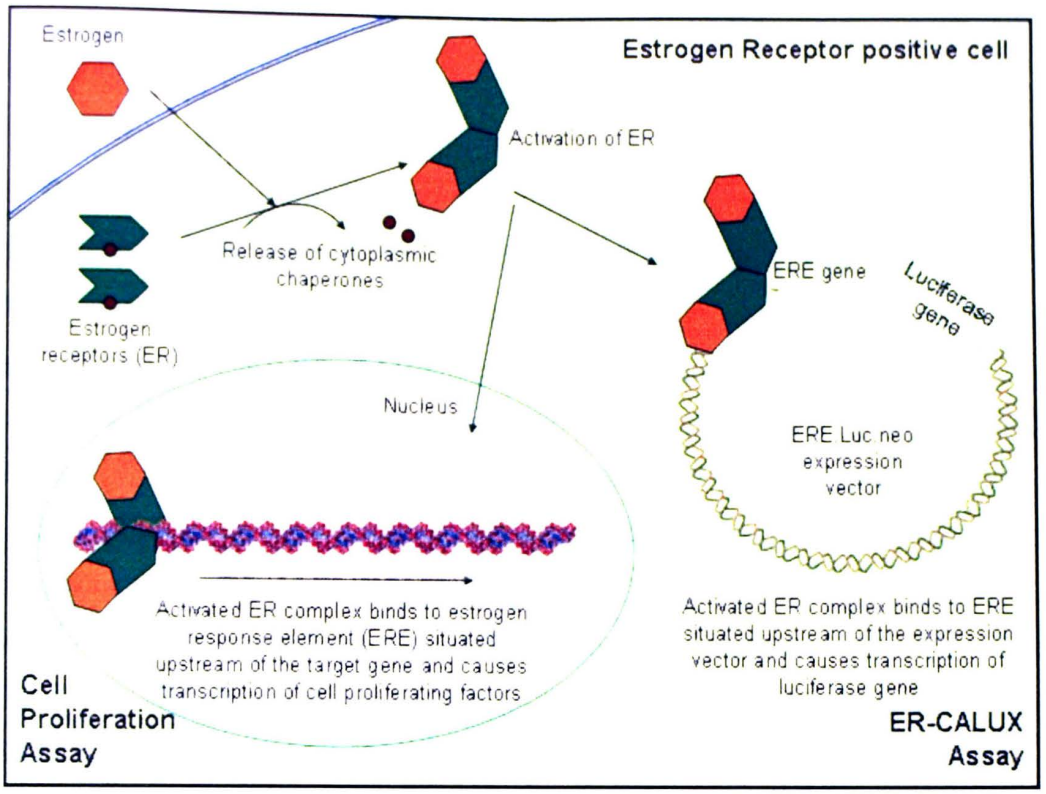

Figure 5. Principle of cell proliferation assay and estrogen-receptor mediated chemically activated luciferase expression reporter gene bioassay. Cell proliferation assay measures the proliferative effect of estrogen or xenoestrogens, on estrogen responsive cells in a hormone-stripped medium. The total number of viable cells after the assay is directly proportional to the effect of chemicals on the estrogen responsive cells. ER-CALUX assay is based on the principle of estrogens (and/or xenoestrogens) binding to and activating endogenous ER. The activated ER binds ERE 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.

Chapter 3. Materials and Methods

3.1 Chemicals

RPMI 1640 containing glutamine, antibiotic/antimycotic solution (15240-096), Dulbecco's phosphate buffered saline (DPBS), and geneticin were all purchased from Invitrogen/Gibco, Gaithersburg, MD. Fetal bovine serum (FBS) and trypsin were purchased from Atlanta Biologicals, Lawrenceville, GA. Bradford reagent was obtained from Bio-Rad Laboratories, Hercules, CA. 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, MO. 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% solvent concentration.

The *puc9.neo* plasmid vector was gifted by Dr. Phillip Hartig, U.S. EPA, Research Triangle Park, NC. The Panomics Translucent Reporter Vector (*LR0020*) was purchased from Promega, Madison, WI. Restriction enzymes, *HindIII* (10656313001, 10 U/μl) and *BamHI* (10220612001, 10 U/μl), and were obtained from Roche Diagnostics, Indianapolis, IN. FuGENE 6 Transfection Reagent (11815091001) was purchased from Roche. Luciferase Assay System (E1500) was purchased from Promega.

3.2 Culture of T47D Cells

Estrogen-receptor positive T47D breast cancer cells (ATCC, HTB 133) were obtained from the American Type Culture Collection, Manassas, VA. T47D cells were cultured in RPMI 1640 supplemented with 10% v/v heat-inactivated-FBS, 0.2 U/ml porcine insulin and 1 ml/ 50 ml medium antibiotic/antimycotic solution (growth medium). 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 re-suspended in fresh medium. Trypsinization medium consisted of 0.25% trypsin plus .53 mM EDTA in DPBS, pH 7.2.

3.3 Charcoal dextran stripped fetal calf serum

FBS (100 ml) and 275 mg charcoal-dextran were mixed and stirred for 24 h at 4° C. The mixture was then centrifuged at 3300X g for 30 minutes, at 4° C. The serum was decanted and fresh charcoal-dextran was added to the serum. The mixture was stirred for an additional 60 minutes and centrifuged 2-3 times at 3300X g. The serum was sterilized by filtration (pore size 0.2μm). On the day of every independent experiment, 50 ml of charcoal stripped FBS was heat inactivated by incubating at 56° C for 30 minutes.

3.4 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 $4.0x10^4$ cells per well in phenol red free RPMI 1640 supplemented with 5% v/v heat inactivated-FBS, 0.2 U/ml porcine insulin and 1 ml/ 50 ml antibiotic/antimycotic solution. After 24 h, the medium was changed to phenol red free RPMI 1640 supplemented with 5% v/v charcoal-stripped and heat inactivated –FBS and 1 ml/ 50 ml antibiotic/antimycotic solution and either E2, tartrazine, erythrosin B, or sudan I (0, 0.001, 0.01, 0.1, 1, and 10 nM), in presence or absence of tamoxifen (1 μM). For all assays, E2 at concentrations similar to its physiological concentrations (0.001 to 10 nM) was used as the positive control. For antagonist assays, the antagonist (tamoxifen) was added 1 hr before treatment application (Makela *et al.* 1994). Growth medium devoid of any treatment was used as the negative control for all assays. Each treatment was performed in quadruplicate and each compound was tested at the five concentrations given above. The

medium was changed every 48 h. Following 96 hours of treatment, the experiment was terminated. Cells were washed twice with phosphate buffered saline. Cells were solubilized in 0.1 N NaOH and 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.

3.5 Reporter gene construct

Restriction maps of *puc9.neo* vector and Panomics Translucent Reporter Vector (*LR0020*) vector were examined to determine the compatibility of restriction sites present in both the vectors. Restriction endonucleases were carefully chosen to ensure (1) digestion of the plasmids at their multiple cloning sites, and (2) absence of the restriction sites on neomycin (*Neo*) gene cassette (in *puc9.neo* vector) or Luciferase (*Luc*) and *ERE* gene cassettes (in *LR0020* vector). The plasmid, *puc9.neo* was amplified and the neomycin gene cassette (1.8 kb) was removed with *BamHI* restriction digestion. Simultaneously, the *LR0020* vector was linearized with *BamHI*. The vectors were incubated at 80° C for 10 minutes 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 (10X buffer) in a 20 µl reaction mixture at 16°C overnight. The ligated DNA was stored at -20°C and subjected to purification with Genopure Plasmid Midi Kit (Roche, 3143414). The resulting plasmid was *ERE.Luc.neo* expression vector.

3.6 Estrogen receptor-mediated chemically Activated Luciferase reporter gene expression (ER-CALUX) assay

Transient transfection was performed as described by Fugene 6™ manufacturer's protocol. Briefly, T47D cells were plated in a 24 well plate at a

density of 5.0×10^5 cells, in phenol red free - growth medium 24 h prior to transfection. On the day of transfection, the cells were washed twice with sterile phosphate buffered saline and once with serum-free and antibiotic-free growth medium. Medium was replaced with fresh serum-free and antibiotic-free growth medium. The cells were transfected with 5 µg *ERE.Luc.neo* plasmid vector at a ratio of 3:1 (Fugene6: DNA). After 6 h, the transfection-medium was replaced with phenol red free RPMI medium 1640 supplemented with 5% v/v heat inactivated – charcoal stripped FBS and either negative control (0 nM), E2 (0.1 nM), tartrazine (0.1 nM), erythrosin B (0.01 nM) or sudan 1 (1 nM). Antibiotic/Antimycotic was not added to the treatments as the mixture creates background levels of luciferase activity (Wilson *et al.* 2004). A negative control for each luciferase assay in every independent experiment was non-treated transfected T47D cells. An additional negative control to assess efficacy of transient transfection was non-treated non-transfected T47D cells. After 48 h of incubation, the cells were washed twice with phosphate buffered saline and lysed with 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 read within 1min after the addition of substrate and six wells were read at a time. Each assay consisted of a minimum of two replicates per treatment. Each independent experiment was repeated a minimum of three times.

3.7 Fugene-mediated-stable-Transfection of T47D cells with *ERE.Luc.Neo* plasmid Vector

Stable transfection was performed as previously described in section 3.6. After 6 h, transfection-medium was replaced with normal growth medium. After 24 h, the growth medium was replaced with growth medium supplemented with 250 µl/50 ml medium selective antibiotic geneticin. The selection medium was replaced every 48 h until sufficient cells were observed in the culture plate. The

surviving clone was trypsinized and transferred to a 75mm flask. The cells were sub-cultured until 100% confluence was reached.

3.8 Statistical analysis

Cell proliferation is reported as proliferative effect (PE) over negative control. Proliferative effect was calculated as the ratio between total-protein quantified from cell proliferation assay and hormone-free negative control. Data (mean ± S.E.M) were analyzed by two–way Analysis of variance (ANOVA), where time (independent experiment) was the blocking factor and concentration was the treatment factor; $p \leq 0.05$ was taken as the statistically significant level. A significant ANOVA was followed by post-hoc Tukey Kramer HSD (significance level of $p \leq 0.05$). Additionally, R^2 adjusted, which calculates R^2 with reference to degrees of freedom of the statistical analysis was calculated. Percentage of E2 response for the food colorant was calculated by dividing the proliferative effect of food colorant by maximal proliferative effect of E2 (Table 1). Relative proliferative effect (RPE) was measured as ratio between maximal PE achieved by test compound relative to E2 (0.1 nM) multiplied by 100 (Soto *et al.* 1995).

Luciferase activity (Relative Light Units, RLU) of each treatment was converted into fold induction over the negative control and was calculated by dividing the RLU of the test compound by the RLU of the negative control. Percentage of E2 response of each food colorants was calculated by dividing the RLU of food colorant by RLU of 0.1nM E2. Additionally, RLU was normalized as log of percentage of E2 (positive control) for each replicate for statistical analysis. Luciferase activity of treatments was compared with the negative control or positive control (0.1 nM E2-treated transfected cells), in a two-way ANOVA, where time (independent experiment) was the blocking factor and concentration was the treatment factor; $p \leq 0.05$ was taken as significant level. Results from a significant ANOVA were analyzed with Dunnett's test (significance level, $p \leq 0.05$).

All statistical analyses were performed in JMP® 6 Statistical Software (SAS Institute Inc.). Graphs and tables were prepared using Microsoft Excel.

4.1 Proliferative effect of Tartrazine, Erythrosin B and Sudan I in T47D cells

Cell proliferation assay measures the proliferative effect of estrogen or xenoestrogens, on estrogen responsive cells in a hormone-stripped medium (Soto *et al.* 1995). The proliferative effect (PE) of different concentrations of E2 (positive control) and synthetic food colorants (tests) are demonstrated in Figure 6 and Figure 7. Data are represented as mean± sem of n independent experiments. E2 (n=9) induced highly significant proliferation of T47D cells over the negative conrol ($p<0.0001$, R^2 adj=0.86). The three food colorants tartrazine (n=3 for 0.001 and 0.01nM; n=4 for 0.1, 1 and 10 nM; $p=0.0004$; R^2 adjusted=0.76), erythrosin B (n=3; $p<0.0001$; R^2 adj=0.88) and sudan I (n = 3; $p=0.0005$; R^2 adj =0.81) induced significant proliferation of T47D cells over negative control.

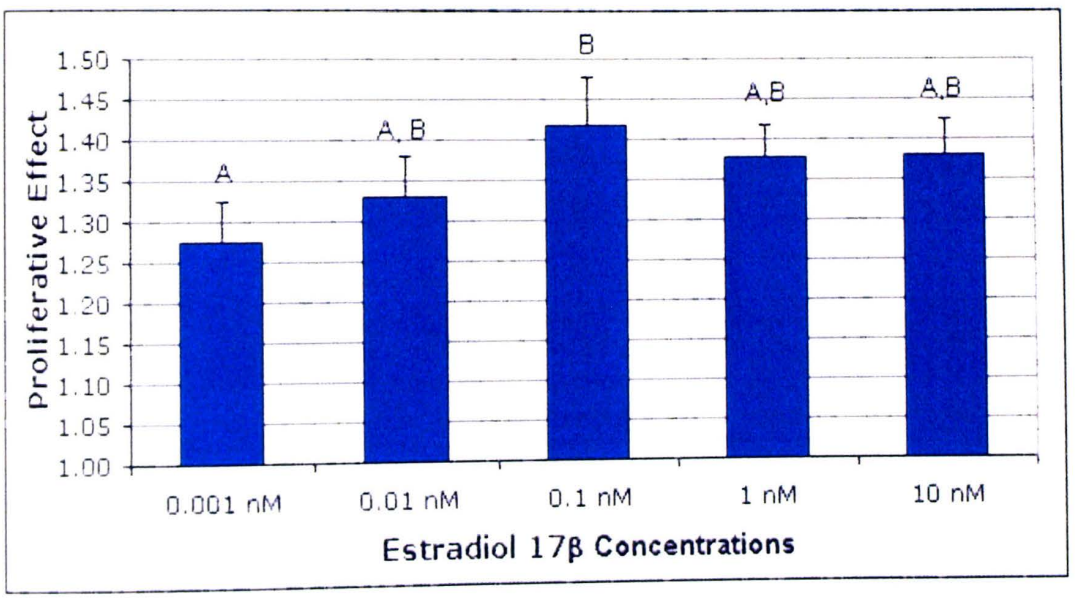

Figure 6. Proliferative effect of E2 in T47D cells treated for 96 hr (mean±sem of 9 independent experiments and four replicates per experiment). E2 significantly stimulated the proliferation of T47D cells at all concentrations over the negative control ($p<0.0001$). Bars with different letters are significantly different from one another. Proliferative effect (PE) = (total protein of treatment)/(total protein of negative control).

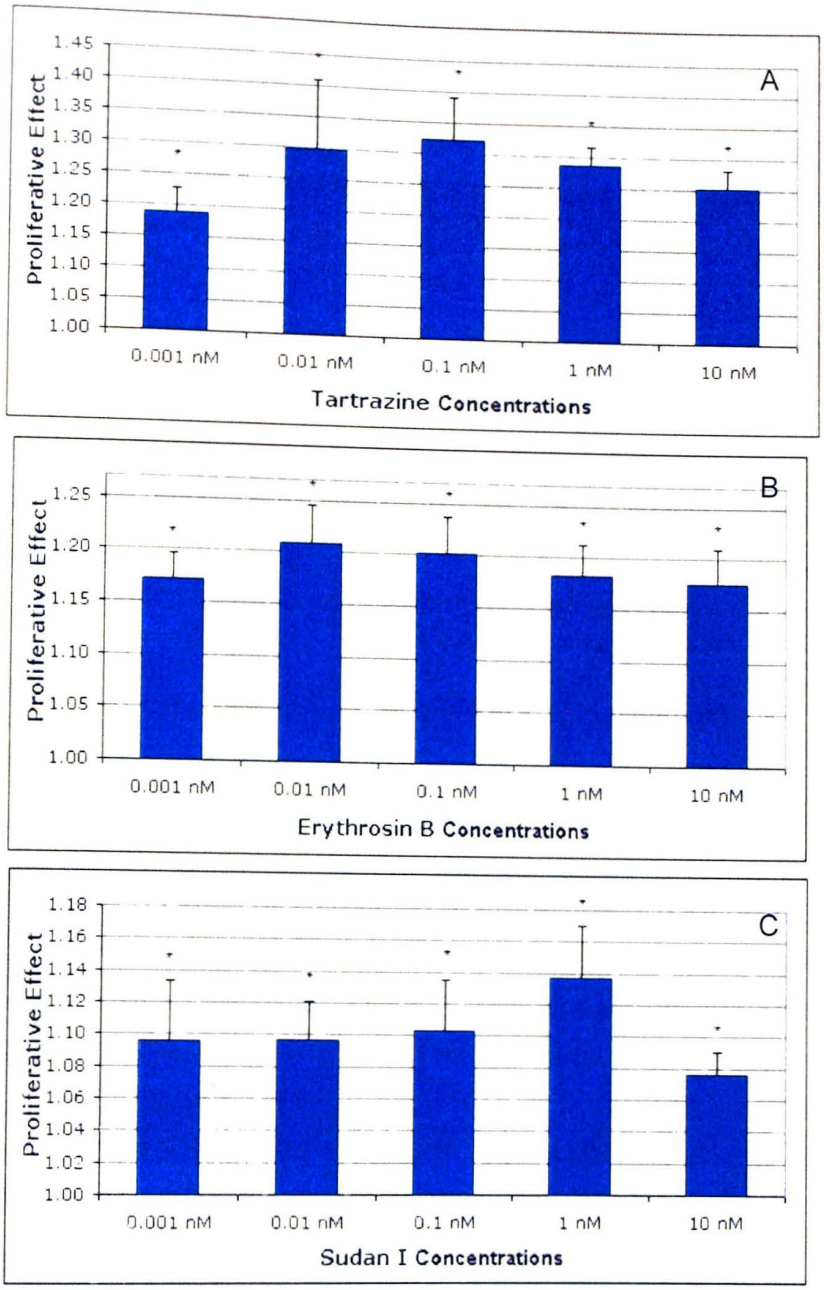

Figure 7. Proliferative effect of (A) Tartrazine, (B) Erythrosin B and (C) Sudan I in T47D cells treated for 96 hr (mean±sem of n independent experiments and four replicates per experiment). (A) Tartrazine (n=3 for 0.001 and 0.01nM; n=4 for 0.1, 1 and 10nM); (B) Erythrosin B (n=3) and (C) Sudan I (n=3). Tartrazine ($p=0.0004$), erythrosin B ($p<0.0001$) and sudan I ($p=0.0005$) significantly stimulated the proliferation of T47D cells at all concentration tested. The proliferative effect elicited by the various concentrations of each test food colorant was similar. Proliferative effect (PE) = (total protein of treatment)/(total protein of negative control).

The maximal PE achieved by investigated colorants with their respective concentration and their relative proliferative effect (RPE) is tabulated in Table 2. The RPE of positive control (E2) was calculated as 100. In our studies, the highest RPE was elicited by tartrazine (78.41) followed by erythrosin B (49.25) and sudan I (38.98).

Table 2. Relative proliferative effect (RPE) and Percentage of E2 response of Tartrazine, Sudan I and Erythrosin B in cell proliferation assay

Test Compounds	Maximal PE [1] achieved	Concentration [2]	RPE [3]	Percentage of E2 [4]
E2	1.42	0.10 nM	100.00	100.00
Tartrazine	1.32	0.10 nM	78.41	93.23
Erythrosin B	1.21	0.01 nM	49.25	85.27
Sudan I	1.14	1.00 nM	38.98	80.20

1. Proliferative effect (PE) = (total protein of treatment)/(total protein of negative control).
2. Concentration corresponding to consistently maximal average PE achieved.
3. Relative proliferative effect (RPE) = {(PE-1) of the food colorant/(PE-1) of E2}X100.
4. Percentage of E2 (% of E2) = (PE of the food colorant)X100/PE of E2.

4.2 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 presence of E2 or its analogues and inhibits cell proliferation (Jordan *et al.* 2001). The difference in proliferative effect of E2 and food colorants in presence and absence of tamoxifen (1 μM) is shown in Figure 8. The assay was performed with concentrations, corresponding to the maximal PE achieved by E2 (0.1 nM), tartrazine (0.1 nM), erythrosin B (0.01 nM) and sudan I (1 nM). Statistical analysis proved that tamoxifen (1 μM) significantly blocks the cell proliferation of E2, tartrazine ($p=0.0013$, R^2 adj=0.77), erythrosin B ($p=0.0003$, R^2 adj=0.82) and sudan I ($p<0.0001$, R^2 adj=0.95).

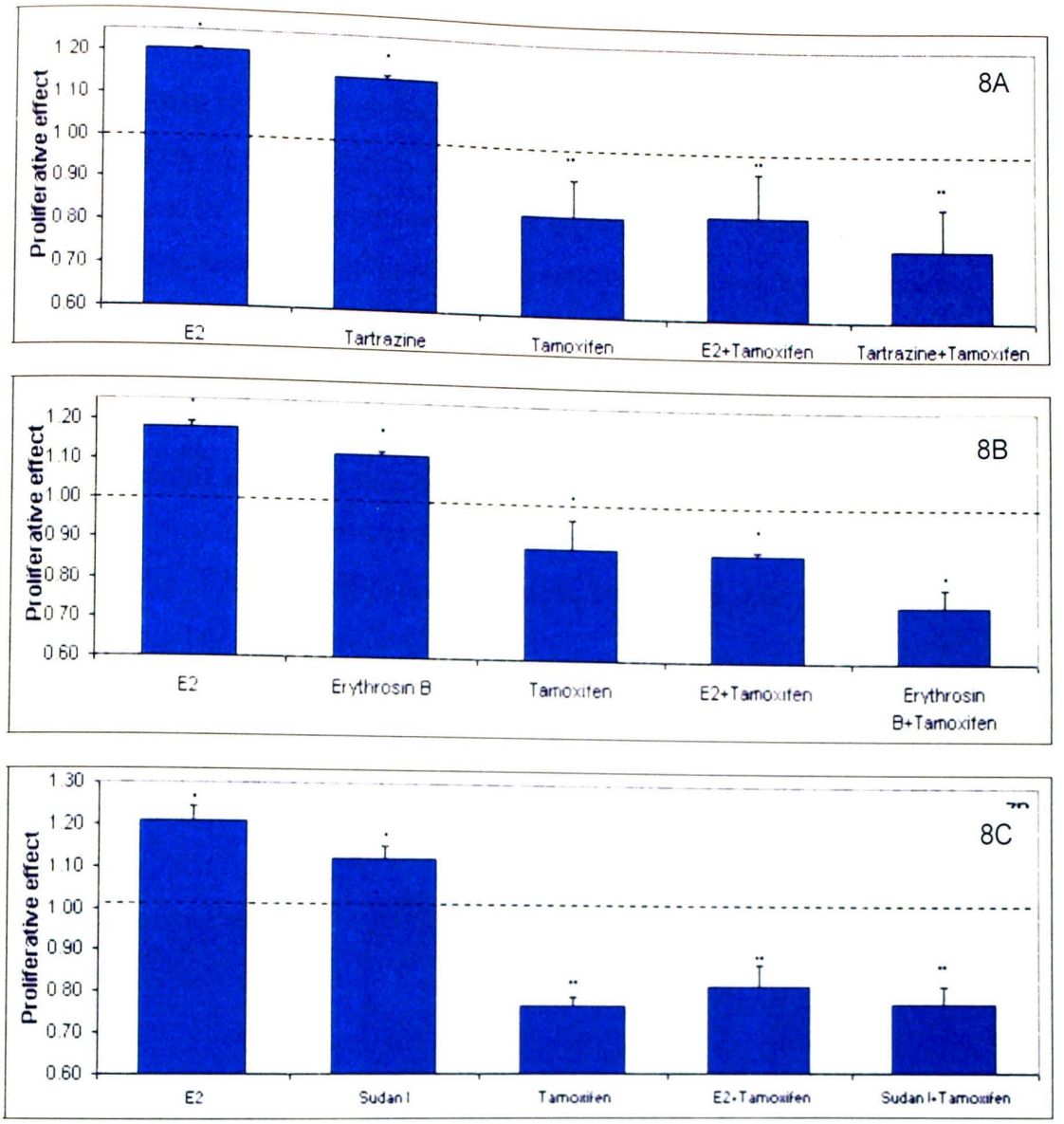

Figure 8. Difference in proliferative effect of 0.1 nM E2 and (8a) Tartrazine (0.1 nM), (8b) Erythrosin B (0.01 nM) and (8c) Sudan I (1 nM), in presence of antagonist tamoxifen (1 μM) in T47D cells treated for 96 hr (n=3, data = mean±sem of three independent experiments and four replicates per experiment). Two way Anova was significant for tartrazine ($p=0.0013$), erythrosin B ($p=0.0003$) and sudan I ($p<0.0001$). Asterisk (*) denotes significant difference from the negative control ($p=0.05$). Double asterisks (**) denote significant difference between tamoxifen, E2+tamoxifen and food colorant+tamoxifen from E2 and food colorants. The dashed line represents negative control (no treatment). Proliferative effect (PE) = (total protein of treatment)/(total protein of negative control).

4.3 Reporter gene construct

The aim of the experiment was to modify pER-reporter vector having *neo*-gene cassette for the selection of stably transfected T47D cells. The restriction digestion and gel-electrophoresis of pER reporter vector, *puc9.neo* and *ERE.Luc.Neo* is shown in Figure 9. The pER reporter vector was double-digested with *HindIII* and *BamHI*, producing bands of 3 kb and 2 kb. The restriction digestion of *puc9.neo* with *BamHI* yielded two bands that were approximately 3 kb and 1.8 kb in size. The restriction digestion of *ERE.Luc.neo* gave two bands approximately 6 kb and 7 kb due to partial digestion of the plasmid. The expected size of *ERE.Luc.neo* was around 6.8 KB (Figure 10). The intensity of the band (9c) was less compared to (9a) and (9b), due to the lesser amount of DNA template.

A. Panomics pER vector				B. *puc9.neo* vector					C. *ERE.Luc.neo* vector				
1	2	3	4	1	2	3	4	5	1	2	3	4	5

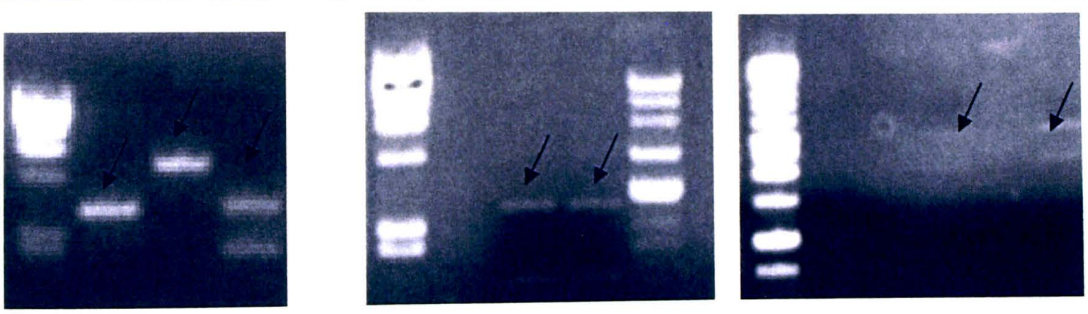

Figure 9. Restriction digestion and gel-electrophoresis. (A)Panomics pER Translucent Reporter Vector, (B) *puc9.neo* and (C) *ERE.Luc.neo* digests are shown. (A) Lane 1: standard Lambda phage ladder; Lane 2: *BamHI* digested plasmid (3 kb); Lane 3: undigested plasmid (3 kb); Lane 4: *HindIII* and *BamHI* digested plasmid (3 kb and 2 kb) (B) Lane 1 and 5: Lambda phage ladder; Lane 3 and 4: HindIII digested plasmid (3 kb and 1.8 kb). (C) *ERE.Luc.neo* plasmid. Lane 1: 1kb Ladder; Lane 3 and 5: HindIII digested plasmid (6.6 kb).

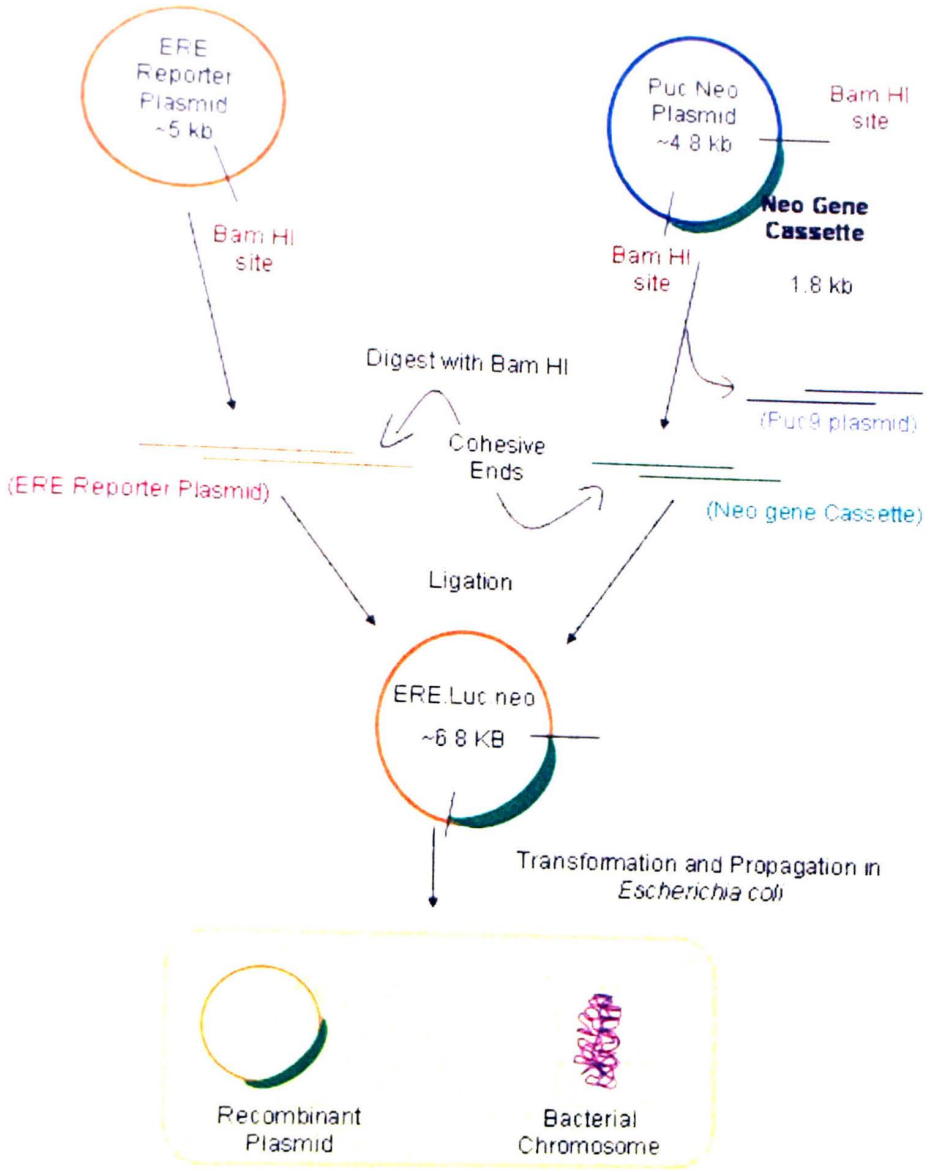

Figure 10. Schematic representation of the *ERE.Luc.neo* recombinant plasmid construct. The Panomics pER Translucent Reporter Vector (ERE Reporter plasmid) has *ERE*, and *Luc* gene cassettes. The plasmid *puc9.neo* has a *neo* gene cassette (1.8 kb) which ensures resistance against neomycin (in *E. coli*) and geneticin (in eukaryotic cell lines) (Wilson *et al.* 2004). Neomycin gene cassette was inserted in the multiple cloning site (MCS) of the Panomics pER Translucent reporter vector.

4.4 Activation of estrogen-receptor mediated luciferase reporter gene expression by Tartrazine, Erythrosin B and Sudan I in T47D cells, transiently transfected with *ERE.Luc.neo*

ER-CALUX assay evaluates whether xenoestrogens are act via the classical nuclear hormone pathway (Wilson *et al.* 2004). The luciferase induction of food colorants, relative to the E2 response in the ER-CALUX assay with T47D cells that were transiently transfected with *ERE.Luc.neo* and treated for 48 hr is demonstrated in Figure 11. Luciferase assays were performed after *ERE.Luc.neo* transfected T47D cells were treated with E2 (0.1 nM), tartrazine (0.1 nM), erythrosin B (0.01 nM) and sudan I (1 nM) for 48 h. Three independent experiments with two or more replicates per experiment performed on each colorant. The data were normalized to log of percentage of E2 for statistical analysis. All three food colorants stimulated significant luciferase induction over negative control ($p=0.0236$, R^2 adjusted=0.61). Luciferase induction of tartrazine, erythrosin B and sudan I were significantly different over the negative control ($p=0.05$).

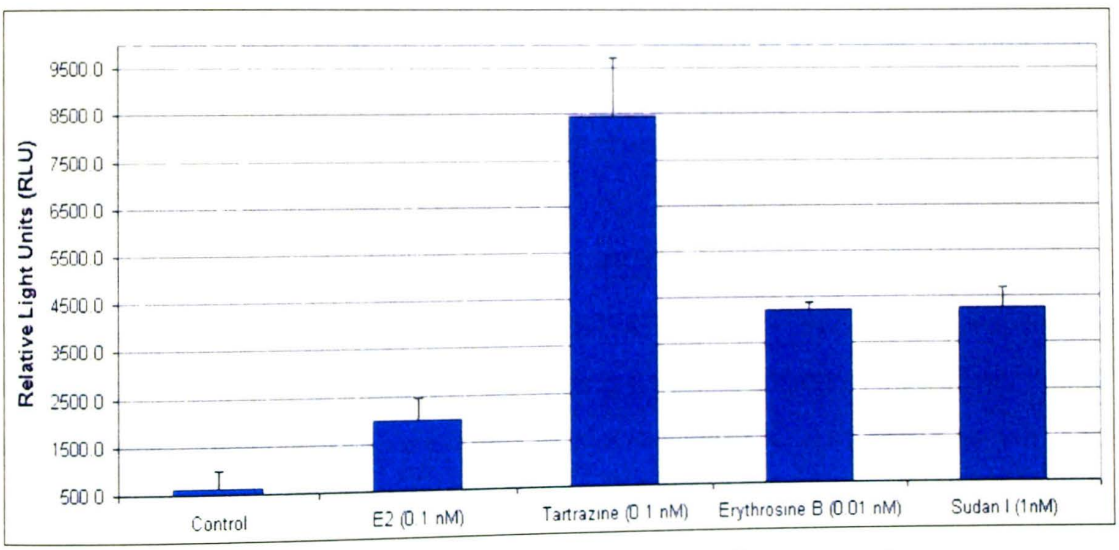

Figure 11. Activation of estrogen-receptor mediated luciferase reporter gene expression by tartrazine, erythrosin B and sudan I in T47D cells, transiently transfected with *ERE.Luc.neo*. The data shown are representative of an independent experiment having three replicates.

The percent estrogenicity achieved by investigated colorants with their respective concentration is tabulated in Table 3. The percent estrogenicity of positive control (E2) was calculated as 100. In our studies, the highest percent of E2 effect was elicited by tartrazine (235.53±95.67) followed by erythrosin B (128.24±44.27) and sudan I (128.97±43.74). Data are represented as mean±sem.

Table 3. Percentage of E2 response of Tartrazine, Sudan I and Erythrosin B in ER-CALUX assay

Test Compounds	Concentration	RPE	Percentage of E2[1]
E2	0.10 nM	100.00	100.00
Tartrazine	0.10 nM	78.41	235.53
Erythrosin B	0.01 nM	49.25	128.24
Sudan I	1.00 nM	38.98	128.97

1. Percentage of E2 (% of E2) = (PE of the food colorant)X100/PE of E2.

4.5 Fugene-mediated-stable-transfection of T47D cells with *ERE.Luc.Neo* expression vector

Stable transfection of T47D cells with *ERE.Luc.neo* expression vector was developed to establish a stably transfected T47D cell-line that will be used for standardized-reporter gene assay for testing potential estrogen mimicking compounds. T47D cells were stably transfected with *ERE.Luc.neo* plasmid vector, as shown in Figure 12. After three weeks only a single colony survived and that colony was allowed to grow to 80% confluence. The cells were trypsinized and plated in 75 mm flask till 100% confluency was reached. The culture was trypsinized and split and one portion was frozen in liquid nitrogen and rest was sub-cultured. The stably transfected cells will be used for ER-CALUX assay for testing potential estrogen mimicking compounds.

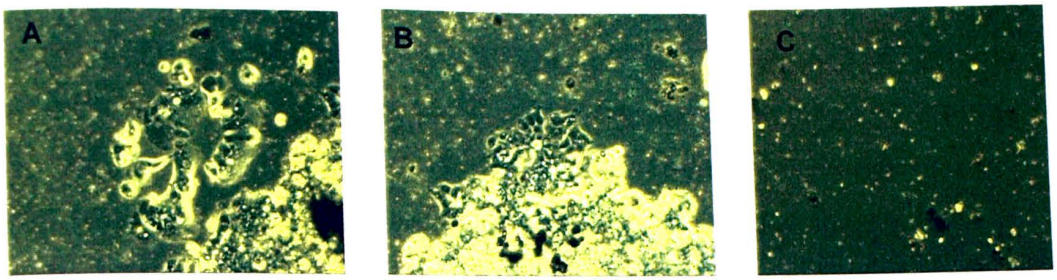

Figure 12. Stable Transfection of T47D cells with *ERE.Luc.neo*. (a) and (b) represent successful transfected T47D cells after 2 weeks of geneticin treatment. (c) Negative control for transfection: non transfected cells, treated with geneticin for 2 weeks.

The goal of our research project was to evaluate estrogenicity of synthetic food colorants chemically similar to E2. We evaluated tartrazine, erythrosin B, and sudan I with cell proliferation and ER-CALUX assay. The 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 (Figure 6) was consistent with Wilson *et al.* (2004). As demonstrated in Figure 6, 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. As demonstrated in the Table 2, tartrazine induced the greatest RPE, followed by erythrosin B and sudan I at concentrations of 0.1, .01, and 1 nM, respectively. A number of well-known estrogen mimicking compounds: *p,p'*–DDE (Fang *et al.* 2000; Soto *et al.* 1995), endosulfan (Fang *et al.* 2000; Soto *et al.* 1995), methoxychlor (Fang *et al.* 2000; Soto *et al.* 1995), *p,p'* –DDT (Fang *et al.* 2000; Soto *et al.* 1995), 3-(4-methylbenzylidene) camphor (Schlumpf *et al.* 2001), bisphenol A (Fang *et al.* 2000; Soto *et al.* 1995) and genistein (Fang *et al.* 2000; Soto *et al.* 1995) have been shown to have similar RPE as our food colorants in a MCF7 cell proliferation assay. Interestingly, exposure to DDT (o,p'-DDT), DDE (hormonally active metabolite of DDT) and methoxychlor has been linked to feminization of male California gull embryos (Fry and Toone, 1981). High levels of DDE have also been linked to lactation failure in women (Rogan *et al.* 1987). Recently, The International Programme on Chemical Safety (IPCS) provided a global assessment of EDCs such as DDT, DDE and polychlorinated biphenyls, and have associated these chemicals with decreased reproductive function of marine animals in the Baltic and the Dutch Wadden Seas, and egg shell thinning

in colonial water birds (http://www.who.int/ipcs /publications/new_issues/endocrine_disruptors/en/index.html). Studies conducted by Timwell *et al.* (2002) have shown the weak uterotrophic activity of 3-(4-methylbenzylidene) camphor in immature rats. They have further confirmed the mitogenic activity of the compound in MCF7 cells, previously observed by Schlumpf *et al.* (2001). Recently, bisphenol A exposure in fetal Wistar rats has been shown to stimulate development of ductal hyperplasias and carcinoma *in situ* (Murray *et al.* 2006). Bisphenol A has been linked to induction of preneoplastic and neoplastic lesions in mammary glands of the fetus, leading to increased risk of breast cancer during adult life (Murray *et al.* 2006; Durando *et al.* 2007). Relative proliferative effect of these proven xenoestrogens is similar to or less than the RPE of the colorant compounds tested in this study establishing a further need to evaluate their xenoestrogenic potential.

To compliment and substantiate the cell proliferation results, additional experiments were performed to study the effects of the food colorants in the presence of tamoxifen. Tamoxifen is an antagonist in estrogen responsive breast cancer cells (Jordan *et al.* 2001; Zhang *et al.* 2005); if a compound was stimulating cell proliferation via the ER binding and activation, then such binding will be competitively inhibited by tamoxifen. In our research, cell proliferation induced by the food colorants was completely inhibited by tamoxifen and cell proliferation induced by E2 was partially inhibited by tamoxifen (Figure 8). These data indicate that the positive control (E2) and food colorants stimulated cell proliferation at least in part via the estrogen receptor. The fact that tamoxifen treated cells had lower proliferative effect than the negative control indicates that there may be undefined estrogenic activity in the control medium (Bondy and Zacharewsky, 1993).

The ER-CALUX assay was performed to demonstrate the competence of the food colorants to activate the nuclear ER directly. ER-CALUX assay is based on the principle of estrogens (and/or xenoestrogens) binding to and activating endogenous ER in T47D cells. The activated ER binds the ERE 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. In our research, all the three investigated compounds significantly stimulated luciferase induction over the negative control ($p=0.05$) and further establishes a nuclear ER mediated effect of the food colorants. Interestingly, the luciferase activity stimulated by the tested food colorants correlate well with other estrogenic compounds reported in T47D cells ER-CALUX assays; estrogenic compounds, like methoxychlor (Wilson *et al.* 2004), polybrominated diphenyl ethers (Meerts *et al.* 2001), 4-nonylphenol (Wilson *et al.* 2004), bisphenol A (Meerts *et al.* 2001), and genistein (Wilson *et al.* 2004) showed similar luciferase induction (calculated as percentage of E2) as the food colorants tested in this study. Furthermore, it has also been demonstrated by Leglar *et al.* (1999) that their ER-CALUX relative potency values were in correlation with MCF7 cell proliferation assays performed by Soto *et al.* (1991, 1994, 1995). This further demonstrates the consistency of our food colorants in inducing estrogen-like activity *in vitro*.

Comparison of the cell proliferation data with the ER-CALUX data shows that all the three synthetic food colorants induced significant cell proliferation and luciferase induction. The rank order of the activity differed. In the cell proliferation assay, sudan I induced 80.20% of E2 response followed by erythrosin B (85.27% of E2 response) and tartrazine (93.23% E2 response) as demonstrated in Table 2. In the ER-CALUX assay, erythrosin B (128.24%) stimulated similar luciferase induction to sudan I (128.97%), while tartrazine stimulated maximal luciferase induction (235.53 %) as demonstrated in Figure 11. Interestingly, tartrazine, sudan I and erythrosin B stimulated luciferase induction greater than E2 in the ER-CALUX assay. As previously, observed by others (Leglar *et al.* 1999; Meerts *et al.* 2001, Wilson *et al.* 2004) this phenomenon may be due to effects on the stability of luciferase or due to stimulated receptor and/or coactivator protein renewal (Leglar *et al.* 1999).

Our research has revealed that a frequently used synthetic food colorant tartrazine (FD&C Yellow 5) is estrogenic, *in vitro*, and supports a previous report of the estrogenicity of erythrosin B (FD&C Red 3) by Dees *et al.* (1997). 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. In addition, we have shown that the suspected carcinogen, sudan I, acts at least in part through activation of the ER. Currently, sudan I and tartrazine do not appear to have been considered as potential EDC. However, erythrosin B is considered a xenoestrogen *in vitro* (Dees *et al.* 1997). Tartrazine induced maximal relative proliferative effect ($p=0.0004$) and luciferase activity ($p=0.05$) among the three colorants. Erythrosin B was estrogenic *in vitro* which is consistent with the work of Dees *et al.* (1997). Sudan I induced highly significant cell proliferation ($p=0.0005$) and luciferase activity ($p=0.0236$). The role of these synthetic food colorants as potential xenoestrogens correlates with the potential adverse physiological effects of food additives in human diet. Currently, few data are available regarding the presence of synthetic food colorants in environment and body burden in humans and wildlife. Future studies will focus on the molecular mechanism of action and physiological toxicokinetics of the three synthetic dyes, tartrazine, erythrosin B and sudan I.

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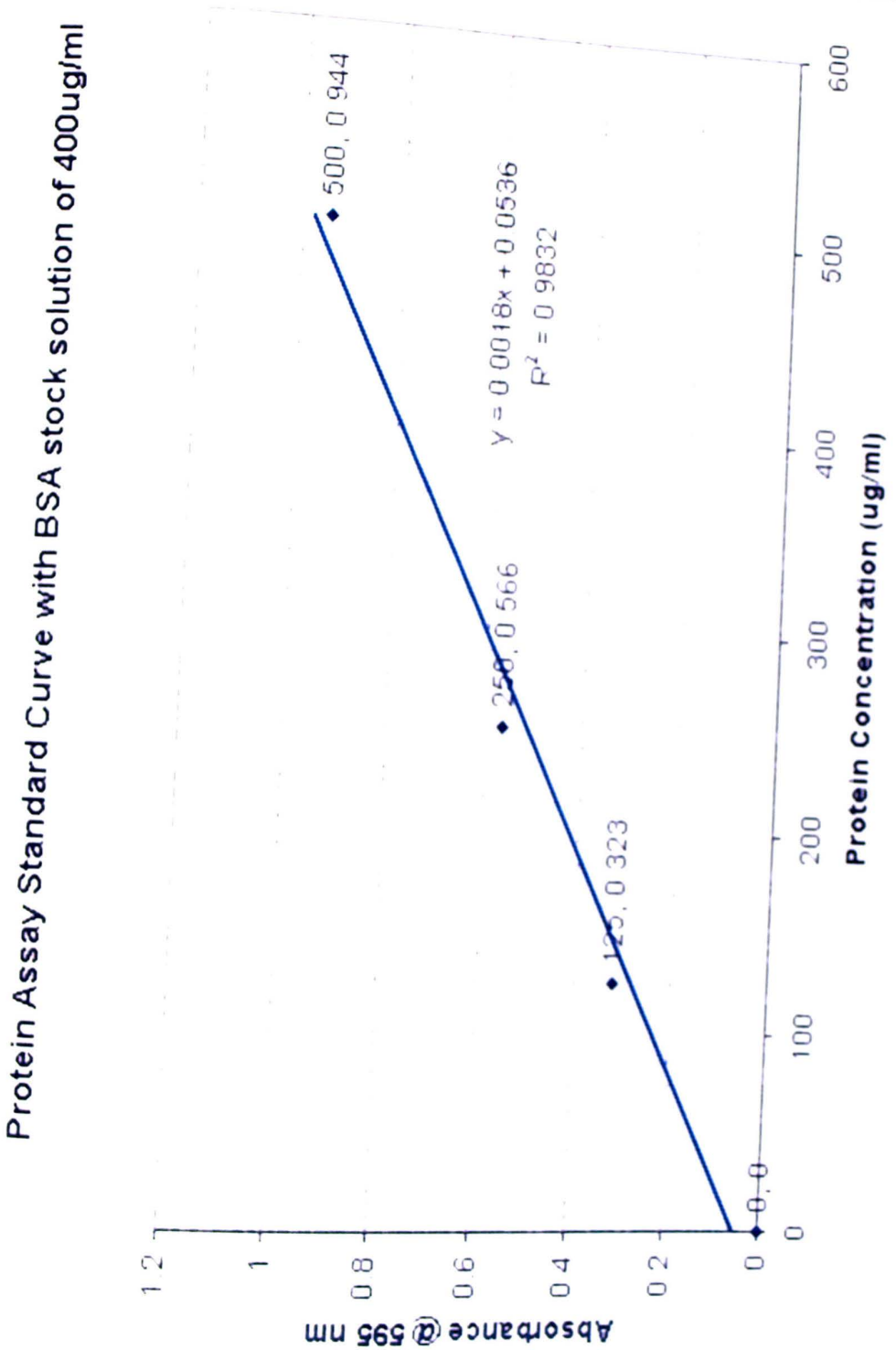
Appendices

APPENDIX I

Raw Data: Sample Protein Standard Curve with Bovine Serum Albumin (BSA)



APPENDIX II

Raw Data: Cell Proliferation Assay

		Replicate1(R1)	R2	R3	R4	R5	R6	R7	R8	R9	Average	STDV	SEM
Erythrosin B	Control	164 93	229 40	238 35							210 89	40 05	23 13
	001 nM EB	192 77	259 65	289 74							247 39	49 63	28 65
	01 nM EB	206 92	260 84	294 67							254 14	44 25	25 55
	1 nM EB	205 33	259 46	293 21							252 67	44 33	25 59
	1 nM EB	204 19	260 34	280 22							248 25	39 43	22 76
	10 nM EB	202 72	254 34	283 14							246 73	40 75	23 53
Sudan I	Control	167 50	139 91	158 84							155 42	14 11	8 15
	.001 nM S	193 81	154 25	163 10							170 39	20 76	11 99
	01 nM S	190 63	153 44	167 59							170 55	18 77	10 84
	1 nM S	192 73	156 09	165 82							171 55	18 98	10 96
	1 nM S	198 01	161 90	170 38							176 76	18 88	10 90
	10 nM S	184 77	149 99	167 44							167 40	17 39	10 04
Tartrazine	Control	179 32	111 82	373 14	138 10						200 60	118 34	59 17
	001 nM T	209 49	141 16	424 53							258 39	147 88	73 94
	01 nM T	221 36	169 40	426 89							272 55	136 16	68 08
	1 nM T	238 41	166 16	430 92	182 07						254 39	121 70	60 85
	1 nM T	234 26	148 37	446 26	181 71						252 65	133 83	66 92
	10 nM T	236 08	142 34	439 74	171 26						247 35	134 11	67 05
E2	Control	112 22	131 16	147 81	152 55	207 28	230 08	248 90	196 47	139 51	174 00	47 90	15 97
	0 001 nM	138 75	191 38	157 07	185 56	230 78	316 8194	309 94	246 41	210 33	220 78	62 18	20 73
	0 01 nM	140 23	201 24	191 56	182 43	249 9	317 6528	311 75	247 89	223 83	229 61	58 89	19 63
	0 1 nM	151 02	232 71	207 51	206 47	258 5875	322 38	321 40	255 78	229 39	242 81	55 00	18 33
	1 nM	150 51	203 74	189 35	205 84	258 65	320 92	322 93	257 03	225 26	237 14	58 38	19 46
	10 nM	146 82	207 41	194 13	207 32	257 84	320 92	319 67	254 36	227 64	237 34	57 47	19 16

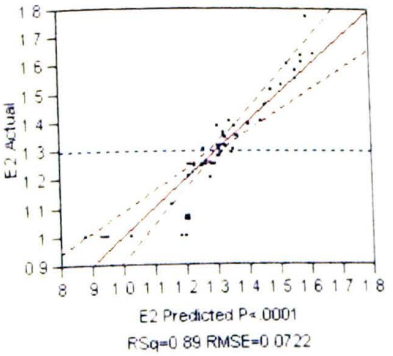
APPENDIX III

Statistical analysis on Cell Proliferation Data of E2

Response E2

Whole Model

Actual by Predicted Plot



Summary of Fit

RSquare	0 890806
RSquare Adj	0 855318
Root Mean Square Error	0 072191
Mean of Response	1 295926
Observations (or Sum Wgts)	54

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	13	1 7006407	0 130819	25 1015
Error	40	0 2084630	0 005212	Prob > F
C Total	53	1 9091037		< 0001*

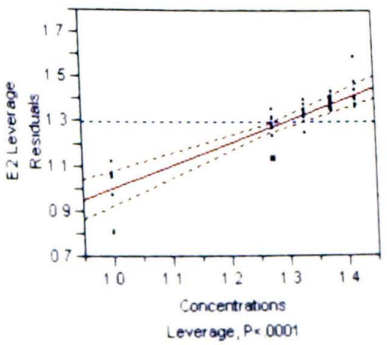
Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Concentrations	5	5	1 0499704	40 2938	< 0001*
Replicate	8	8	0 6506704	15 6064	< 0001*

Concentrations

Leverage Plot



LSMeans Differences Tukey HSD

α= 0 050 Q= 2 99223

Level		Least Sq Mean
1 nM	A	1 4166667
10 nM	A B	1 3766667
1 nM	A B	1 3755556
01 nM	A B	1 3311111
001 nM	B	1 2755556
Control	C	1 0000000

Levels not connected by same letter are significantly different

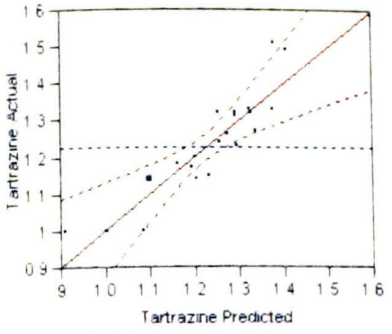
APPENDIX IV

Statistical analysis on Cell Proliferation Data of Tartrazine

Response Tartrazine

Whole Model

Actual by Predicted Plot



P=0 0004 RSq=0 84 RMSE=0 0731

Summary of Fit

RSquare	0 842091
RSquare Adj	0 744917
Root Mean Square Error	0 073063
Mean of Response	1 223182
Observations (or Sum Wgts)	22

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	0 37008005	0 046260	8 6658
Error	13	0 06939722	0 005338	Prob > F
C Total	21	0 43947727		0 0004*

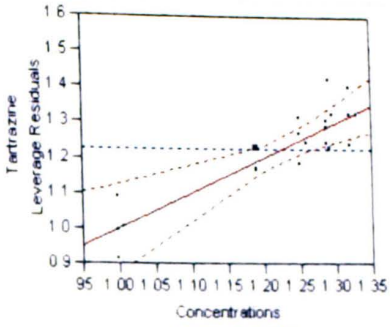
Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Concentrations	5	5	0 27808611	10 4186	0 0003*
Replicate	3	3	0 09201944	5 7459	0 0100*

Concentrations

Leverage Plot



Leverage, P=0 0003

LSMeans Differences Tukey HSD

α= 0 050 Q= 3 31611

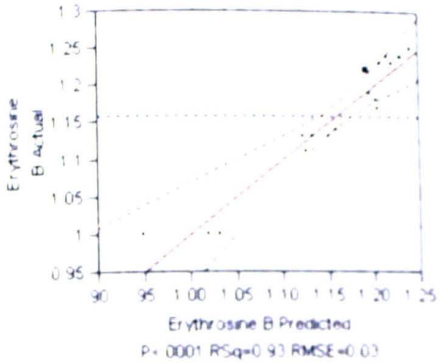
Level		Least Sq Mean
1 nM	A	1 3225000
01 nM	A	1 2945833
1 nM	A	1 2900000
10 nM	A	1 2525000
001 nM	A	1 1912500
Control	B	1 0000000

Levels not connected by same letter are significantly different

APPENDIX V

Statistical analysis on Cell Proliferation Data of Erythrosin B

- Response Erythrosine B
- Whole Model
  - Actual by Predicted Plot



  - Summary of Fit

RSquare	0.927719
RSquare Adj	0.877123
Root Mean Square Error	0.029881
Mean of Response	1.157222
Observations (or Sum Wgts)	18

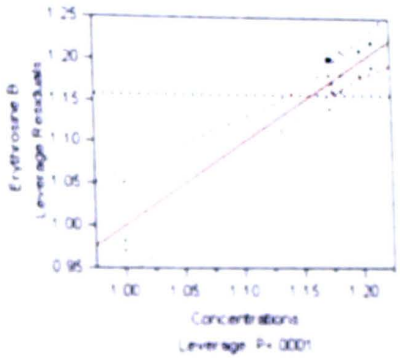
  - Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	0.11537222	0.016482	18.3357
Error	10	0.00898889	0.000899	Prob > F
C. Total	17	0.12436111		<.0001*

  - Parameter Estimates
  - Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Concentrations	5	5	0.09196111	20.4811	<.0001*
Replicate	2	2	0.02341111	13.0222	0.0016*

- Concentrations
  - Leverage Plot



  - LSMeans Differences Tukey HSD

α= 0.050 Q= 3.47331

Level		Least Sq Mean
01 nM	A	1.2100000
1 nM	A	1.2000000
1 nM	A	1.1833333
10 nM	A	1.1766667
001 nM	A	1.1733333
Control	B	1.0000000

Levels not connected by same letter are significantly different

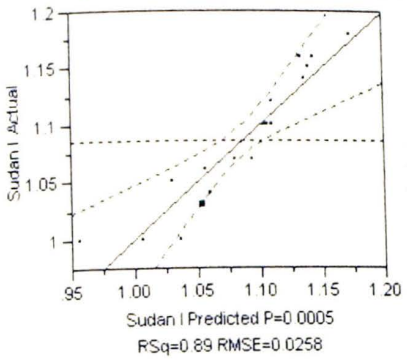
APPENDIX VI

Statistical analysis on Cell Proliferation Data of Sudan I

Response Sudan I

Whole Model

Actual by Predicted Plot



Summary of Fit

RSquare	0.885942
RSquare Adj	0.806102
Root Mean Square Error	0.02582
Mean of Response	1.085
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	0.05178333	0.007398	11.0964
Error	10	0.00666667	0.000667	Prob > F
C. Total	17	0.05845000		0.0005*

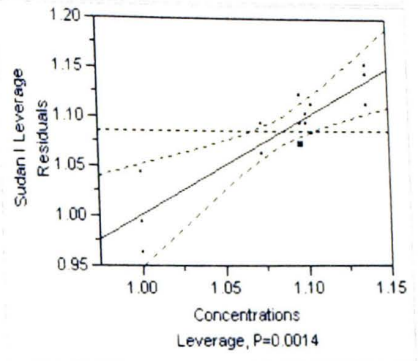
Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Concentrations	5	5	0.03218333	9.6550	0.0014*
Replicate	2	2	0.01960000	14.7000	0.0011*

Concentrations

Leverage Plot



LSMeans Differences Tukey HSD

α= 0.050 Q= 3.47331

Level		Least Sq Mean
1 nM	A	1.1366667
.1 nM	A	1.1033333
.01 nM	A	1.1000000
.001 nM	A	1.0966667
10 nM	A	1.0733333
Control	B	1.0000000

Levels not connected by same letter are significantly different.

APPENDIX VII

Raw Data: Cell proliferation in presence of Tamoxifen

Treatments	Replicate1	R2	R3	Average	STDV	SEM
Control (negative control for ER-CALUX assay)	618 67	2011 33	2566 00	1732 00	1003 27	579 24
E2 (0 1 nM)	1979 33	5177 00	4672 00	3942 78	1719 04	992 49
Sudan I (1nM)	4253 00	5193 00	3351 00	4265 67	921 07	531 78
Tartrazine (0 1 nM)	8448 33	7406 67	6385 67	7413 56	1031 35	595 45
Erythrosine B (0 01 nM)	4236 33	3435 50	4873 67	4181 83	720 63	416 06
Control (vehicle, negative control for transfection)	189 00	194 00	235 33	206 11	25 43	14 68

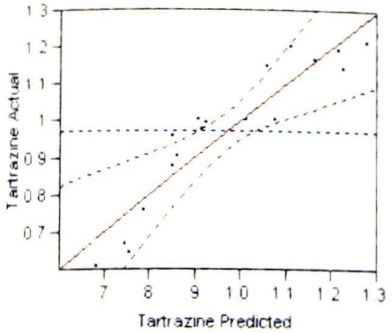
APPENDIX VII

Statistical analysis on Cell Proliferation Data of Tartrazine in presence of Tamoxifen

Response Tartrazine

Whole Model

Actual by Predicted Plot



Summary of Fit

RSquare	0 863099
RSquare Adj	0 767269
Root Mean Square Error	0 094177
Mean of Response	0 968679
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	0 55917051	0 079882	9 0065
Error	10	0 08869290	0 008869	Prob > F
C Total	17	0 64786341		0 0013*

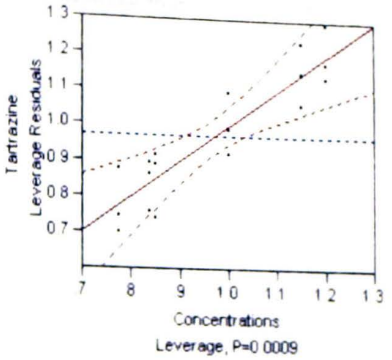
Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Concentrations	5	5	0 47263629	10 6578	0 0009*
Replicate	2	2	0 08653422	4 8783	0 0332*

Concentrations

Leverage Plot



LSMeans Differences Tukey HSD

α= 0 050 Q= 3 47331

Level		Least Sq Mean
E2	A	1 2021029
Colorant	A	1 1493863
Control	A B	1 0000000
E2+Tamoxifen	B	0 8485061
Tamoxifen	B	0 8382681
Colorant+Tamoxifen	B	0 7738097

Levels not connected by same letter are significantly different

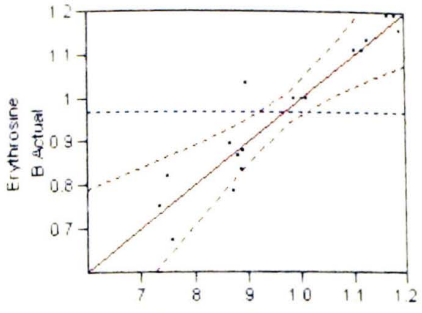
APPENDIX VIII

Statistical analysis on Cell Proliferation Data of Erythrosin B in presence of Tamoxifen

Response Erythrosine B

Whole Model

Actual by Predicted Plot



P=0.0003 RSq=0.90 RMSE=0.0677

Summary of Fit

RSquare	0.896285
RSquare Adj	0.823684
Root Mean Square Error	0.067704
Mean of Response	0.968594
Observations (or Sum Wgts)	18

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	0.39612119	0.056589	12.3454
Error	10	0.04583779	0.004584	Prob > F
C. Total	17	0.44195898		0.0003*

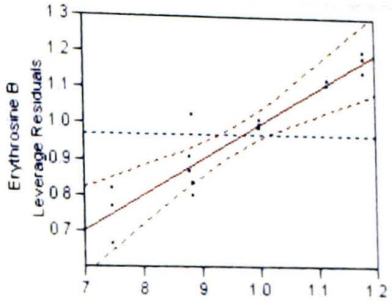
Parameter Estimates

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Concentrations	5	5	0.39447153	17.2116	0.0001*
Replicate	2	2	0.00164965	0.1799	0.8380

Concentrations

Leverage Plot



Leverage, P=0.0001

LSMeans Differences Tukey HSD

α= 0.050 Q= 3.47331

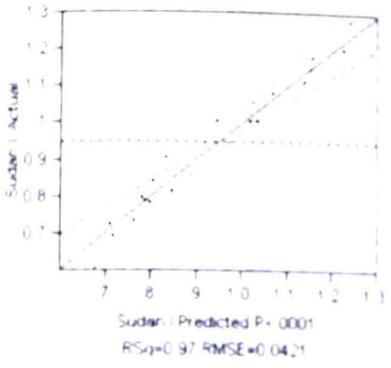
Level		Least Sq Mean
E2	A	1.1802875
Colorant	A	1.1180152
Control	A B	1.0000000
Tamoxifen	B C	0.8853315
E2+Tamoxifen	B C	0.8795777
Colorant+Tamoxifen	C	0.7483514

Levels not connected by same letter are significantly different.

APPENDIX IX

Statistical analysis on Cell Proliferation Data of Sudan I in presence of Tamoxifen

- Response Sudan I
- Whole Model
  - Actual by Predicted Plot



- Summary of Fit

RSquare	0.969932
RSquare Adj	0.948884
Root Mean Square Error	0.042143
Mean of Response	0.946749
Observations (or Sum Wgts)	18

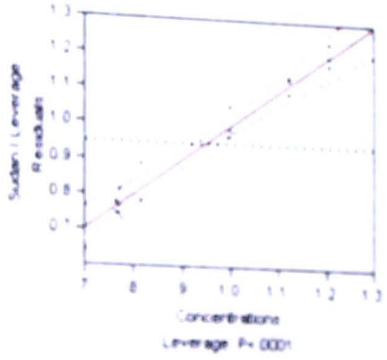
- Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	0.57290508	0.081844	46.0825
Error	10	0.01776025	0.001776	Prob > F
C. Total	17	0.59066533		<.0001*

- Parameter Estimates
- Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Concentrations	5	5	0.54712888	61.6128	<.0001*
Replicate	2	2	0.02577620	7.2567	0.0113*

- Concentrations
  - Leverage Plot



- LSMeans Differences Tukey HSD

α= 0.050 Q= 3.47331

Level		Least Sq Mean
E2	A	1.2067402
Colorant	A	1.1222411
Control	B	1.0000000
E2+Tamoxifen	C	0.8165715
Colorant+Tamoxifen	C	0.7708664
Tamoxifen	C	0.7650749

Levels not connected by same letter are significantly different

APPENDIX XI

Raw Data: ER-CALUX assay

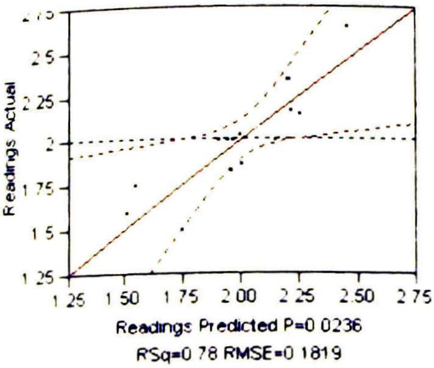
	Treatments	Replicate1	R2	R3	Average	STDV	SEM
Sudan I	Control	232 15	294 90	262 89	263 31	31 38	18 12
	E2	278 26	338 03	335 27	317 19	33 74	19 48
	Sudan I	257 84	318 09	309 53	295 15	32 60	18 82
	Tamoxifen	184 46	212 59	204 99	200 68	14 55	8 40
	E2+Tamoxifen	209 84	215 21	213 74	212 93	2 78	1 60
	Sudan I+Tamoxifen	181 78	203 09	221 07	201 98	19 67	11 36
Tartrazine	Control	225 78	270 15	269 31	255 08	25 38	14 65
	E2	269 28	324 39	326 64	306 77	32 49	18 76
	Tartrazine	262 71	309 18	307 03	292 98	26 23	15 14
	Tamoxifen	197 78	179 88	262 03	213 23	43 20	24 94
	E2+Tamoxifen	204 09	174 81	267 83	215 57	47 56	27 46
	Tartrazine+Tamoxifen	171 21	164 32	257 15	197 56	51 72	29 86
Erythrosine B	Control	237 78	254 04	243 57	245 13	8 24	4 76
	E2	283 84	293 69	290 10	289 21	4 99	2 88
	Erythrosin B	264 09	287 72	270 56	274 12	12 21	7 05
	Tamoxifen	186 59	263 00	203 63	217 74	40 11	23 16
	E2+Tamoxifen	212 46	222 79	211 47	215 57	6 27	3 62
	Erythrosin B+Tamoxifen	178 90	184 74	199 76	187 80	10 76	6 21

APPENDIX XII

Statistical analysis on ER-CALUX Data

Whole Model

▼ Actual by Predicted Plot



▼ Summary of Fit

RSquare	0.78081
RSquare Adj	0.616417
Root Mean Square Error	0.181874
Mean of Response	2.007333
Observations (or Sum Wgts)	15

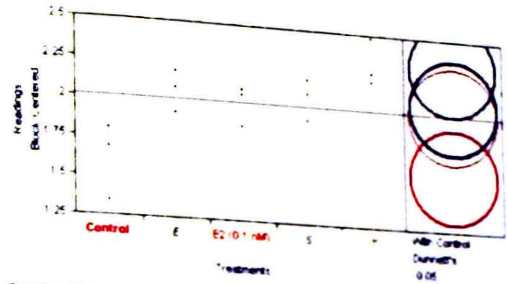
▼ Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	6	0.9426667	0.157111	4.7497
Error	8	0.2646267	0.033078	Prob > F
C. Total	14	1.2072933		0.0236*

▶ Parameter Estimates

▼ Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatments	4	4	0.77329333	5.8444	0.0168*
Time (Block)	2	2	0.16937333	2.5602	0.1382



Block: Time (Block)

▼ Means Comparisons

▼ • Comparisons with a control using Dunnett's Method

Control Group = Control

|d|	Alpha
3.02284	0.05

Level	Abs(Dif)-LSD	p-Value
[illegible]	0.254	0.0048*
S	0.008	0.0483*
E	0.001	0.0495*
E2 (0.1 nM)	-0.08	0.0864
Control	-0.46	1.0000

Positive values show pairs of means that are significantly different