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Boric Acid Causes ER Stress and Activates the eIF2alpha/ATF4 and ATF6 Branches of the Unfolded Protein Response in Prostate Cancer Cells and Using Toxicology in the Public Interest

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A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Toxicology

by

Sarah Ellen Kobylewski

2012

ABSTRACT OF THE DISSERTATION

Boric Acid Causes ER Stress and Activates the eIF2alpha/ATF4 and ATF6 Branches of the Unfolded Protein Response in Prostate Cancer Cells and Using Toxicology in the Public Interest

by

Sarah Ellen Kobylewski

Doctor of Philosophy in Molecular Toxicology

University of California, Los Angeles, 2012

Professor Curtis D. Eckhert, Chair

Nutritional chemoprevention is a growing area in the field of toxicology. What we do and do not eat has a major impact on the development of cancer. However, it is difficult to show a causal relationship between a natural product and cancer prevention because mechanistic biochemical data are often missing and animals studies can be inconclusive. Both determining and elucidating molecular mechanisms that modulate pathological endpoints are necessary components in the risk assessment process used to determine if chemicals that show chemoprevention properties in the laboratory are safe for public use. The research presented in this dissertation focuses on chemoprevention through the consumption of a nutrient or the avoidance of toxic food additives. Part I presents research that elucidated a molecular pathway activated by boric acid (BA), an essential plant nutrient, which may provide insight into the

inhibition of cell proliferation of prostate cancer cells and reduced risk of prostate cancer. Part II consists of molecular toxicology research in application to public interest and health. It is a critical analysis of two commonly consumed FDA-approved food additives, rebaudioside A, an artificial sweetener, and artificial food dyes. In Part I, it was shown that BA is not an isoform-specific antagonist to the ryanodine receptor (RyR), a calcium (Ca^{2+}) channel, but is a RyR antagonist that functions by interacting and competing with the receptor's only known endogenous agonist. This results in altered Ca^{2+} signaling that induces ER stress and the eIF2α/ATF4 and ATF6 branches of the unfolded protein response (UPR) in DU-145 prostate cancer cells. ER stress and the UPR are tightly associated with cell proliferation. The specific pathway that we have unfolded in BA-treated DU-145 cells is correlated with cell survival and an inhibition of cell proliferation. In Part II, we describe how *in vivo* and *in vitro* studies on rebaudioside A and food dyes demonstrated their toxicity. The assessment of toxicology studies on food dyes showed they do present an increased health risk and this is important given their widespread use by the public. The research presented here thus presents both the molecular mechanistic and public health sides of molecular toxicology.

The dissertation of Sarah Ellen Kobylewski is approved.

Richard J. Jackson

Wendie A. Robbins

Robert H. Schiestl

Curtis D. Eckhert, Committee Chair

University of California, Los Angeles

2012

For mom and dad.

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Acknowledgments

Chapter 1 is a version of the following published paper: Kobylewski SE, Henderson KA, Eckhert CD. Identification of ryanodine receptor isoforms in DU-145, LNCaP, and PWR-1E cells. *Biochem Biophys Res Commun.* 2012: 425(2): 431-435. Co-authors include Kimberly Henderson, Ph.D. (Molecular Toxicology, UCLA) and Curtis D. Eckhert, Ph.D (Principle Investigator, Molecular Toxicology and Environmental Health Sciences faculty, UCLA). Chapter 2 is a version of a paper in preparation for submission. Co-authors include Kimberly Henderson (see above), Kristin Yamada (Ph.D. candidate, Molecular Toxicology, UCLA), and Curtis D. Eckhert (Principle Investigator, see above). Chapter 3 is a version of an internal publication written for the Center for Science in the Public Interest (CSPI). Curt D. Eckhert (see above) was co-author. Chapter 4 is a version of a CSPI publication that has been accepted for publication in the International Journal of Occupational and Environmental Health. Michael F. Jacobson, Ph.D. (co-first author, Executive Director CSPI). This work was funded by personal funds (Curt Eckhert) and in part by the University of California Toxic Substances Research and Training Program (TSR&TP) for partial support of Sarah Kobylewski. Sarah Kobylewski was partially funded by the UCLA Dissertation Fellowship, the Ruth F. Richards Memorial Student Award, and UCLA Affiliates. Curt Eckhert is the doctor mentor for Sarah Kobylewski. TSR&TP, Dissertation Fellowship, and student awards funding was for student support and had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Sarah Kobylewski would also like to thank Kristin Yamada for her help with editing this dissertation.

Biographical Sketch

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University of California, Los Angeles, Dr. Curt Eckhert (03/06-Present). Molecular Toxicology graduate student; Research the molecular mechanism of boric acid-induced inhibition of prostate cancer.

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Scientific Assistant, Dr. John Froines. Science writer (08/11-Present); Review, critique, and write on the pesticide registration process in California using methyl iodide as a paradigm. Facilitating support staff (03/09-2010); Assist in the organization and collection of data for the scientific review committee for the review of methyl iodide registration in California.

US Army Corps of Engineers, Construction Engineering Research Laboratory (CERL), Dr. Donald Cropek (05/03-06/05). Engineering aid; environmental chemistry laboratory

Beckman Institute, University of Illinois Urbana-Champaign, Dr. Kenneth Watkin (08/03-05/04). Research assistant

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Technical Skills
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Kobylewski SE, Henderson KA, Eckhert CD. Identification of ryanodine receptor isoforms in DU-145, LNCaP, and PWR-1E cells. *Biochem Biophys Res Commun.*2012: 425(2): 431-435.

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Kobylewski SE, Henderson KA, Yamada K, Eckhert CD. Boric acid induces ER stress and the eIF2α/ATF4 and ATF6 branches of the unfolded protein response in DU-145 prostate cancer cells. *J Biol Chem.* 2012.

Abstracts
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Kobylewski SE and Jacobson MF. Food Dyes: Colorful or Harmful? A toxicology review. Prepared for the Center for Science in the Public Interest (2010).

Lectures
Guest lecturer. Environmental Health Sciences 100, UCLA. "Methyl Iodide: To register or not to register." Nov. 2009.

Part I: Molecular Toxicology

Part I Introduction

Prostate cancer

Worldwide, prostate cancer accounts for about 15.3 and 4.3% of cancers afflicting men in developed and developing countries, respectively (1). In the United States, prostate cancer is the most common and second deadliest cancer in men (2). Though the reasons are unclear, prostate cancer incidence and mortality is 1.6 and 2.3 times higher, respectively, in African-Americans compared to Caucasians and Hispanics (3). Risk factors include increasing age, race, and family history (3).

Due to a the widespread use of screening tools, such as the prostate specific antigen (PSA) blood test, developed countries such as the United States have a high incidence of low-risk as well as more advanced cases of prostate cancer. Because of the wide range of diagnosed stages of prostate cancer, treatments range from "watchful waiting" to radical prostatectomy. Radiotherapy, which is sometimes used in combination with cryotherapy or androgen-ablation therapy, is another commonly used active treatment (4). The more conservative, active treatments have side effects including sexual and urinary dysfunction and incontinence (5). The high incidence of prostate cancer and the severity of the therapeutic side effects make the search for an effective chemopreventatitve agent highly desired.

For years, scientists have been seeking a chemopreventative nutrient that would help lower men's risk of prostate cancer. In 2001, a federally funded prospective randomized trial called SELECT (Selenium and Vitamin E Cancer Trial) enrolled over 35,000 men to test if selenium and vitamin E, individually and in combination, prevented prostate cancer. In 2008, the study

had to be stopped because of the increase in prostate cancer in men receiving vitamin E supplements (p=0.6) and potential increased risk of diabetes in men receiving selenium supplements (6). Boric acid (BA) is another micro-nutrient that has been targeted as a potential chemoprevention to prostate cancer. Epidemiological evidence demonstrates that prostate cancer risk is inversely proportional to dietary intake of BA in humans (7-9). The biological plausibility of this has been supported by experiments showing BA reduced the growth of prostate tumors and prostate specific antigen (PSA) levels in nude mice and prostate cell proliferation (10-14).

Boric acid

Boron is a metalloid found ubiquitously in nature. Boron's average concentration in the earth's crust and sea water is approximately 10 and 4.6 ppm, respectively (9). With a pKa of 9.2, BA, $B(OH)_3$, is the primary form of boron found in physiological fluids (pH 7.4) (Fig. 1). The anionic borate form, $B(OH)_4^-$, is found in natural systems at less than 2% (15). People consume boron every day in food and water. Greater than 95% of BA is absorbed by the gastrointestinal tract and it is excreted unchanged in the urine (16). The five highest contributors of boron to the American diet are coffee (6.7%), milk (5.1%), apples (5.1%), beans (4.8%) and potatoes (4.8%) due to their high consumption rather than high boron concentration (17). Boron intake in men and women in the United States ranges from 0.3-9.46 mg/day. BA plasma concentrations range in people with an American diet from 2-9 μM, with an average of 4.6 and 3.8 μM in men and women, respectively (17-19).

Boron has been used by nature for a variety of functions. It binds molecules with *cis*-diols and creates complexes with several biological molecules, including NAD^+ and nucleosides (20).

Boron is necessary for the stability of the plant cell wall, crosslinking two rhamnogalacturonan II monomers, a pectin polysaccharide, through a borate bridge (22). In bacteria, the quorum-sensing signal, AI2, is a boron-containing stable complex produced by a variety of bacteria (23). BA is chemopreventative at low, dietary doses and a reproductive toxin at high doses (7, 9). Our lab found that in animals, boron is essential for maximum growth of rainbow trout embryos and the early stage of cleavage in fertilized zebrafish eggs (24, 25). Others have shown BA is essential for embryonic development in *Xenopus laevis*. Several studies have demonstrated the positive effects of boron in bone metabolism (26-28). There is also evidence that B plays a role in immune function, enzyme function, plasma membrane structure and processes, the inflammatory response, oxidative stress relief, and the functioning of serine proteases (15).

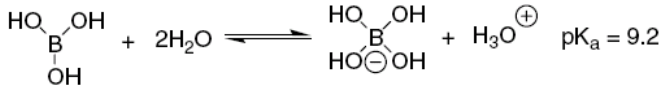

Figure 1. Boron is found in physiological fluids in the form of boric acid (21).

BA's effects in prostate cancer cells

Our lab demonstrated that BA causes a cell death-independent inhibition of prostate cancer cell lines DU-145 and LNCaP in a dose-dependent manner. Significant inhibition occurred at 60 and 100 μM BA in DU-145 (androgen-independent) and LNCaP (androgen-dependent) cell lines, respectively. In contrast, proliferation in the non-tumorgenic prostate cell lines, RWPE-1 and PWR-1E were not inhibited until concentrations reached 500 and 1000 μM, respectively. These results demonstrated that the BA-sensitivity of prostate cancer and non-cancer cell lines is dependent on one or several physiological differences between cell lines (10).

The coenzymes NAD^+ and $NADP^+$ involved in intermediary metabolism can be released into the extracellular space by cell death and active secretion where they act as paracrine signals. NAD+ and NADP+ are converted by a multifunctional enzyme on the plasma membrane called CD38 into cADPR and NAADP, respectively. cADPR and NAADP are both agonists of intracellular calcium (Ca^{2+}) release, but for different Ca^{2+} stores (18, 29, 30). We showed that BA binds to NAD^+ and later demonstrated that NAD^+- and $NADP^+$-induced Ca^{2+} store release was inhibited by 250 and 1,000 μM BA, respectively (18, 20). This data led us to hypothesize that BA acts as an antagonist to an intracellular Ca^{2+} channel to inhibit Ca^{2+} release in response to external stimuli.

Using nanoSIMS imaging we have shown that BA localizes to specific regions in DU-145 cells, which indicates that it is probably in a bound state. The localization is within close proximity to the nucleus, most likely the endoplasmic reticulum (ER) and mitochondria (31). We also found that BA acts as an antagonist to the ryanodine receptor (RyR), a Ca^{2+} channel, in response to three different RyR agonists including caffeine, cADPR, and 4-chloro-m-cresol (32). It became clear that BA was having an effect on the Ca^{2+} homeostasis of DU-145 cells and understanding BA's effect on cellular Ca^{2+} could help elucidate the mechanism by which BA inhibits DU-145 proliferation.

Cell proliferation is controlled by a variety of exogenous and endogenous signals, Ca^{2+} being among the major contributing factors (33). There is a vast difference in Ca^{2+} concentration in the extracellular space (~1.2mM), cytosol (100 nM), and intracellular stores such as the ER/SR (~100-200 μM) which allows for controlled Ca^{2+} signaling. The cell's complex Ca^{2+} signaling

system consists of numerous receptors, transducers, channels, buffers, effectors, enzymes, pumps and exchangers that can be used in a variety of combinations allowing for the transmission of unique Ca^{2+} signals (34). Cells usually receive the signal to proliferate in response to growth factors and other external stimuli. The stimulus then causes the assembly of a signal transducing complex which, through a cascade of events, increases cytosolic Ca^{2+}. This free Ca^{2+} functions to signal gene transcription through the activation of cytosolic (NF-AT, NF-κB) and nuclear (CREB) transcription factors (35). In prostate cancer, the Ca^{2+} plasma membrane channels, TRPV6 and TRPM8, are upregulated and this increases cytosolic Ca^{2+} which in turn increases the Ca^{2+}-regulated proliferation signal (36).

Current work

The work described in the following two chapters describes the molecular pathway activated by BA following its initial impact on Ca^{2+} signaling in DU-145 cells. Since BA is a RyR antagonist, our first goal was to characterize RyRs in the three cell lines we use as models for BA sensitivity: DU-145, LNCaP, and PWR-1E. Next, we studied the possibility that BA-treated DU-145 cells go through ER stress and the unfolded protein response (UPR), a cellular coping mechanism that is tightly linked to cell proliferation. Our work in these two studies demonstrated that BA is not a RyR isoform-specific antagonist but rather is probably exerting its effects through one of the RyR's many accessory proteins. This action on such an important Ca^{2+} channel is inducing ER stress and certain branches of the UPR. As a result, stress granules form, an event that is tightly linked to cell survival and proliferation.

Chapter 1: Characterization of ryanodine receptor isoforms in prostate cell lines[1]

Abstract

The ryanodine receptor (RyR) is a large, intracellular calcium (Ca^{2+}) channel that is associated with several accessory proteins and is an important component of a cell's ability to respond to changes in the environment. Three isoforms of the RyR exist and are well documented for skeletal and cardiac muscle and the brain, but the isoforms in non-excitable cells are poorly understood. The aggressiveness of breast cancers in women has been positively correlated with the expression of the RyR in breast tumor tissue, but it is unknown if this is limited to specific isoforms. Identification and characterization of RyRs in cancer models is important in understanding the role of the RyR channel complex in cancer and as a potential therapeutic target. The objective of this report was to identify the RyR isoforms expressed in widely used prostate cancer cell lines, DU-145 and LNCaP, and the non-tumorigenic prostate cell line, PWR-1E. Oligonucleotide primers specific for each isoform were used in semi-quantitative and real-time PCR to determine the identification and expression levels of the RyR isoforms. RyR1 was expressed in the highest amount in DU-145 tumor cells, expression was 0.48-fold in the non-tumor cell line PWR-1E compared to DU-145 cells, and no expression was observed in LNCaP tumor cells. DU-145 cells had the lowest expression of RyR2. The expression was 26- and 15-fold higher in LNCaP and PWR-1E cells, respectively. RyR3 expression was not observed in any of the cell lines. All cell types released Ca^{2+} in response to caffeine showing they had functional RyRs. We also used live-cell confocal miscroscopy to determine the functionality of the RyRs in these cell lines as well as the necessity of the RyR accessory protein, FKBP12, in

[1] This chapter is a modified version of a recently published paper 37. Kobylewski SK, Henderson KA, Eckhert CD. Identification of ryanodine receptor isoforms in prostate DU-145, LNCaP, and PWR-1E cells. Biochemical and Biophysical Research Communications2012;425(2):431-5.

DU-145's response to boric acid, a nutritient that is thought to be chemopreventative to prostate cancer. Total cellular RyR-associated Ca^{2+} release is determined by both the number of activated RyRs and its accessory proteins which modulate the receptor. Our results suggest that the correlation between the expression of the RyR and tumor aggression is not related to specific RyR isoforms, but may be related to the activity and number of receptors.

Introduction

It has been nearly 40 years since Balk and his colleagues reported a difference in Ca^{2+} stimulated proliferation between transformed and non-transformed fibroblasts (38). Since this discovery, major advances have been made in elucidating Ca^{2+}'s role in the cell cycle and proliferation (39, 40). Recently, an evaluation of breast cancer tissue arrays from the National Cancer Institute found a positive correlation between RyR immunostaining and tumor aggressiveness (41). Three isoforms of the RyR are known to exist (RyR1, RyR2, and RyR3) in skeletal muscle, cardiac muscle, and the brain (42), but the identification of the isoforms in ryanodine-sensitive non-excitable epithelial and exocrine cells is limited to only a few cell types (43).

The RyR is a large, transmembrane, homotetrameric Ca^{2+} channel consisting of 4 ~550 kDa subunits, totaling approximately 2.3 MDa, making it the largest cellular ion channel (44, 45). It is the major Ca^{2+} channel linking the rich Ca^{2+} stores of the endoplasmic reticulum (ER) and cytoplasm of non-excitable cells. A high conductance ion channel, the RyR allows for rapid and precise release of Ca^{2+} across a 20,000-fold gradient between the ER (~0.2 to 20 mM [Ca^{2+}]) and cytoplasm (~100 nM [Ca^{2+}]) enabling cells to activate Ca^{2+}-dependent cellular processes in response to changes in the cellular environment (46). RyR-associated Ca^{2+} release occurs when

the receptors are stimulated, either through an agonist or calcium induced calcium release (CICR) (42). RyRs serve a major role in regulating several cellular functions including differentiation, apoptosis, secretion, muscle contraction, and neurotransmitter release (45, 47).

Mammalian RyRs exist in three distinct isoforms, approximately 70% homologous in sequence and residing on three different chromosomes (45, 47). Isoforms 1 and 2 function in excitation-contraction coupling to stimulate contraction of striated muscle (48). RyR1 is the primary isoform in skeletal muscle and RyR2 in cardiac muscle (45, 47). RyR3 is preferentially expressed in the brain, especially in the hippocampus and striatum (49).

RyRs are associated with a variety of diseases. Defects in RyR1 cause malignant hyperthermia and a spectrum of myopathies in skeletal muscle (50). RyR2 dysregulation can result in fatal cardiac arrhythmias and heart failure (50). RyR3 knockout mice are hyperactive and exhibit decreased social contact with other mice (51). Altered RyR gating (opening and closing of the channel's pore) has been implicated in a range of other diseases, including epilepsy, neurodegeneration, pain, and cancer (50). Consequently, these channel complexes represent potential therapeutic targets for treatment of numerous diseases, including cancer.

RyRs interact with a range of substances of public health concern and are more susceptible to modulation in tumor cells than normal cells (52). In differentiated normal cells, Ca^{2+} is highly regulated both spatially and temporally, but in tumor cells there is a sustained elevation in whole cytoplasmic Ca^{2+} (global Ca^{2+} signaling) (53, 54). RyRs, unlike the inositol triphosphate receptor (IP3R), are modulators of global Ca^{2+} (55). Normal and tumor models of prostate

cancer have been reported to respond differently to RyR agonists and inhibitors (10). This may indicate a potential mechanism of tumorigenicity.

Several cell models are used to study prostate cancer. Among the most common are DU-145 and LNCaP tumors cells and PWR-1E non-tumor cells. DU-145 is an epithelial, androgen-independent line originally derived from the brain tumor of a man with prostate cancer. It was the first cell line used to study prostate cancer *in vitro* and is still widely used today (56). LNCaP cells are an androgen-dependent prostate cancer epithelial cell line. It was originally derived from the lymph nodes of a prostate cancer patient (56). PWR-1E cells are an immortalized, non-tumorigenic, epithelial prostate cell line that is used to provide a normal prostate cell control (57). The objective of this study was to identify RyR isoforms in DU-145, LNCaP, and PWR-1E cell lines in order to determine if RyR isoform expression differs between these cell models.

Materials and methods

Cell culture

DU-145 prostate cancer cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were maintained in RPMI Media 1640 (Gibco-Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (200 mM) (Gemini Bio-Products, Sacramento, CA). LNCaP cells were obtained from ATCC and cultured without androgen supplementation in the same media described for DU-145. When LNCaP cells are grown in androgen-depleted media they are referred to as subline LNCaP CL1 (56). PWR-1E cells were obtained from ATCC, maintained

in KSFM media supplemented with bovine pituitary extract (0.05 mg/ml), human recombinant epidermal growth factor (5 ng/ml) (Gibco-Life Technologies), penicillin (100 U/ml), and streptomycin (100 µg/ml). A172 cells were obtained from ATCC, maintained in DMEM media supplemented (Gibco-Life Technologies) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (200 mM) (Gemini Bio-Products, Sacramento, CA). Cells were plated on 10 or 15 cm plates (Corning Life Sciences, Corning, NY) incubated at 37ºC in a humidified chamber containing 5% CO_2 and 95% air and grown to 80% confluency. All treatment groups used media that had been stripped of boron by shaking 2 grams of Amberlite IRA 743 exchange resin (Sigma-Aldrich, St. Louis, MO) for 12 hours at 4ºC.

Semi-quantitative PCR

Semi-quantitative PCR used primers designed by the authors using Primer Express 3.0 (Applied Biosystems (ABI), Foster City, CA) and specific for each isoform (Table 1.1). RNA was isolated from cells using an RNeasy mini kit (Qiagen, Valencia, CA). Total RNA (2 µg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) with random hexamer primers (Invitrogen) at a final volume of 20 µl at 25°C, 10 minutes (10:00); 50°C, 45:00; and 70 °C, 15:00. PCR reactions for RyR isoform identification were 50 µl total: 5 µl cDNA, 10 µl 5x green GoTaq® reaction buffer, 5 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTPs, 0.25 µl 100 µM forward primers, 0.25 µl 100 µM reverse primers, 0.25 GoTaq® Flexi DNA polymerase (Invitrogen). Reactions were run on a PTC-200 (MJ Research, Ramsey, MN) using the following protocol: 95°C, 2:00; 95 °C, 0:30-1:00; 55-60°C, 0:30-1:00; 72 °C, 1:00; repeat steps 2-4 35 times; 72 °C, 5:00; 4 °C, forever.

Table 1.1. Primers and enzymes used to identify RyR isoforms.

	Forward Primer	Reverse Primer	Restriction Enzyme
RyR1	TGTCAAGCGCAAGGTCCTGG	TGTCCAGGAGATGGGCAGCAA	BglII (Invitrogen)
RyR2	AAGGAGCTCCCCACGAGAAGT	CAGATGAAGCATTTGGTCTCCAT	BsmI (New England Biolabs, Ipswich, MA)
RyR3	AAGAGGAAGAAGCGATGGT	CTCCAAGCTTCCAGATATGG	BglII

Restriction enzyme digestion

PCR products were digested with a restriction enzyme chosen for the gene of interest (Table 1.1). Digestion reaction consisted of 20 µl PCR product, 2 µl enzyme, 2 µl corresponding reaction buffer, and 6 µl autoclaved deionized water. 2 µl enzyme were replaced by autoclaved deionized water as a negative control. Digestion products were run on 2% agarose gels pre-stained with ethidium bromide (Sigma-Aldrich). Products were run adjacent to a 1 Kb Plus DNA ladder (Invitrogen). The gel was run at 100V for 1 hour using an EC4000P power source (Thermo Electron Corporation, Waltham, MA). Gels were viewed on a Typhoon 9410 Variable Mode Imager (Amersham Biosciences, Piscataway, NJ).

SybrGreen real-time PCR (RT-PCR)

cDNA was created from total cellular RNA according to the above protocol. Primers specific to RyR isoforms were designed using Primer Express 3.0 (ABI) and are listed in Table 1.2. 10 µl reactions included 5 µl RT^2 Real Time™ SYBR Green/ROX PCR master mix (SA Biosciences, Frederick, MD), 3 µl cDNA, 0.9 µl forward primer, 0.9 µl reverse primer (Invitrogen), and 2 µl autoclaved ddI water. Reactions were added to MicroAmp™ fast optical 96-well reaction plates (ABI). Each plate contained reactions for the gene of interest as well as the internal

housekeeping gene, GAPDH. Plates were covered with MicroAmp^{TM} optical adhesive film (ABI) and read by a 7500 Fast Real Time PCR System using the 7500 Fast System Software v1.4.0 (ABI). The delta delta ct method was used to analyze the data.

Table 1.2. RT-PCR primers

Gene	Forward Primer	Reverse Primer
RyR1	GTCATCCTGTTGGCCATCATC	GGTCTCGGAGCTCACCAAAAG
RyR2	TTTTTTTGCCGCTCACCTTCT	CTGAGGACAAGATGGTTCTTAATGTC
GAPDH	CCTGTTCGACAGTCAGCCG	CGACCAAATCCGTTGACTCC

Measuring and Analysis of Ca^{2+} release

Ca^{2+} release was measured as previously described by Henderson *et al.* (32). The following is a modified version of that protocol: Ca^{2+} release was monitored in DU-145 cells treated with caffeine, boric acid, and/or rapamycin (Sigma-Aldrich) using the Ca^{2+}-sensitive dye, Rhod-2, AM ester (Biotium, Hayward, CA). This dye was chosen because it compartmentalizes well into cellular organelles. ER tracker green (Molecular Probes, Carlsbad, CA) was used to stain the ER and identify areas of ER Ca^{2+}. Ca^{2+} changes were measured by selecting regions of interest in cells where the dyes overlapped. Rhod-2, AM ester was prepared as a 1 mM stock solution in DMSO and diluted in cellular media. Cells were incubated with 5 µM Rhod-2, AM ester and 0.5 µM ER Tracker for 30 minutes at 37°C. Caffeine, boric acid, and rapamycin were diluted in Ringers solution at a final concentration of 20 mM, 50 µM, and 100 nM, respectively. Images were collected with a Zeiss 510 LSM 5 Pascal mounted to an upright microscope (Zeiss Axioplan 2) equipped with an Axoplan X63 (NA 0.95) water immersion objective. A HeNe laser was used to excite Rhod-2, AM ester at 543 nm. ER Tracker was excited at 488 nm from a laser diode. The emission was collected on a photomultiplier tube through a 560 nm LP filter (Rhod-2) and a 505 LP filter (ER Tracker). Additional magnification, time series, and

background subtraction were controlled using Zeiss LSM acquisition software. All images were acquired as 12 bit.

Statistical Analysis of the Data

Images of semi-quantitative PCR products of all 3 isoforms are representative of 3 biological replicates. All RT-PCR data was analyzed using 6 biological replicates of each cell line. The paired Student's t-test was used to measure significance between DU-145 and the other cell lines. Ca^{2+} release data is representative of 3 biological replicates and analyzed using the unpaired Student's t-test. Ca^{2+} levels were analyzed as a measure of fluorescence intensity (F). The formula $((F_o-F)/F_o)$ was used to calculate Ca^{2+} level. Time points pre-treatment were chosen randomly while the treatment time was chosen using the peak value. The unpaired Student's t-test was used to calculate significance.

Results

RyR isoform identification

The expression of RyR isoforms in DU-145, LNCaP, and PWR-1E cells was determined by semi-quantitative PCR using primers specific for each isoform (Table 1.1). Restriction enzyme digestion was performed on PCR products in order to confirm that the band was the expected sequence. A172 human glioblastoma cells were used as a positive control because, like many brain cells, it expresses all 3 RyR isoforms (48, 58). RyR1 is only expressed in DU-145 and PWR-1E cells but not at all in LNCaP cells (Fig. 1.1). RyR 2 is expressed in all three cell lines (Fig. 1.1). RyR3 is not expressed in any of the cells lines (Fig.1.1).

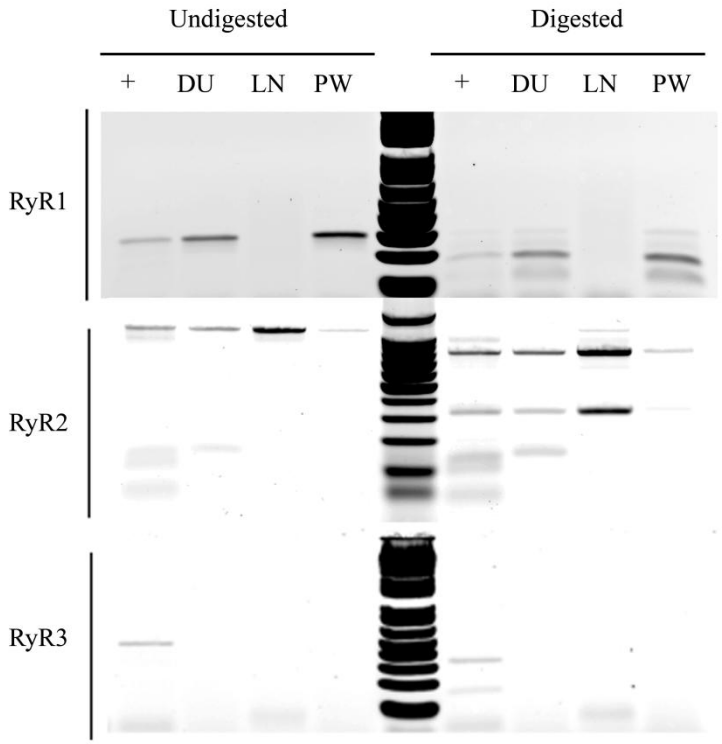

Figure 1.1. RyR isoform identification in DU-145, LNCaP, and PWR-1E cells. The positive control (+) cell line is A172, a glioblastoma cell line. RyR1 (290 base pairs (bp) undigested; 171 and 119 bp digested) is expressed in DU-145 (DU) and PWR-1E (PW) cells (top). RyR2 (1082 bp undigested; 763, 319 bp digested) is expressed in DU-145, LNCaP (LN), and PWR-1E cells (middle). RyR3 (477 bp undigested; 323 and 154 bp digested) is not expressed in any of the examined cell lines (bottom). Images are representative of three individual experiments.

Relative RyR expression

We performed RT-PCR in order to determine the relative expression levels of each RyR isoform. We used SybrGreen because pre-designed assays gave inconsistent results and the SybrGreen method allowed us to design our own primers. DU-145 cells were chosen arbitrarily as the "control" cell line to compare the other lines to. RT-PCR results confirmed that only DU-145 and PWR-1E cells express RyR1 (Fig. 1.2A) and all three cell lines express RyR2 (Fig. 1.2B). RT-PCR was not performed on RyR3 since semi-quantitative-PCR showed that none of the cell lines express this isoform (Fig. 1.1). PWR-1E cells expressed approximately 0.48 the level as DU-145 cells (Fig. 1.2A). LNCaP and PWR-1E cells expressed approximately 26 and 15 times the amount of RyR2 than DU-145 cells, respectively (Fig. 1.2B). It is clear from these results, as

well as the isoform identification, that tumorigenicity was not correlated with RyR isoform in our prostate cells.

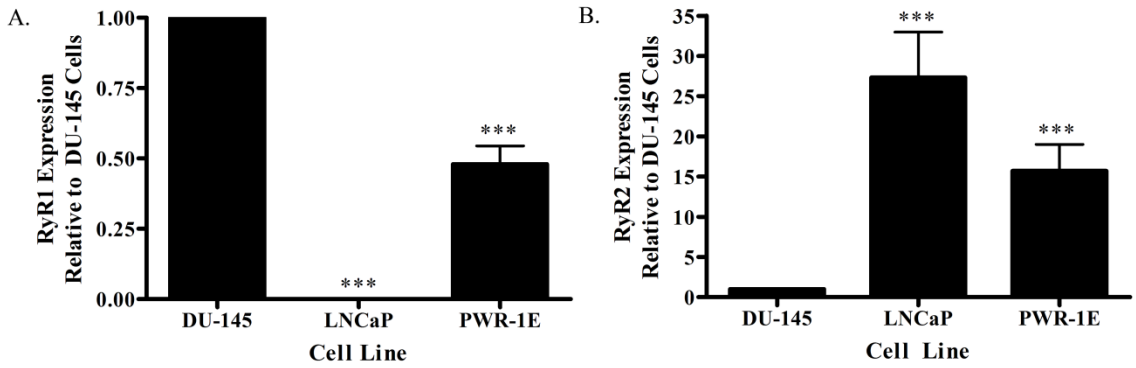

Figure 1.2. Relative expression of RyR isoforms in DU-145, LNCaP, and PWR-1E cells. RT-PCR was performed on cDNA created from all 3 cell lines. LNCaP and PWR-1E cells were compared to DU-145. DU-145 was arbitrarily chosen as the control line. A) RT-PCR confirmed the absence of RyR1 in LNCaP cells. PWR-1E expression was approximately 0.48 that of DU-145. B) Expression of RyR2 in LNCaP and PWR-1E cells was approximately 26 and 15 times that of DU-145 cells, respectively. Analysis was performed on 6 biological replicates per cell line. RT-PCR was not performed on RyR3 as semi-quantitative PCR revealed the absence of expression in all 3 cell lines.

Ca^{2+} Release from RyRs

In order to determine if DU-145, LNCaP, and PWR-1E cells translate RyRs into functional proteins, we used caffeine to stimulate Ca^{2+} release from the RyR-sensitive ER Ca^{2+} stores ($[Ca^{2+}]_{ER}$). Caffeine stimulated significant $[Ca^{2+}]_{ER}$ reduction in DU-145 (Fig. 1.3A), LNCaP (Fig. 1.3B), and PWR-1E (Fig. 1.3C) cells, showing that RyR expression resulted in functional protein in all three cell lines.

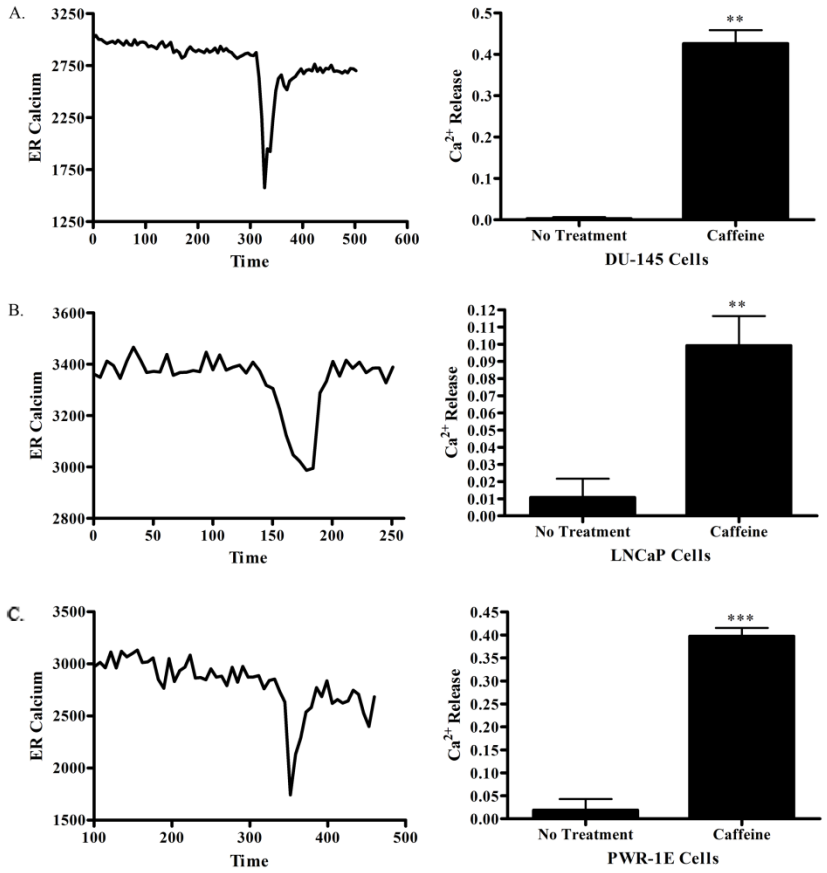

Figure 1.3. Ca^{2+} release from functional RyRs in DU-145, LNCaP, and PWR-1E cells. Caffeine stimulated ER Ca^{2+} release from RyRs in (A) DU-145, (B) LNCaP, and (C) PWR-1E cells. Graphs are representative of 3 experiments.

FKBP12 is necessary for BA-induced inhibition of Ca^{2+} release

FKBP12 is an accessory protein that stabilizes the RyR in the closed position. The FKBP12/RyR interaction is not completely understood, but upon FKBP12 release, the RyR channel is activated to a state of probable Ca^{2+} release (42). Rapamycin is a drug that dissociates FKBP12 from the RyR, causing a release of Ca^{2+} from ER stores into the cytoplasm in the presence of an agonist (42). BA has been shown to inhibit agonist-induced Ca^{2+} release from the RyR which was most pronounced in DU-145 cells (32). In order to determine if the presence of

FKBP12 is necessary for BA to inhibit Ca^{2+} release from cellular stores in the presence of an agonist, we used confocal microscopy to measure Ca^{2+} release in cells treated with the known RyR agonist, caffeine, and caffeine plus BA. As expected, BA inhibited Ca^{2+} release from the RyR in DU-145 cells (Fig. 1.4A). Interestingly, pre-treatment with rapamycin not only prevented BA-induced inhibition, but caused a significant increase in Ca^{2+} release (Fig. 1.4B).

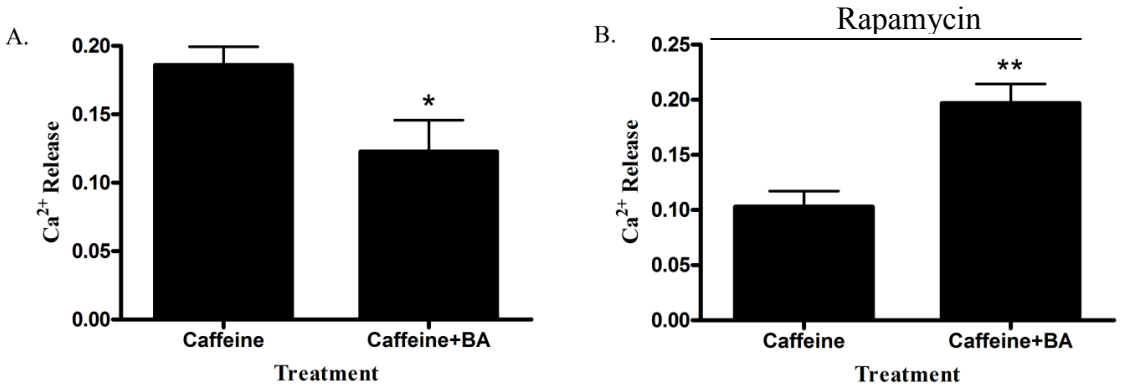

Figure 1.4. Rapamycin rescues BA-induced Ca^{2+} release inhibition from RyRs in DU-145 cells. (A) DU-145 cells were treated with 20 mM caffeine followed by treatment with 20 mM caffeine plus 50 µM BA. (B) DU-145 cells treated continuously with 100 nM rapamycin while given the same caffeine and BA treatment as (A). Analysis calculated with n=5.

Discussion

The differences in the expression of RyR isoforms was most pronounced between tumor cell lines, DU-145 and LNCaP (Fig. 1.1 & 1.2). The dominant isoform in DU-145 was RyR1 whereas RyR2 was most highly expressed in LNCaP. PWR-1E expressed both RyR1 and RyR2. RyR3 was not expressed in any of the cell lines. All three cell lines express functional RyRs as seen by caffeine-induced Ca^{2+} release (Fig. 1.3). RyR1 has been identified in MCF-7 breast cancer carcinoma cells (59) and prostate cancer LNCaP cells (60), but this is the first time it has been shown in prostate DU-145 cancer cells and normal PWR-1E cells. RyR2 has previously

been reported in HeLa (48, 58) and melanoma cells (61). Our results in LNCaP cells differ from Mariot and colleagues who reported finding RyR1 and RyR2 in LNCaP cells (60). The difference between our results may be explained by the use of different oligoprimers. We used different forward and reverse primers to the transmembrane region of the receptor that were specific to the individual isoforms whereas they used the same reverse primer for all 3 isoforms. Their set of primers for RyR2 was also specific for RyR3. In addition, we used a positive control which was not mentioned in the Mariot paper. Differences in RyR isoform expression have also been reported in HeLa cells due to the use of different nucleotide sequences (48, 58). It is also possible that our laboratories used a different LNCaP cell line, as LNCaP has many sublines (56).

In tumor cells there is a shift from low to high and sustained global cellular Ca^{2+} concentrations compared to normal cells (54). Both the IP3R and RyR control spatially localized Ca^{2+} but Westcott has reported that only the RyRs control global Ca^{2+}concentrations (55). Inhibition of DU-145 and LNCaP cell proliferation has been reported to be associated with an inhibition of RyR Ca^{2+} release (32). The chemopreventative agent boric acid inhibits RyR Ca^{2+} release in DU-145 and LNCaP cells and slows proliferation within a concentration range that can be achieved by a boron rich diet. However, it requires a 4-fold higher concentration to inhibit the proliferation and 15-fold higher concentration to inhibit RyR Ca^{2+} release in non-tumorigenic PWR-1E cells (10, 32). When DU-145 cells are treated with rapamycin, a FKBP12-binding drug, BA's inhibitory effects were not only eliminated, but Ca^{2+} release significantly increased (Fig. 1.4). Caffeine and BA are both compounds that deplete the ER of Ca^{2+} (32, 62). When FKBP12 is available, BA acts as an RyR antagonist in the presence of caffeine (32). However,

when FKBP12 is removed, these Ca^{2+} depleting compounds have an additive effect, showing that FKBP12 is a necessary component of BA's antagonistic properties. We hypothesized that the difference in BA sensitivity between the tumor cell lines and a normal prostate cell line was due to the expression of different RyR isoforms or to differences in the response of one of the accessory proteins that modulate the activity of the receptor. The results of the present study rule out the first possibility since the major difference in RyR1 and RyR2 expression occurred between tumor cell lines, whereas PWR-1E cells, the non-tumorigenic and least sensitive to BA, expressed both RyR 1 and RyR2. The second hypothesis now seems the most likely to explain the differences in BA response. The RyR is a macromolecular complex that serves as a scaffold for proteins that modulate Ca^{2+} channel function (50, 63) and offer attractive targets that may explain BA's unique chemopreventive effect.

Chapter 2: Boric Acid induces ER stress and the eIF2α/ATF4 and ATF6 branches of the unfolded protein response in DU-145 cells[2]

Abstract

Boric acid (BA) is a ubiquitous dietary component that we and others have reported reduces the incidence and mortality of prostate and lung cancer in a dose-dependent manner. In cultured cells BA, at concentrations achievable in the blood by diet, inhibit proliferation of DU-145 prostate cancer cells. Physiological levels of BA (1 to 50 μM) inhibit release of calcium (Ca^{2+}) from ryanodine receptor (RyR) channels on endoplasmic reticulum (ER) stores in response to cADPR, the only endogenous agonist of the RyR, and by the pharmaceutical agonists, caffeine and 4-chloro-m-cresol. BA inhibition occurs within seconds and is followed by a 32% reduction in ER Ca^{2+} concentrations ($[Ca^{2+}]_{ER}$). The ER is the site of synthesis of secretory proteins and high Ca^{2+} concentrations are required for proper protein folding. Low $[Ca^{2+}]_{ER}$ leads to an accumulation of unfolded and misfolded proteins resulting in ER stress. This activates the unfolded protein response (UPR), a cellular coping mechanism that activates pathways that reduce overall protein load until the stress is removed or, if too severe, apoptosis. The objective of the current study is to identify if physiological levels of BA induce ER stress and the UPR in DU-145 cells. This response may be the key to understanding the mechanism by which BA slows the proliferation of these cells and BA's chemopreventative nature. Transmission electron microscopy of DU-145 cells treated with BA indicates the presence of ER stress with a dose-dependent increase in ER swelling and vacuolization. The appearance of stress granules as indicated by the accumulation of TIA-1 foci in the cytoplasm of BA-treated DU-145 cells is further evidence that ER stress is occurring. The UPR consists of three branches named for the

[2] Chapter 2 is currently in submission to the Journal of Biological Chemistry and is undergoing its first set of revisions.

transmembrane proteins that initially trigger each pathway: PERK, ATF6, and IRE1. Our analysis of the three UPR branches demonstrated that physiological doses of BA decreased protein synthesis, activated the eIF2α/ATF4 and ATF6 branches of the UPR but did not activate the IRE1 branch. UPR gene and protein markers were significantly induced by BA treatment. Grp78, or BiP, protein was significantly upregulated and multiple genes, including calreticulin, HERP, and EDEM1, which are controlled by the ER stress response element I (ERSEI) and II (ERSEII) and the UPR response element (UPRE), respectively, were significantly upregulated by BA treatment. CHOP, a pro-apoptotic protein commonly increased during UPR, decreased with BA treatment. However, this is not surprising given that DU-145 cells slow proliferation when treated with BA but do not undergo apoptosis. The formation of stress granules, which has been correlated with cell survival, in BA-treated DU-145 cells may be the reason we do not see apoptosis. Overall, our data indicate that physiological doses of BA induced ER stress, reduced global protein synthesis, and induced the eIF2α/ATF4 and ATF6 branches of the UPR in DU-145 prostate cancer cells.

Introduction

Borates, the natural form of boron, are found in high concentrations in the earth's soil and ocean water (64). Boron's role in biological systems has been one of the most difficult of the elements to study. It does not have a stable radioisotope, is not removed from water using common laboratory ultra-purification systems, is added to many reagents as a surfactant and buffering agent, and is poorly controlled in laboratory animal diets. It has taken several decades to uncover boron's role as a nutrient in both plants and animals. It was first shown to be essential for plant growth in 1923, but it was not until 1996 that a molecular role was identified (65).

Borate esters were shown to link polysaccharide chains of rhamnogalactanuran II, thereby reinforcing cell walls against the extreme hydrostatic pressures incurred during cell elongation (66). In subsequent years it was shown to be required for optimum embryonic growth in rainbow trout, initial stages of cleavage of the zebrafish zygote, and normal development of *Xenopus laevis* embryos (24, 25, 67). Human studies conducted over several months identified deficits in cognitive function as the primary symptom of boron deficiency (68). These studies, together with numerous animal studies, have identified the endocrine, immune, nervous, and skeletal systems as responsive to boron intake (68-71).

We turned to the use of epidemiology as a screening tool to determine if boron intake was associated with a human disease that could be used as an endpoint for probing its mechanism of action (8). Screenings found that boron intake was inversely associated with the risk of prostate cancer and other studies showed it reduces the risk of lung cancer in a dose-dependent manner (8, 72). Importantly, the protective effect remained when the source of exposure was water, thus eliminating the possibility of dietary confounders (7).

Studies in prostate cancer models showed that BA reduced growth rates of human prostate tumor implants in nude mice and cell proliferation in cultured prostate cancer cells in a dose-dependent manner without inducing apoptosis (10, 13). These studies, along with the epidemiology data, led us to explore BA's chemopreventative role in prostate cancer. And understanding how BA reduced cancer risk required learning more about its molecular partners.

Spectroscopy studies conducted in the 1970s showed that borates interacted with NAD^+ but at 1000 times physiological concentrations (21). Using this as a starting point we used mass spectrometry to determine that two borate molecules bind to the ribose moiety of NAD^+ and the binding affinity was greatly reduced by phosphorylation and reduction in charge (20, 21). In addition to its role as a coenzyme in intermediary metabolism, cells use NAD^+ as a paracrine signaling molecule. It is released into the extracellular environment and binds to the extracellular domain of CD38, a multifunctional enzyme that converts it into cADPR and releases it into the cytoplasm (29). There, cADPR acts as an endogenous agonist to the ER's RyR, a high-conductance Ca^{2+} channel that is a key component of the cell's ability to maintain Ca^{2+}-dependent processes, such as the creation of proteins and cell proliferation (33, 34). We showed BA bound to and was a reversible competitive inhibitor of cADPR (32, 73). This response occurred within seconds in a live cell and was followed by a 32% decrease in $[Ca^{+2}]_{ER}$ (32).

In a healthy cell, $[Ca^{+2}]_{ER}$ is much higher than cytosolic Ca^{+2} concentrations (100-500 µM verses 20-100 nM, respectively), allowing for fast and precise cellular signaling (74, 75). High $[Ca^{+2}]_{ER}$ is required for the proper folding of proteins. A disruption in $[Ca^{+2}]_{ER}$ can lead to an accumulation of misfolded and unfolded proteins, which results in ER stress (76). The cell is capable of both sensing and responding to ER stress brought on by low $[Ca^{+2}]_{ER}$ and the subsequent protein overload in the ER by activating the UPR (77). The UPR decreases global protein translation while increasing the transcription and translation of chaperones and other proteins which function to adapt to and ameliorate the stress (76). If successful, cells remain viable, but if the stress is insurmountable, apoptosis is initiated through CHOP (78).

BiP, also known as Grp78, is a major ER Ca^{2+} binding protein contributing to 25% of stores (79). BiP is a molecular chaperone and binds to three ER transmembrane proteins, PERK (PKR-like ER kinase), ATF6 (activating transcription factor 6), and IRE1 (inositol requiring enzyme 1), all of which are key proteins in UPR signaling. However, BiP binds to the hydrophobic region of misfolded and unfolded proteins with a higher affinity than to these ER proteins. When there is an accumulation of improperly folded proteins in the ER, BiP leaves PERK, ATF6, and/or IRE1 to aid in the folding process, thus relieving the stress on the cell (80). BiP leaving one or more of the three ER transmembrane proteins signals one, two, or all three branches of the UPR (76, 80).

IRE1 is a kinase and endoribonuclease that cleaves a 26 nucleotide intron from X-box binding protein 1 (XBP1) mRNA. The resulting spliced mRNA translates a protein (XBP1p) that acts as a potent transcription factor which upregulates genes involved in protein folding, ER-associated degradation (ERAD), and lipid synthesis (81, 82). It is the only UPR pathway that is conserved among higher eukaryotes (80).

When ATF6 α or β is activated it translocates to the Golgi where it is cleaved from a 90 kDa protein to a 50 kD fragment. The 50 kD fragment translocates to the nucleus where it acts as a transcription factor for a number of UPR-related genes, including XBP1 (83).

Different kinases can phosphorylate eIF2α but only PERK is induced via ER stress (76). PERK phosphorylates the α subunit of the eukaryotic translation initiation factor 2 (eIF2α) at serine 51 resulting in inhibition of cap mediated protein translation, thereby reducing the ER protein load

(84). A decrease in global protein translation is a direct result of eIF2α phosphorylation (80). During translation, polysomes assemble to create protein from mRNA. Polysomes are composed of 40S, 60S, and 80S monosomal subunits (85). When global translation is halted, polysomes disassemble and there is an increase in the monosome/polysome ratio (86). Phosphorylation of eIF2α also initiates the selective translation of ATF4 via an upstream open reading frame (uORF) (87). ATF4 induces transcription of several important UPR-related genes, including GADD34, a phosphatase that removes the phosphate from eIF2α, thus creating a negative-feedback-loop (76, 80).

UPR can activate all three transducers or differentially activate one depending on the stressor and cell type (88-90). The objective of the present study was to determine if BA induces ER stress and/or the UPR in DU-145 prostate cancer cells at concentrations that occur in human blood from diet.

Materials and methods

Chemicals

BA, thapsigargin, Tris, NaCl, $MgCl_2$, sucrose, DTT, methanol, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Gluteraldehyde, cacodylic acid, lead citrate, and uranyl acetate were purchased from Electron Microscopy Supplies (Hatfield, PA). Paraformaldehyde was purchased from Affymetrix/USB Corporation (Cleveland, OH). TritonX-100, Tween-20, NP40, and cyclohexamide were purchased from Fisher Scientific, Pittsburg, PA). BSA was purchased from Santa Cruz biotechnologies (Santa Cruz, CA). FBS was purchased from Gibco-Life

Sciences (Grand Island, NY). Protease and phosphatase inhibitors were purchased from Calbiochem (San Diego, CA)

Cell culture

DU-145 prostate cancer cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were maintained in RPMI Media 1640 (Gibco-Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (200 mM) (Gemini Bio-Products, Sacramento, CA). Cells were plated on 10 cm or 15 cm plates (Corning Life Sciences, Corning, NY) incubated at 37ºC in a humidified chamber containing 5% CO_2 and 95% air and grown to 80% confluency. All treatment groups used media that had been stripped of boron by shaking 2 grams of Amberlite IRA 743 exchange resin (Sigma-Aldrich) for 12 hours at 4ºC.

Transmission Electron Microscopy (TEM)

TEM was performed as previously described by Henderson *et. al.* (31). The following is a modified version of that protocol: Cells were grown on plastic cover slips (Nalgene, Rochester, NY) to no more than 80% confluency and treated with BA-supplemented complete media at concentrations of 0, 10, 50, and 250 µM for 24 hours, followed by fixation with 2% gluteraldehyde in 0.1 M sodium cacodylate buffer. The samples were then dehydrated with increasing concentrations of ethanol followed by infiltration and embedding in RL White acrylic resin (Ted Pella, Redding, CA). The resin embedded cells were sectioned in 100 nm thick slices and placed on copper grids. The sections were counter stained with 2% aqueous uranyl acetate

at 57ºC for 1 hour followed by 4% lead citrate staining for 1 minute. Imaging by TEM was performed using a JEOL 100CX transmission electron microscope.

Immunoblot analysis (Western)

DU-145 cells grown on 15 cm plates (Corning) to 80% confluency were treated with 10 µM BA for varying time points. Cells were washed with ice cold PBS supplemented with 0.1% Tween (PBST) prior to adding 100 µl RIPA lysis buffer. Cell lysates were scraped from plates using a spatula (Corning) and passed through a 23 gauge needle (BD, Franklin Lakes, NJ) 8-10 times on ice. The protein was quantitated using the Bradford Assay (Thermo-Scientific,Waltham, MA). 30-35 µg of protein were run on a 4-12% gradient SDS-polyacrylamide gel (Bio-Rad, Hercules, CA) at 200V for 30 minutes along with a molecular weight ladder (Bio-Rad). Protein was transferred to a nitrocellulose membrane in transfer buffer with 20% methanol at 40V for 1.5 hours. The membranes were then blocked in 3% BSA with Tris (pH 8.8), 5M NaCl, and 0.1% Tween 20 for at least 4 hours. Following blocking, the membranes were incubated with the primary antibody for 1 hour in PBST, washed in PBST, and incubated with the appropriate secondary antibody with a horse radish peroxidase (HRP) tag, followed by washing 3 times with 0.1% PBST. The membranes were exposed to ECL Plus (Amersham/GE Healthcare, Pittsburg, PA) for 2-5 minutes and imaged using a Typhoon 9410 Variable Mode Imager (Amersham). Densitometry was performed using ImageQuant 5.2 software (Molecular Dynamics, Pittsburg, PA). All secondary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). We used the following primary antibodies from Santa Cruz Biotechnology: BiP, Actin, GAPDH, ATF4, GADD34. Total eIF2α and ph-eIF2α antibodies were purchased from Cell Signaling (Danvers, MA).

Polysome profile

Prior to harvesting, cells were incubated in culture media containing 50 µg/ml cycloheximide for 10 minutes at 37°C. Plates were transferred to an ice tray, the media removed, and cells were rinsed 2-3 times with ice cold PBS containing 50µg/ml cycloheximide. The cells were lysed in 500 µl lysis buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 0.4% NP-40, 50 µg/ml cycloheximide, and protease and phosphatase inhibitors). The lysate was scraped with a spatula (Corning) and transferred to a microcentrifuge tube. The lysate was passed through a sterile 23 gauge needle (BD) 8-10 times and incubated on ice for 10 minutes. The lysate was spun at 8000 x g for 10 minutes. The supernatant was used for the polysome profile. 10 µl of lysate was used for an OD measurement at 260 nm. 10 OD of lysate was used for the profile. A wide needle was used to fill the SW41 centrifuge tube approximately halfway with 10% gradient solution (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/mL cycloheximide, and 10% sucrose (w/v)). The remainder of the tube (minus a portion at top for lysate) was filled with 50% gradient solution (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml cycloheximide, and 50% (v/w) sucrose) by inserting the needle to the bottom of the tube and gently expunging the contents. A gradient maker was used to create a 10-50% gradient. 10 OD (up to 800 µl) of lysate was gently added to the top of the gradient. The tubes were carefully balanced before centrifugation. The tubes were gently placed in a pre-cooled SW41 rotor (Beckman Coulter, Brea, CA) and spun for 3 hours at 35,000 rpm. When the centrifuge was done, we gently removed the tubes and poked a small hole with a needle in the bottom of the tube. 50 fractions were collected from the tube and immediately placed on ice. The absorbance of each fraction was measured at 254 nm.

Taqman real time PCR (RT-PCR)

DU-145 cells were grown on 10 cm plates (Corning) to 80% confluency at least 24 hours prior to treatment. Cells were treated with BA-supplemented RPMI 1640 media (Gibco Life Sciences) for varying time points. RNA was isolated from cells using an RNeasy mini kit (Qiagen, Valencia, CA). Total RNA (2 µg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) with random hexamer primers (Invitrogen) at a final volume of 20 µl at 25°C, 10 minutes (10:00); 50°C, 45:00; and 70 °C, 15:00. Applied Biosystems (ABI, Foster City, CA) Taqman predesigned assays were used for all genes as well as GAPDH (internal housekeeping gene). Plates were read by a 7500 Fast Real Time PCR System using the 7500 Fast System Software v1.4.0 (ABI). Quantitation of gene expression level was calculated from a standard curve created from reactions containing a combination of cDNA from all treatments for each gene.

Immunofluorescent microscopy

DU-145 cells were grown to 70-80% confluency on glass coverslips and treated with either BA-free media, 10 µM BA, or 1 µM thapsigargin. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% TritonX-100 in PBS. Fixed cells were blocked with 10% FBS in PBS overnight. Next, they were incubated in a humidity chamber with anti-ATF6 (Imgenex, San Diego, CA) or anti-TIA-1 (Santa Cruz Biotechnology) monoclonal antibody at concentrations of 1:200 and 1:50, respectively, followed by secondary Alexa 488 or FITC at 1:500 and 1:100, respectively. Coverslips were mounted with a mixture of Vectashield with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and regular HardSet Vectashield (Vector Laboratories) mounting mediums at 1:5 respectively. Images were captured

with an Olympus DP72 camera (Olympus America, Center Valley, PA) connected to an Olympus BX51 fluorescence microscope (Olympus America) using an Olympus UIS2 UPlanFLN 100X/1.30 OilPh3 objective (Olympus America) and FITC and DAPI filters. Either Olympus DP2-BSW (Olympus America) or Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA) software was used to merge and crop images. All treatments were done in triplicate.

XBP1 Cleavage Analysis

Total RNA was extracted from BA (0-250 μM) or thapsigargin (10 μM) treated DU-145 cells using RNeasy Mini Kit (Qiagen). RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). XBP1 cDNA was amplified with GoTaq Flexi DNA Polymerase (Promega, Madison WI) using the forward primer 5'CACCTGAGCCCCGAGGAG3' and reverse primer 5'TTAGTTCATTAATGGCTTCCAGC3'. 50 μl PCR reactions were run under the following amplification conditions: initial denaturation step of 95°C for 2 minutes; 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, all for 25 cycles; 72°C for 5 minutes (91). PCR products were run on 2% agarose E-gels with SybrSafe (Invitrogen).

Statistical Analysis

All immunoblot and RT-PCR data was analyzed using the unpaired Student's t-test. All timepoints were analyzed using 3-6 replicates. The polysome profile was analyzed by adding the trapezoidal area under the curve method. The unpaired Student's t-test was using to analyze the monosome/polysome ratios.

Results

BA causes ER ultra-structural changes in DU-145 cells

Transmission electron images of DU-145 cells treated with varying doses of BA for 24 hours show dose-dependent ultra-structural changes. The ER became increasingly swollen and vacuolized as BA treatment was increased from 0-250 µM (Fig. 2.1) (31).

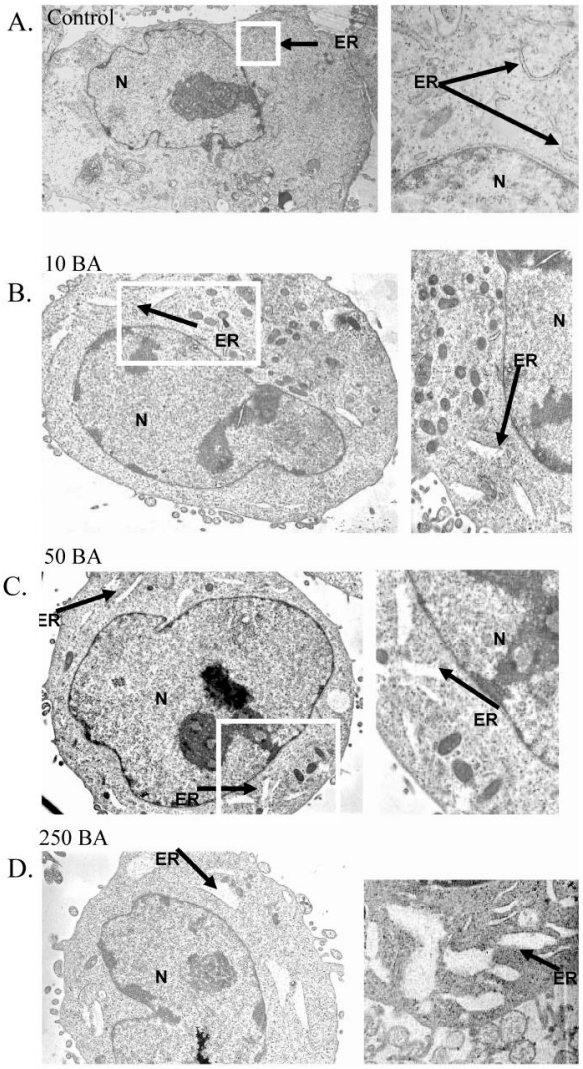

Figure 2.1. Transmission electron images of DU-145 cells treated for 24 hours with (A) 0 µM BA (B) 10 µM BA (C) 50 µM BA, and (D) 250 µM BA. Cells treated with 10-250 µM BA for 24 hours exhibit swollen and vacuolized ER. N=nucleus, ER =endoplasmic reticulum. White boxes indicate areas of the cell that have been expanded (31).

BA increases BiP (Grp78) translation

BiP (Grp78) dissociation from the 3 transmembrane proteins, PERK, ATF6, and IRE1, and its interaction with unfolded and misfolded proteins is the first signal that begins the UPR cascade. A hallmark marker of the UPR is an increase in BiP translation (92). BiP translation is increased significantly at 0.5, 1, 2, 3, and 4 hours of treatment with 10 µM BA (Fig. 2.2A). As a positive control, DU-145 cells were treated with 1 µM thapsigargin in DMSO. Thapsigarin decreases $[Ca^{2+}]_{ER}$ and induces UPR by

inhibiting SERCA, an ATPase that pumps Ca^{2+} into the ER (93). Thapsigargin significantly induced BiP translation (Fig. 2.2B).

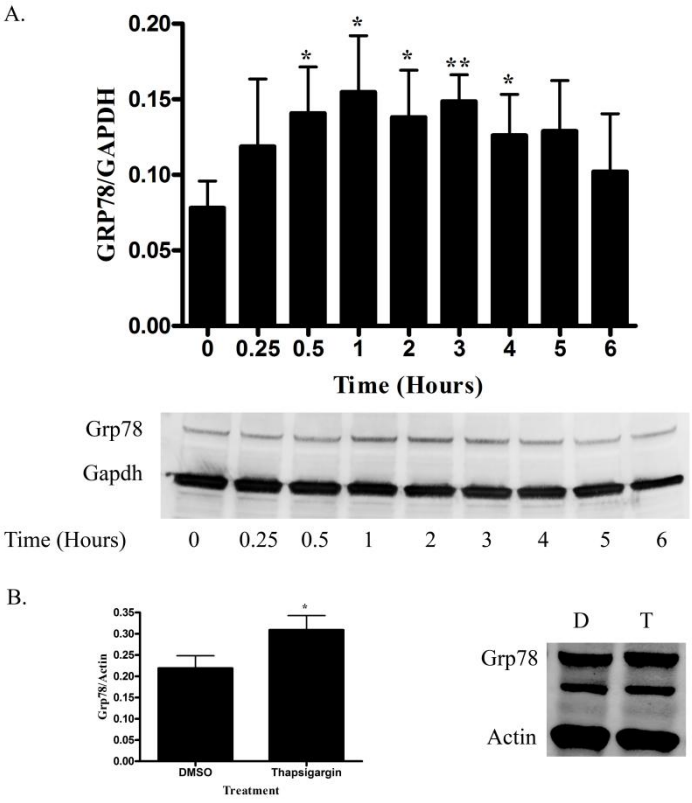

Figure 2.2. Western blot of BiP (Grp78) in DU-145 cells. (A) DU-145 cells treated with 10 µM BA for 0, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hours. Translation increased in cells treated for 0.5, 1, 2, 3, and 4 hours. Timepoints represent n=4. (B) DU-145 cells treated with 1 µM thapsigargin (T) or DMSO (D) (vehicle) for 1 hour. Treatments represent a positive control with n=3.

BA induces phosphorylation of eIF2α

In order to determine if BA induces the eIF2α/ATF4 branch of the UPR, our first step was to look at the phosphorylation of eIF2α. Although PERK phosphorylation is the most upstream event in this pathway, its analysis is often skipped as westerns on phosphorylated PERK can be

difficult and inconsistent. In DU-145 cells treated with 10 μM BA over 6 hours, phosphorylation of eIF2α at serine 51 increased at 0.5, 1, and 2 hours of treatment (Fig. 2.3A). As a positive control, DU-145 cells were treated with 1 μM thapsigargin for 1 hour. Thapsigargin significantly induced the phosphorylation of eIF2α (Fig. 2.3B).

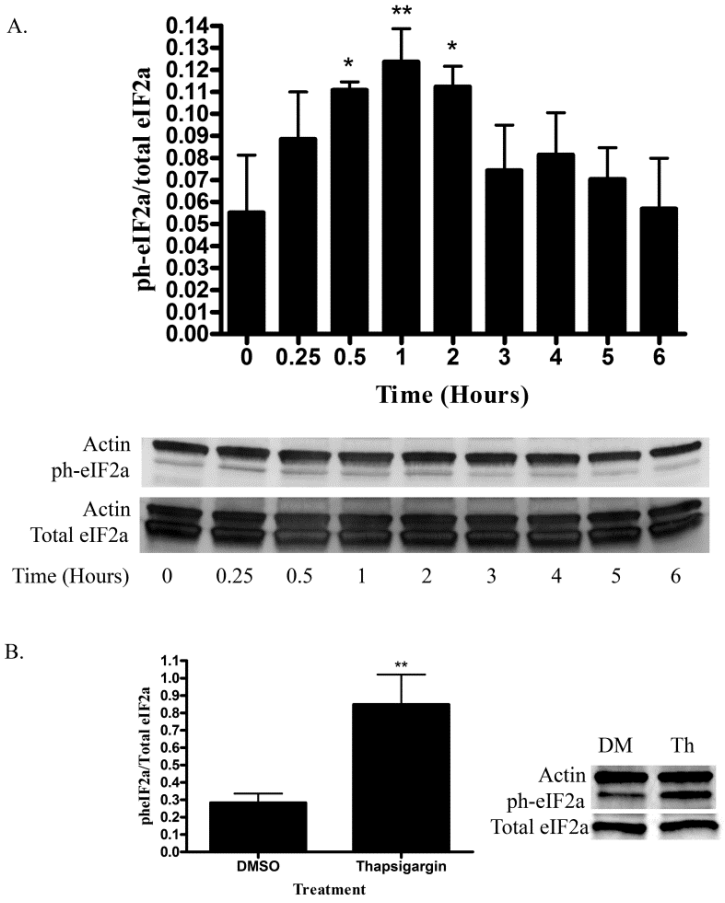

Figure 2.3. Western blot of phosphorylated-eIF2α in DU-145 cells. (A) DU-145 cells treated with 10 μM BA for 0, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hours. BA induced phosphorylation of eIF2α in DU-145 cells at 0.5, 1, and 2 hours of treatment. Phosphorylation levels were determined by the following formula: (ph-eIF2α/actin)/(total eIF2α/actin). Timepoints represent n=3-5. (B) DU-145 cells treated with 1 μM thapsigargin (T) or DMSO (D) for 1 hour. Treatments represent a positive control with n=3.

BA causes a decrease in global protein translation

In order to analyze if protein translation is being affected by BA, we performed a polysome profile, specifically looking at the monosome/polysome ratio in BA treated DU-145 cells. The monosome/polysome ratio doubles in cells treated with 10 μM BA for 2 hours compared to

untreated cells (Fig. 2.4). This indicates that BA causes a decrease in the universal translation in DU-145 cells.

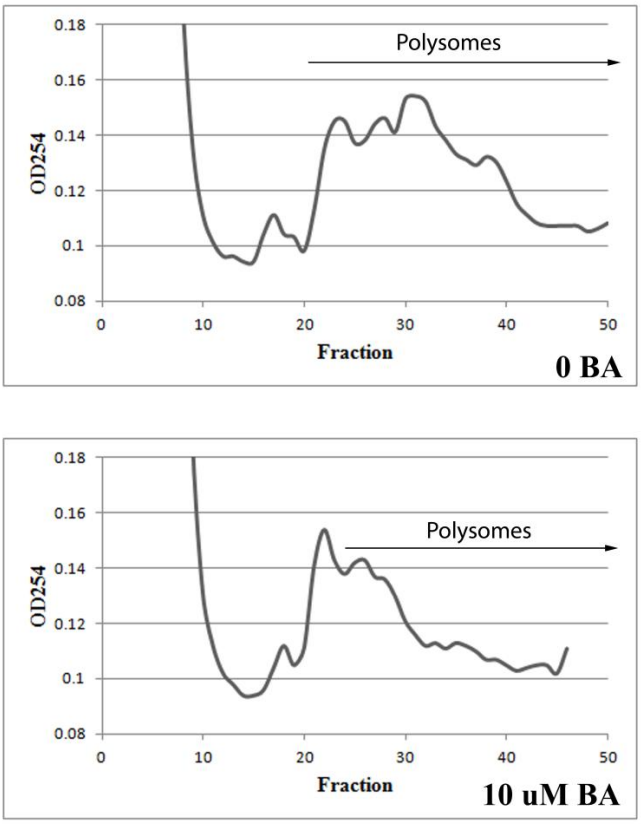

Figure 2.4. Polysome profile of DU-145 cells. DU-145 cells treated with 10 µM BA for 2 hours had a significantly higher monosome/polysome ratio than DU-145 cells treated with 0 µM BA for 2 hours. Analysis was performed on 3 replicates. Each replicate was an area under the curve analysis of 48-52 sucrose gradient fractions. Graphs of the fractions shift slightly based on the number of fractions collected.

BA induces ATF4 translation and transcription

ATF4 is a transcription factor selectively activated by phosphorylated eIF2α. An increase in ATF4 translation is an UPR hallmark. ATF4 translation is increased in DU-145 cells treated with 10 μM BA for 1, 2, and 3 hours (Fig. 2.5A). Interestingly, translation of ATF4 is significantly decreased at 4 and 5 hours of treatment (Fig. 2.5A). Again, as a positive control, DU-145 cells were treated with 1 μM thapsigargin for 1 hour. Thapsigargin significantly induced the translation of ATF4 (Fig. 2.5B) in DU-145 cells.

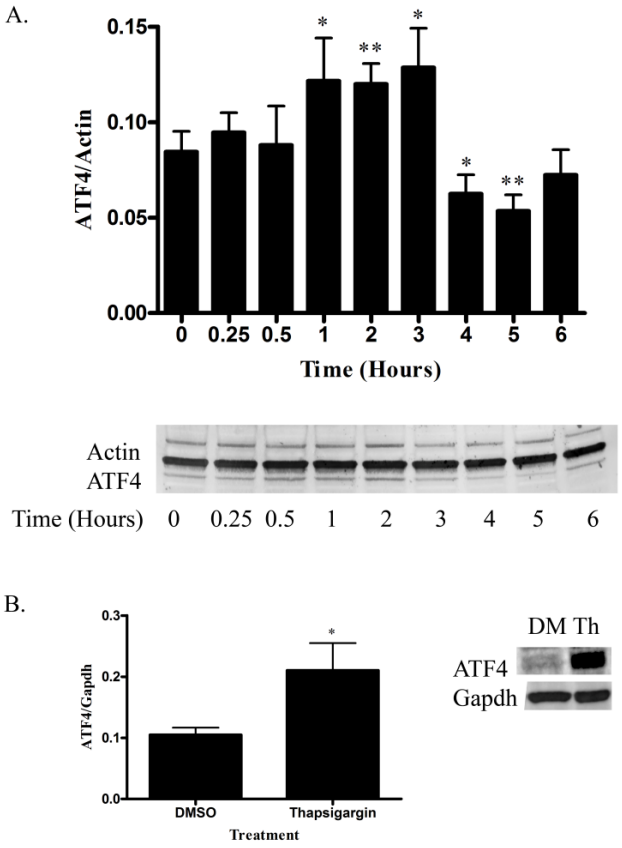

Figure 2.5. Western blot of ATF4 in DU-145 cells. (A) DU-145 cells treated with 10 μM BA for 0, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hours. ATF4 translation is increased in cells treated for 1, 2, and 3 hours. ATF4 protein is significantly decreased at 4 and 5 hours of treatment. Timepoints represent n=3-5. (B) DU-145 cells treated with 1 μM thapsigargin (T) or DMSO (D) for 1 hour. Treatments represent a positive control with n=3.

Although ER stress-induced activation of ATF4 usually occurs via an increase in translation, an increase in ATF4 transcription is also sometimes seen during ER stress and the UPR. We looked at ATF4 transcription using RT-PCR which showed that 10 µM BA induced ATF4 transcription in DU-145 cells treated for 0.5, 1, and 2 hours of treatment (Fig. 2.6).

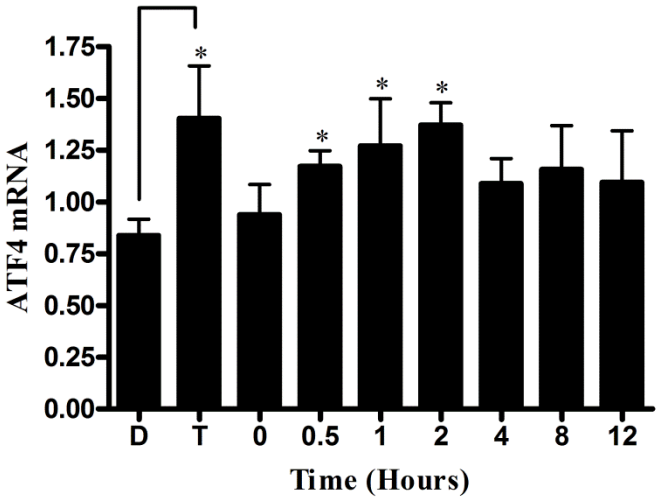

Figure 2.6. RT-PCR analysis of ATF4 transcription in BA-treated DU-145 cells. 10 µM BA induces ATF4 transcription at 0.5, 1, and 2 hours of treatment. 1 µM thapsigargin (T) and DMSO vehicle (D) was used as a positive control and significantly induced ATF4 transcription.

BA induces GADD34 translation

GADD34, a phosphatase which dephosphorylates eIF2α, is necessary for the negative feedback loop that exists in the eIF2α/ATF4 branch of the UPR. During ER stress and the UPR, GADD34 translation typically increases (80). In BA-treated DU-145 cells, GADD34 increased over 3 hours of 10 µM BA treatment but only significantly at 3 hours (Fig. 2.7A). The significant increase in GADD34 protein occurs concurrently with eIF2α dephosphorylation (Fig. 2.3). DU-145 cells treated with 1 µM thapsigargin significantly increased GADD34 translation (Fig. 2.7B).

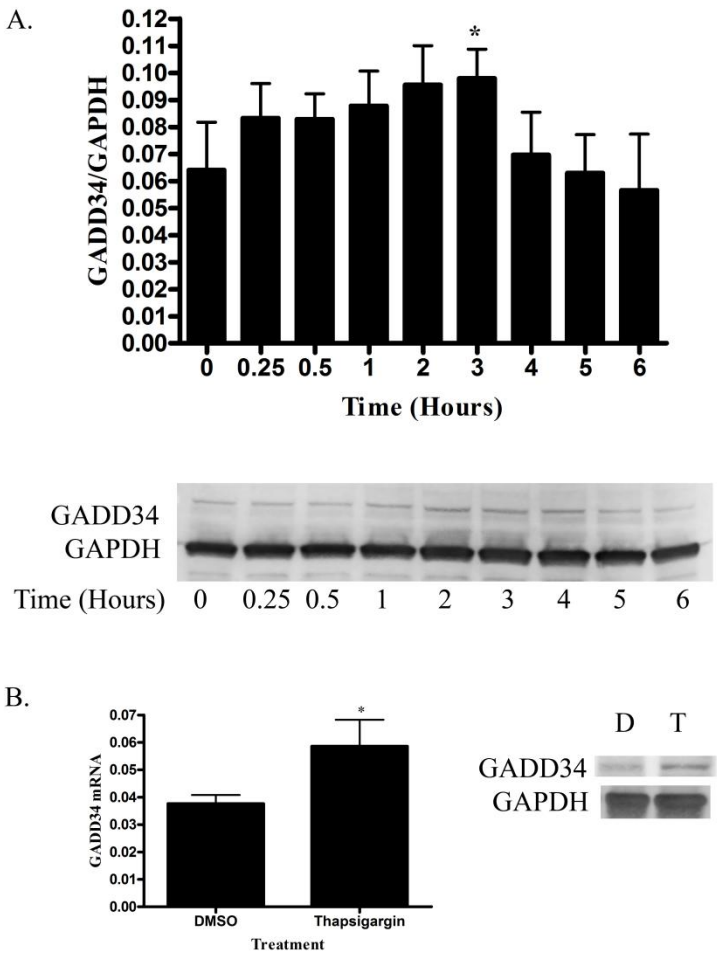

Figure 2.7. Western blot of GADD34 in DU-145 cells. (A) DU-145 cells treated with 10 µM BA for 0, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hours. GADD34 translation is increased in cells treated for 3 hours. Timepoints represent n=3-5. (B) DU-145 cells treated with 1 µM thapsigargin (T) or DMSO (D) for 1 hour. Treatments represent a positive control with n=3.

ATF4 target genes are induced by BA

ATF4's role in the UPR is to upregulate genes that assist in relieving the cell's protein overload (80). GADD34, CHOP(GADD153), and Herp are all ATF4-inducible genes. In order to determine if ATF4 is acting as a transcription factor, we used RT-PCR to analyze the transcription of these genes. 10 µM BA significantly increased the transcription of GADD34 at

0.5, 1, and 2 hours (Fig. 2.8A). Herp is an ERAD protein that is thought to recruit the 26S proteasome component to the ER membrane during ER stress and an ERSEII gene (80). 10 µM BA induced a significant increase in Herp transcription at 15 minutes and 4 hours of treatment (Fig. 2.8B). This is evidence that ATF4 is selectively translated and moves into the nucleus to act as a transcription factor. Though GADD153/CHOP, a pro-apoptotic protein, is commonly induced in cells undergoing ER stress and the UPR, we did not know how it would respond in our cells since DU-145 cells do not undergo apoptosis with BA treatment. Interestingly, GADD153/CHOP expression is intermittently decreased with 10 µM BA treatment (Fig. 2.8C).

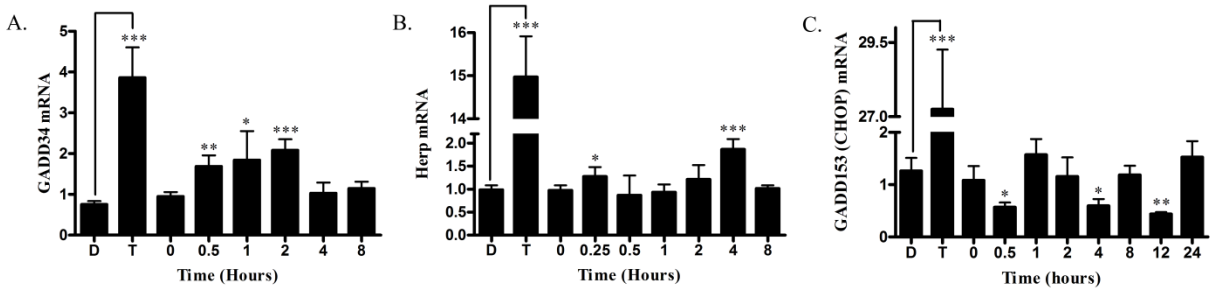

Figure 2.8. RT-PCR of ATF-4 inducible genes. 10 µM BA induced expression of (A) GADD34 at 0.5, 1, and 2 hours and (B) Herp at 0.25 and 4 hours of treatment. 10 µM BA down-regulates expression of GADD153 (CHOP) at 0.5, 4, and 12 hours of treatment. As a positive control 1 µM thapsigargin (T) up-regulated expression of all three genes compared to DMSO vehicle (D). All timepoints are representative of n=3-6.

BA induces the ATF6 branch of the UPR

ATF6 activation can be demonstrated through western immunoblots, however, this technique is known to be difficult and inconsistent. Immunofluorescent microscopy is a more reliable technique for ATF6 activation detection. If ATF6 is being activated, we should see the cleaved protein move from the cytoplasm to the nucleus of the cell. Using microscopy, we were able to detect the movement of ATF6 into the nucleus with 10 µM BA treatment (Fig. 2.9). This occurred starting at 0.5 hours of treatment and began to subside around 4 hours.

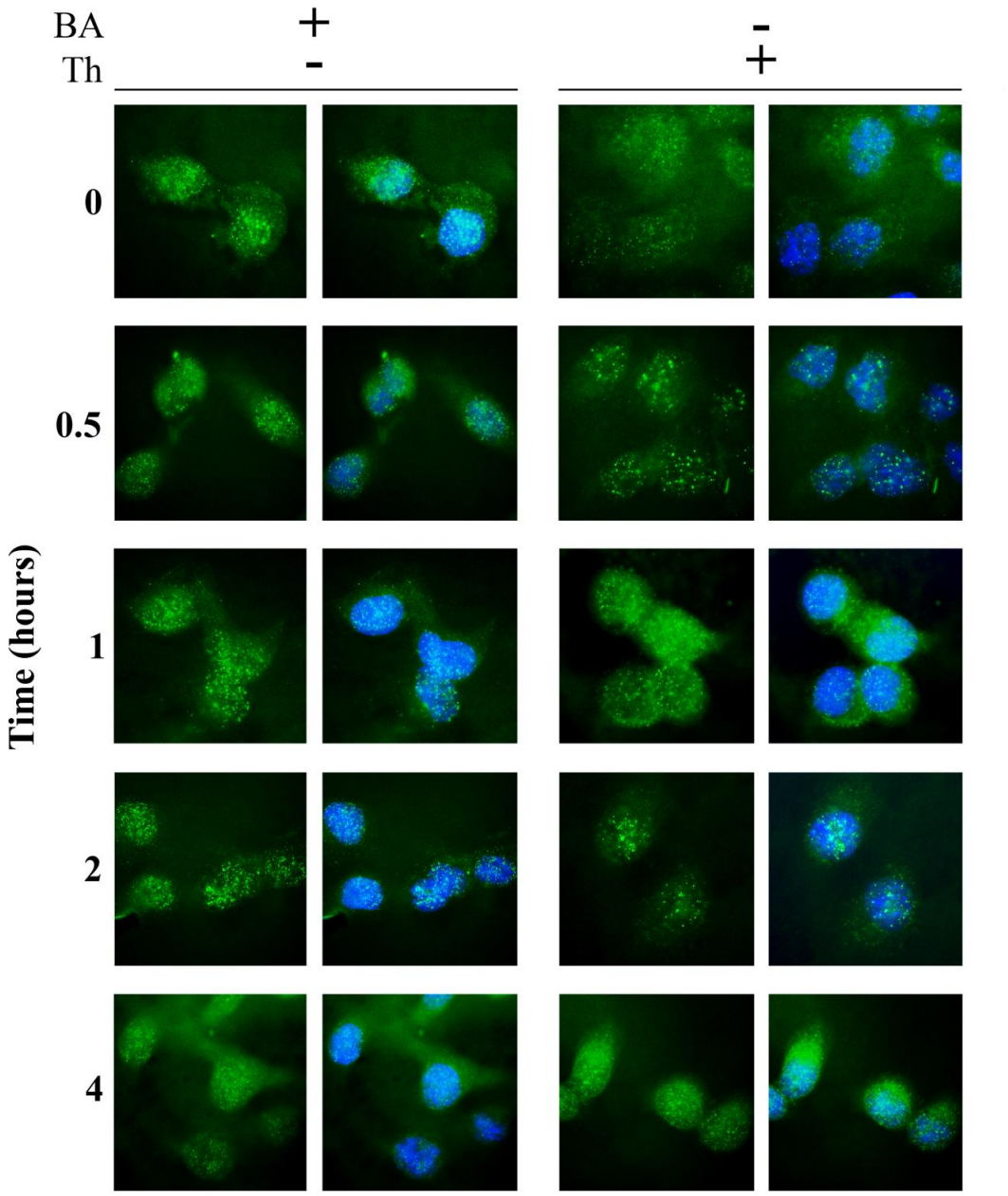

Figure 2.9. Immunofluorescent microscopy of ATF6 in DU-145 cells. 10 µM BA induced ATF6 (green) to cleave and move into the nucleus (blue) of DU-145 cells treated up to 4 hours. 1 µM thapsigargin (Th) was used as a positive control. Pictures are representative of n=3.

BA induces transcription of ATF6 target genes

Cleaved ATF6 is a transcription factor for UPR-related genes that contain the ERSE in their promoter (80). ATF6-inducible genes include BiP (Grp78), Grp94, calreticulin, XBP1, and GADD153 (CHOP) (76). In order to confirm that ATF6 is activated by BA and is acting as a transcription factor, we used RT-PCR to look at BA's effects on these genes. 10 μM BA induced a significant increase in BiP (Grp78) and Grp94 transcription at 1, 2, and 8 hours and 0.25 hours of treatment, respectively (Fig. 2.10A and B). 10 μM BA upregulated expression of calreticulin at 2, 4, 8, and 24 hours of treatment in DU-145 cells (Fig. 2.10C). 10 μM BA also upregulated expression of XBP1 at 24 hours of treatment (Fig. 2.10D). As discussed above, CHOP (GADD153) is also an ATF4-inducible gene and transcription was decreased with BA treatment (Fig. 2.8C). This makes sense since DU-145 cells do not undergo apoptosis.

BA does not activate the IRE1 branch of UPR

The easiest and most common method for analyzing the activation of the IRE1 branch of the UPR is to look at XBP1 splicing. We used primers specific for both spliced and unspliced forms of XBP1 mRNA. When PCR products are run slowly on an agarose gel, the two forms separate into two individual bands if IRE1 is activated and XBP1 mRNA is spliced. We treated DU-145 cells with varying doses of BA for 24 hours and no splicing was seen (Fig. 2.11A). We also treated DU-145 cells with 10 μM BA for varying timepoints and again we did not observe spliced XBP1 mRNA (Fig. 2.11B). From this data we can conclude that BA does not induce the IRE1 branch of the UPR in DU-145 cells.

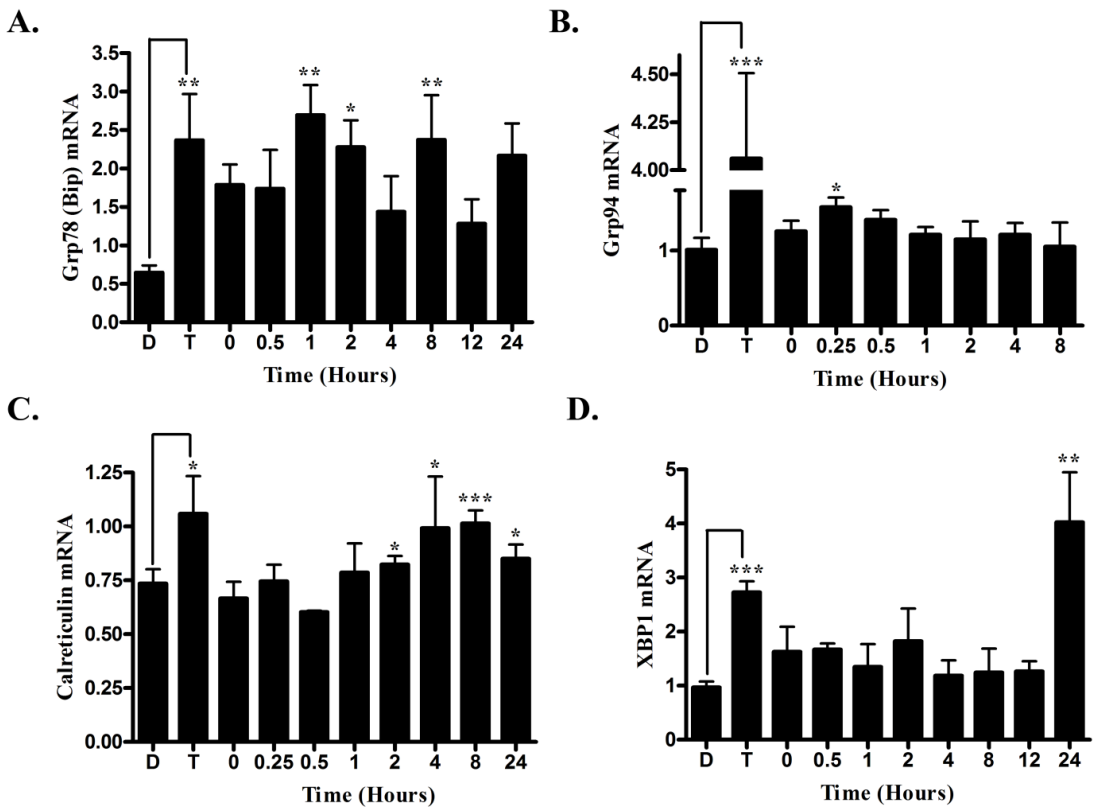

Figure 2.10. RT-PCR of ATF6-inducible genes. 10 µM BA induced expression of (A) BiP (Grp78) at 1, 2 and 8 hours, (B) Grp94 at 0.25 hours, (C) calreticulin at 2, 4, 8, and 24 hours, and (D) XBP1 at 24 hours of treatment. As a positive control 1 µM thapsigargin (T) upregulated expression of all genes compared to DMSO vehicle (D). All timepoints are representative of n=3-6.

Transcription of XBP1 target genes

XBP1 protein (XBP1p) acts as a transcription factor for a number of UPR-related genes. If IRE1 is activated we would expect an increase in the transcription of these genes. In order to confirm that the IRE1 branch of the UPR is not being activated, we used RT-PCR to analyze the expression of these genes. EDEM1 and Hrd1 are UPRE genes that are both XBP1p targets. They are both components of the ERAD pathway. Surprisingly, 10 µM BA increased the transcription of EDEM1 at 2, 4, and 24 hours of treatment (Fig. 2.12A). However, EDEM1 is a gene that is induced by both XBP1p and ATF6. Since there is no other evidence of IRE1

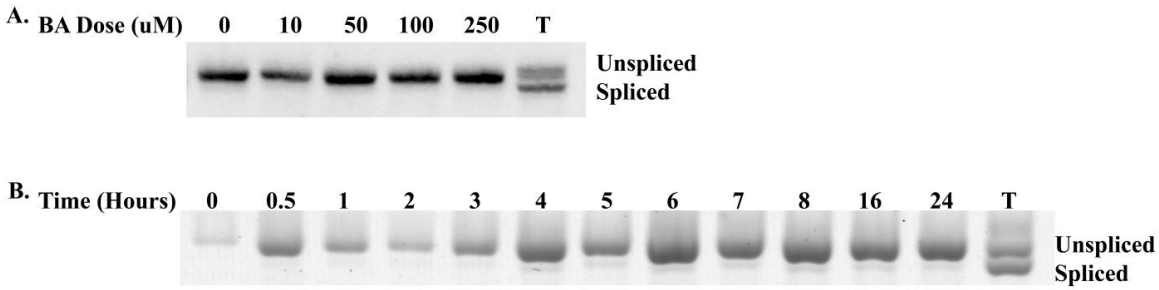

Figure 2.11. Analysis of IRE1 activation in BA-treated DU-145 cells. (A) DU-145 cells were treated with 0, 10, 50, 100, or 250 µM BA or 1 µM thapsigargin (T) for 24 hours. Thapsigargin was used as a positive control. Varying doses over 24 hours did not lead to XBP1 cleavage. (B) DU-145 cells were treated with 10 µM BA for varying time points or 1 µM thapsigargin (T) as a positive control. XBP1 was not cleaved at any timepoint. Gels are representative of n=3.

activation and BA does activate ATF6, we assume that the upregulation of this gene is due to ATF6, not XBP1. Hrd1 is a gene that is specifically controlled by XBP1. 10 µM BA does not increase the transcription of this Hrd1 (Fig. 2.12B).

BA decreases CHOP translation

CHOP (Gadd153) is a pro-apoptotic protein whose translation is typically increased during the UPR (76, 80). However, in DU-145 cells treated with 10 µM BA, translation is decreased at 0.25, 0.5, 3, 4, 5, and 6 hours (Fig. 2.13A). As a positive control, 1 µM thapsigargin did induce an increase in translation, as expected (Fig. 2.13B). This data makes sense given that the same BA dose caused a decrease in CHOP transcription (Fig. 2.8C). Since BA-treated cells do not go through apoptosis, but only have a slowed proliferation, it seems reasonable the CHOP transcription and translation are decreased.

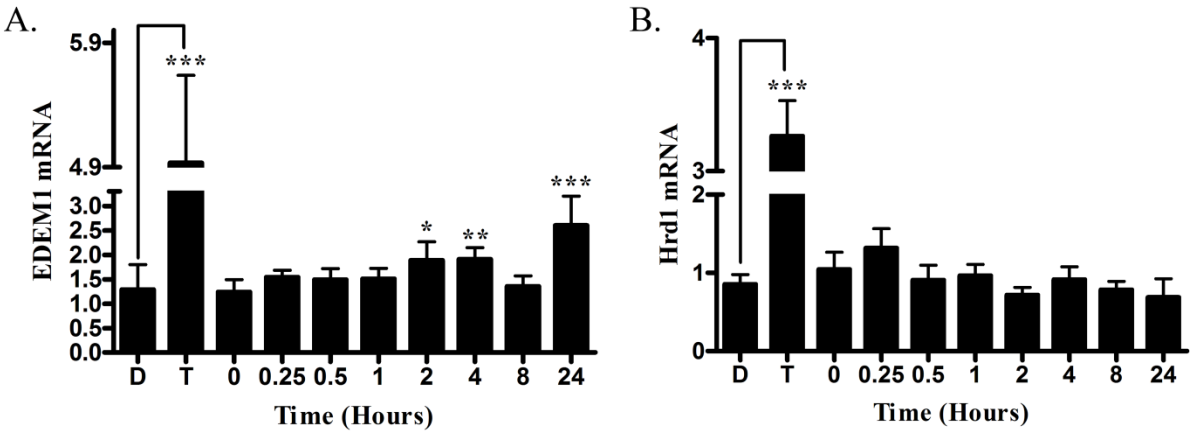

Figure 2.12. RT-PCR of XBP1-inducible genes. 10 μM BA induces expression of (A) Edem1 at 2, 4 and 24 hours. (B) 10 μM BA does not induce expression of Hrd1 in DU-145 cells. As a positive control 1 μM thapsigargin (T) up-regulated expression of both genes compared to DMSO vehicle (D). All timepoints are representative of n=3-6.

Stress granules form in BA-treated DU-145 cells

DU-145 cells treated with BA form small granules. Cells under stress can form stress granules comprised of a variety of RNA and proteins (94). Although stress granules are not well understood, they have been implicated in cell survival during periods of stress. TIA-1 is an RNA-binding protein that is commonly used as a stress granule marker. In unstressed cells, TIA-1 is predominantly located in the nucleus. In stressed cells, TIA-1 appears as foci in the cytoplasm as it accumulates in the stress granules. In DU-145 cells treated with 10 μM BA, fluorescence microscopy shows TIA-1 foci forming in the cytoplasm as early as 15 minutes of treatment and continuing through 1 hour (Figure 2.14).

Discussion

In the present study we show that BA reduced protein synthesis and induced ER stress and the eIF2α/ATF4 and ATF6 branches of the UPR without activating the IRE1 branch in DU-145

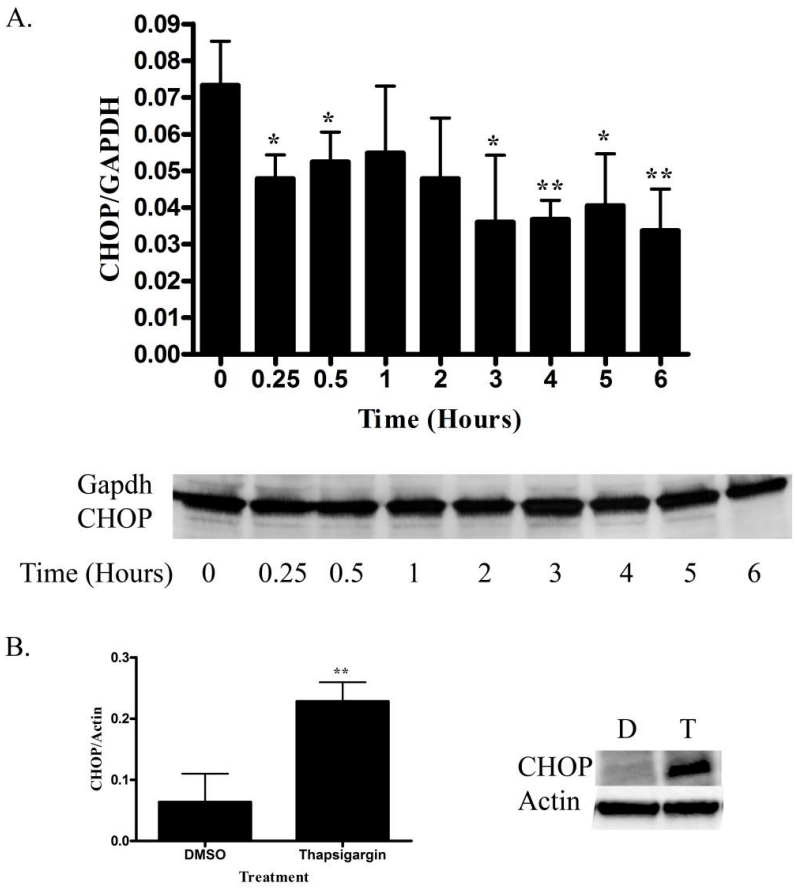

Figure 2.13. Western blot of CHOP (GADD153) in DU-145 cells. (A) DU-145 cells treated with 10 µM BA for 0, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 hours. CHOP (GADD153) translation is decreased in DU-145 cells treated for 0.25, 0.5, 3, 4, 5, and 6 hours. Timepoints represent n=3-5. (B) DU-145 cells treated with 1 µM thapsigargin (T) or DMSO (D) for 1 hour. Thapsigargin increased CHOP (GADD153) translation. Treatments represent a positive control with n=3.

prostate cancer cells. BA treatment induced ultra-structural changes to the ER including swelling, vacuolization, and vesicle production. ER swelling is characteristic of ER stress and has been observed in many cell types that activate the UPR including malignant B cells that have accumulated unfolded proteins and neurons exposed to hypoxic conditions (95, 96). One of the many causes of ER stress is $[Ca^{2+}]_{ER}$ depletion and results in the accumulation of misfolded and

unfolded proteins (97). How $[Ca^{2+}]_{ER}$ depletion arrests protein processing is not well understood, but many of the retained proteins are glycoproteins in the high mannose

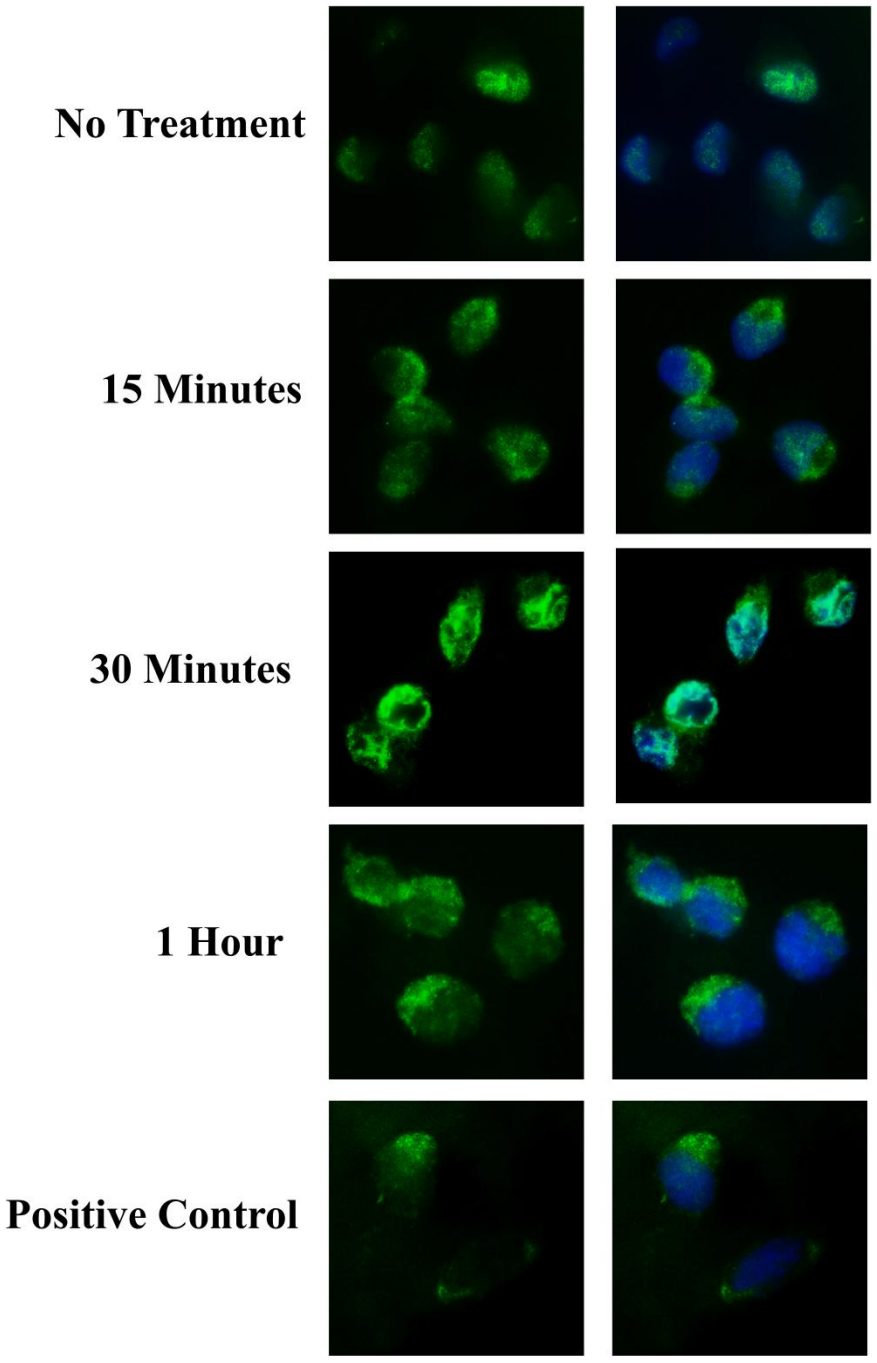

Figure 2.14. Immunofluorescent microscopy of TIA-1 in DU-145 cells. DU-145 cells treated with 10 µM BA formed stress granules at 0.25, 0.5, and 1 hour of treatment. TIA-1 (green) foci form outside of the nucleus (blue) in BA-treated cells (middle pictures) compared to cells that were not treated. 1 µM thapsigargin was used as a positive control (bottom pictures) and TIA-1 foci form in the nucleus after 30 minutes of treatment.

configuration. Agents known to deplete $[Ca^{2+}]_{ER}$ include hormones, chelating agents, ionophores, unsaturated fatty acids such as arachidonic acid, metalloendoprotease antagonists, thapsigargin, and BA (32).

A cell activates the UPR during periods of stress, including ER stress, as a way of ameliorating the protein load, bringing the cell back to its "healthy" homeostasis. The functions of the UPR include inhibiting global protein translation, upregulating proteins such as ER chaperones and foldases that will aid in refolding the misfolded proteins, and the degradation of proteins through the ERAD system (76). Through polysome profile analysis we found that physiological doses of BA significantly attenuated global protein translation in DU-145 cells. BiP (Grp78) is an ER chaperone that binds with a high affinity to misfolded and unfolded proteins due to the exposed hydrophobic regions on their surface. BiP does not help a protein fold but holds it in a folding-competent state (80). Immunoblots of BA-treated cells revealed an increase in BiP (Grp78) translation. We also observed an increase in transcription of BiP (Grp78) and Grp94, both components of the protein folding machinery, and calreticulin and EDEM1, which are important for glycoprotein quality control and glycoprotein degradation, respectively (98). ERAD relieves a stressed cell of its protein burden through the degradation of misfolded and unfolded proteins (98). Transcription of the ERAD protein, Herp, increased with BA in DU-145 cells. From this data it was clear that DU-145 cells had functional UPR responses to BA treatment. The next step was to look at the activation of the UPR's specific pathways.

The eIF2α /ATF4 branch of the UPR is highly conserved from yeast to mammals and because several different environmental stressors converge on this pathway it was named the Integrated

Stress Response (ISR) (99). Protein synthesis consumes energy and substantial quantities of each amino acid and protein folding requires reducing equivalents, particularly glutathione. Folding also requires disulfide bond formation and the mammalian ER utilizes Ero1p, a flavoenzyme that generates disulfide bonds and uses molecular oxygen as its electron acceptor. High rates of protein synthesis therefore result in the formation of reactive oxygen species. ISR protects cells by inhibiting protein translation when single amino acids become limiting and activates antioxidant pathways when deficits in glutathione and other reducing equivalents alter the redox state (99).

BA activates the eIF2α / ATF4 branch of the UPR at concentrations that are present in the blood of normal healthy people who consume diets with boron containing foods. eIF2α was phosphorylated at ser 51 with 10 μM BA treatment. Phosphorylated eIF2α interferes with the formation of the 43S translation-initiation complex and is responsible for the inhibition of global translation in the cell, an effect that is seen in BA-treated DU-145 cells (100). Another function of ph-eIF2α is the preferential translation of genes encoding short upstream open reading frames (uORF), including ATF4, a transcription factor that induces genes important in the UPR process (98). In BA-treated DU-145 cells there is an increase in ATF4 translation as well as an increase in the transcription of ATF4's target genes, GADD34 and Herp. GADD34 regulates the phosphatase activity of protein phosphatase 1 (PP1) which removes the phosphate from eIF2α, creating a negative feedback loop in this branch of the UPR (98). BA increased translation of GADD34 in DU-145 cells with a timing that coincides with the dephosphorylation of eIF2α.

Interestingly, transcription and translation of the ATF4-inducible gene, CHOP (GADD153), is intermittently decreased in DU-145 cells treated with BA. CHOP, an anti-apoptotic protein, is a transcription factor that downregulates the expression of bcl-2 and is usually increased in cells undergoing UPR (98). However, DU-145 cells treated with BA seem to go into a semi-quiescent state with slowed proliferation without induction of apoptosis (10). Apoptosis usually occurs in cells that are undergoing prolonged UPR (101). It seems that our cells recover from the UPR within a 24 hour timeframe. The suppression of CHOP transcription and translation is likely a contributing factor or the result of survival signals in BA-treated DU-145 cells. Though much still remains to be known about stress granules, they are thought to play an important role in a stressed cell's decision to survive (94). Stress granules sequester apoptotic regulatory factors and are likely cell protective as impairing the formation of stress granules leads to cellular death (94, 102, 103). TIA-1 is a pro-apoptotic protein that is a marker in stress granules (94). TIA-1 foci accumulated in the cytoplasm of BA-treated DU-145 cells indicating the presence of stress granules and the sequestration of this apoptotic protein.

Analysis of the remaining two branches of the UPR in DU-145 cells treated with BA revealed that the ATF6 but not the IRE1 branch is activated. Immunofluorescent microscopy showed a clear increase in ATF6 foci in the nucleus of treated cells, indicating that cleavage occurred and the 50 kDa ATF6 fragment moved into the nucleus to act as a transcription factor. This evidence is supported by the fact that the ATF6-inducible genes, Grp78, Grp94, calreticulin, and XBP1, are all upregulated in BA-treated DU-145 cells. In DU-145 cells treated with BA, XBP1 was not spliced, therefore, IRE1 was not activated. Also, the XBP1-inducible gene, Hrd1, is not induced by BA, confirming that IRE1 is no an activated UPR branch. However, EDEM1, another XBP1-

inducible gene, transcription is up-regulated with BA treated. This is explained because EDEM1 is induced by XBP1 but promoted by ATF6. The increase in EDEM1 transcription is probably due to activated ATF6, not XBP1.

The differential activation of some, but not all branches of the UPR plays an important role in the response of cells to environmental stressors. Cigarette smoke induces phosphorylation of eIF2α and activation of IRE1 and ATF6 in a mouse fibroblast cell line, yet only eIF2α phosphorylation in normal and human lung cancer cells.(89, 90) UV light irradiation represses ATF4 and this prevents the activation of CHOP and activation of apoptosis (104). Antigen stimulation of antibody production requires that B cells transform into antibody secreting plasma cells and this depends on the activation of IRE1 and ATF6, but not PERK. Treatment of CHO cells with thapsigargin activates IRE1 and PERK followed by a delayed activation of ATF6 (105, 106).

In summary, we found that physiological concentrations of BA induced ER stress and the eIF2α /ATF4 and ATF6 branches of the UPR in DU-145 prostate cancer cells. Electron micrographs showed BA treatment resulted in an expansion of the ER and an increase in vacuolization and granule formation. These findings are consistent with previous studies using DU-145 and LNCaP prostate cells that showed long-term treatment with BA inhibited cell proliferation without inducing apoptosis or necrosis (10). A recent analysis of genetic models of prostate tumorigenesis determined that all three branches of the UPR were selectively downregulated (107). Most cancer cells have an upregulated UPR system, giving them a survival advantage in a stressful environment (76). This unique characteristic in prostate cancer cells gives them a disadvantage when undergoing stress such as that induced by BA. These models provide a means to test the hypothesis that BA's ability to reduce the risk of cancer is a consequence of its

ability to activate the eIF2α/ATF4 pathway. The present results taken together with the fact that BA has been shown to be effective in decreasing the growth of human prostate tumor implants in the nude mouse model provide encouragement that BA will be effective in delaying the onset and progression of cancer in the genetic models. Finally, eIF2α phosphorylation is important for normal secretory cell function in the endocrine, immune, nervous and skeletal systems (108-111). These are the same systems that have been reported to be responsive to boron (68-71). Further work will determine if BA's ability to modulate this pathway explains many of the diverse effects attributed to boron over the years.

Part I Conclusions

Our lab has been studying boron's role in nature and prostate cancer prevention for many years. We were the first to show boron increased growth of a vertebrate embryo and that it was essential for the early stage of cleavage in fertilized zebrafish eggs (24). In the human, we discovered that boron in the diet and drinking water supply reduced the risk of prostate cancer in a dose-dependent manner (8). We identified BA's molecular binding partners in a physiological system, its inhibitory effects on stored Ca^{2+} release, and its inhibition of cellular proliferation of prostate cancer cell lines (10, 18, 20, 21, 112). Further studies revealed that physiologically relevant doses of BA were acting as an antagonist to the RyRs in prostate cancer cells in the presence of pharmaceutical and endogenous agonists (32). What remained to be studied was the mechanism connecting BA's effects on Ca^{2+} in prostate cancer cells to its inhibitory effects on proliferation. Understanding this mechanism could provide insight into BA's role as a prostate cancer chemoprevention. Since cell proliferation, among many cellular processes, is tightly regulated by Ca^{2+}, our first step was to look at the RyRs.

BA sensitivity differs among cell lines. We considered that the reason for this difference could be the key to understanding BA's mechanism of action in prostate cancer. We hypothesized that BA has a preferential affinity to one of the three RyR isoforms as it acts as an antagonist and expression of different RyR isoforms between the cell lines could explain their difference in BA sensitivity. Studying BA's relationship with specific RyR isoforms would not only help us better understand BA's effects in our cell lines, but could lead to the ability to predict BA's chemo-preventative nature in other cells by characterizing their RyR isoforms. As outlined in the first chapter, we characterized the RyRs in DU-145, LNCaP, and PWR-1E cell lines. RyR

characterization had not been done in DU-145 and PWR-1E cells previous to our study. Another group characterized RyRs in LNCaP cells but their study lacked proper controls (60). RyR characterization in DU-145, LNCaP, and PWR-1E cells showed us that the isoforms of the RyR were not associated with BA's ability to reduce cell proliferation or cancer. However, when we repeated experiments using BA to inhibit Ca^{2+} release from DU-145 cells but in the presence of rapamycin, an FKBP12-binding drug, we found that the inhibitory effect was reversed and the effects of caffeine and BA were additive. This was evidence that BA either directly or indirectly exerts its effects via FKBP12, an RyR accessory protein that is responsible for the channel's gating. Mass spectrometry binding studies are currently being performed by another graduate student to further characterize this relationship. We decided that studying BA's interaction with the RyRs, while important, would not help us further complete the pathway between Ca^{2+} release inhibition from the RyR and cell proliferation inhibition.

TEM images of DU-145 cells treated with BA showed that the ER stress was occurring. This was not surprising since BA causes a significant $[Ca^{2+}]_{ER}$ depletion in DU-145 cells and $[Ca^{2+}]_{ER}$ depletion is one of the major causes of ER stress in cells. The next step was to analyze if the UPR, a cellular response to ER stress, was occurring in our cells. Our studies demonstrated that physiological levels of BA activated the eIF2α/ATF4 and ATF6 branches of the UPR without activating the IRE1 branch. Interestingly, we saw a decrease in the transcription and translation of the pro-apoptotic protein, CHOP, and the appearance of stress granules. A decrease in CHOP and the formation of stress granules are both supportive of cell survival. Prostate cancer cells are unusual from other cancer cells in that their UPR system is normally down-regulated (113). This

is a disadvantage to the cells in stressful environments. The activation of the two branches of the UPR in DU-145 cells confirms that BA is inducing ER stress in these cells.

It is known that Ca^{2+} signaling is essential for controlling cell proliferation and it has been shown that ER stress induced by depletion of Ca^{2+} stores leads to an inhibition of cell proliferation (34, 114, 115). The present research demonstrated that BA's effect on the inhibition of Ca^{2+} release from RyRs is a non-isoform specific interaction that relies on FKBP12. Further, physiological doses of BA induced ER stress and the eIF2α/ATF4 and ATF6 branches of the UPR without activating the IRE1 branch in DU-145 cells. BA-induced ER stress is the most reasonable explanation of why BA inhibits the proliferation of prostate cancer cell line, DU-145, without inducing apoptosis and provides insight into BA's chemopreventative nature in prostate cancer.

Part II: Molecular Toxicology Application in Public Interest

Part II: Introduction

In addition to fulfilling the molecular toxicology laboratory research required for the obtainment of my Ph.D., I have decided to include two chapters that outline work that represents an area of toxicology I am interesting in pursuing in my future career. Based on recent graduates of the UCLA molecular toxicology program, toxicology assessment has an interesting and wide array of job opportunities. My tenure as a Ph.D. student at UCLA has not only consisted of training to become a molecular scientist, as outlined in chapters 1 and 2, I have also been given opportunities to gain experience in the area of toxicity assessment. The following two chapters outline two of the projects I have worked on.

Exposure to toxic substances can lead to adverse health effects, but the risk of these effects is based on the level of exposure and the mode of action of the toxicity. The process of assessing risk is one of the fastest growing areas of toxicology since it bridges the molecular and regulatory aspects of the field and involves translating scientific information into a form assessable to the public. In the second part of this dissertation I have written critical reviews of the toxicological data for the popular all-natural artificial sweetener, rebaudioside A, and for the artificial food dyes used in the U.S. The review of rebudioside A has been published by the Center for Science in the Public Interest and the review on food dyes has been accepted and is being prepared for publication.

Rebaudioside A is a natural, zero calorie sweetener that is extracted from the stevia rebaudiana plant and is ~300 times sweeter than sugar. Several diterpene glycoside sweeteners come from

the stevia rebaudiana plant but rebaudioside A has become the most popular and widely-used (116). For years, rebaudioside A was available as a supplement since they are not regulated by the FDA. In fact, the FDA rejected 3 company requests to use the compound in food (117). In 2008, several international industries petitioned the FDA to approve rebaudioside A as a food additive and expressed their opinion that it be classified as generally recognized as safe (GRAS) (118, 119). A GRAS classification allows a compound to avoid certain FDA requirements and regulations. If a compound is classified as GRAS, it can be put on the public market prior to FDA approval. The FDA approved rebaudioside A, the purified form of stevia, as GRAS in early 2009 even though studies did not meet FDA suggestions for toxicity testing (120, 121).

Artificial food dyes are widely used food additives that most humans and animals consume daily and are among the most controversial of all of the FDA-approved food additives. There are nine food dyes currently approved in the United States. The number of FDA-approved dyes has dwindled over the past few decades because of toxicity. All of the currently approved dyes have been thoroughly tested for chronic toxicity, carcinogenity, allergenicity, and/or genotoxicity. Though some of the results are controversial and often disagreed upon, not one dye was without positive toxicity results. The fact that these dyes are unnecessary should cause the FDA to reconsider their approval, especially since there are safer alternatives.

FDA approval of food additives involves a great deal of studies, government and industry documents, and communications between involved parties. It is important for the public to be well-informed about the potential toxicity of the food additives they consume in order to make

health-protective choices. Communication of scientific data to the public is a key component to applying molecular toxicology to public interest and health.

Chapter 3: Toxicity review of rebaudioside A[3]

Abstract

Rebaudioside A, a purified form of stevia, is an artificial sweetener currently classified as GRAS by the FDA. For decades, stevia was available as a supplement in the United States because the FDA does not regulate supplements. It was only in the last few years that the FDA gave rebaudioside A, but not stevia, a GRAS label as suggested by several interested industries. The two most popular commercial brand names of rebaudioside A products are Truvia® and Pure Via™. Currently, products that contain this artificial sweetener include Sweetleaf®, SoBe Lifewater®, Sprite Green, and Celestial Seasonings® Tea. Toxicity testing for rebaudioside A is lacking but industry investigators pushed the FDA to use studies performed on stevioside, another diterpene glycoside that is extracted from the stevia plant. This review has found that stevioside is an inadequate parallel chemical to rebaudioside A due to differences in metabolism, structure, and chemistry. Also, rebaudioside A's toxicity was only tested in rats, not mice, even though testing in two rodent species is part of the suggested FDA guidelines. This extra testing is particularly important because rebaudioside A and its metabolites were positive in several mutagenicity assays. Although rebaudioside A is an all-natural compound and would make a convenient sugar-substitute, it is the opinions of the authors that enough concerning data and lack of data exist to conclude that rebaudioside A needs further testing to be deemed safe, especially GRAS.

Introduction

[3] This document was published and released publically by CSPI 122. Kobylewski SE, Eckhert CD. Toxicity of Rebaudioside A: A review. CSPI2008. This chapter has been slightly modified from the original version.

Rebaudioside A is a steviol glycoside derived from the herb *Stevia Rebaudiana* (bertoni). Rebaudioside A and stevioside (Fig. 3.1) are the two main steviol glycosides found in the *S. Rebaudiana* herb and are the two predominant derivatives used in high-potency sweeteners. Stevioside differs from rebaudioside A by one less glucose moiety. Steviol glycosides have been used as food and medicine in Japan and South America for many years, but stevia in the leaf or extracted form is permitted to be sold in the U.S. only as a dietary supplement, as defined in section 201(ff)(1) of the Federal Food, Drug, and Cosmetic Act. In 2007, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) specified that steviol glycoside sweeteners must be composed of at least 95% of the known steviol glycosides (123). Products that consist predominantly of rebaudioside A are referred to as rebiana.

The temporary acceptable daily intake (ADI) for steviol glycosides set by JECFA was 0-2 mg/kg bw/day (based on steviol content) with a steviol equivalent of 0-6 mg/kg bw/day of rebaudioside A (steviol equivalent=[stevioside]*(0.4); [rebaudioside A]*(0.33)). JECFA concluded that there was insufficient data to give steviol glycosides a permanent ADI. The FDA has not yet authorized stevia as a food additive nor has the FDA considered it to be GRAS but it has authorized rebaudioside A to be GRAS (120, 124, 125).

The JECFA made the following requests for research before it set a permanent ADI for rebiana and reduced the safety factor to 100 (originally 200): (1) detailed information on specifications; (2) human studies conducted in normotensive and hypotensive subjects to gain information on potential hypotensive effects; (3) human studies with subjects with insulin-dependent and insulin-independent diabetes to gain information on the effects on glucose homeostasis (124).

Methods

Eligibility Criteria

Information was obtained from published, peer-reviewed studies, as well as unpublished studies and other writings, and personal correspondence. Studies were not restricted by date, language, or source. Studies judged to be of inadequate and/or unnecessary for this review were excluded.

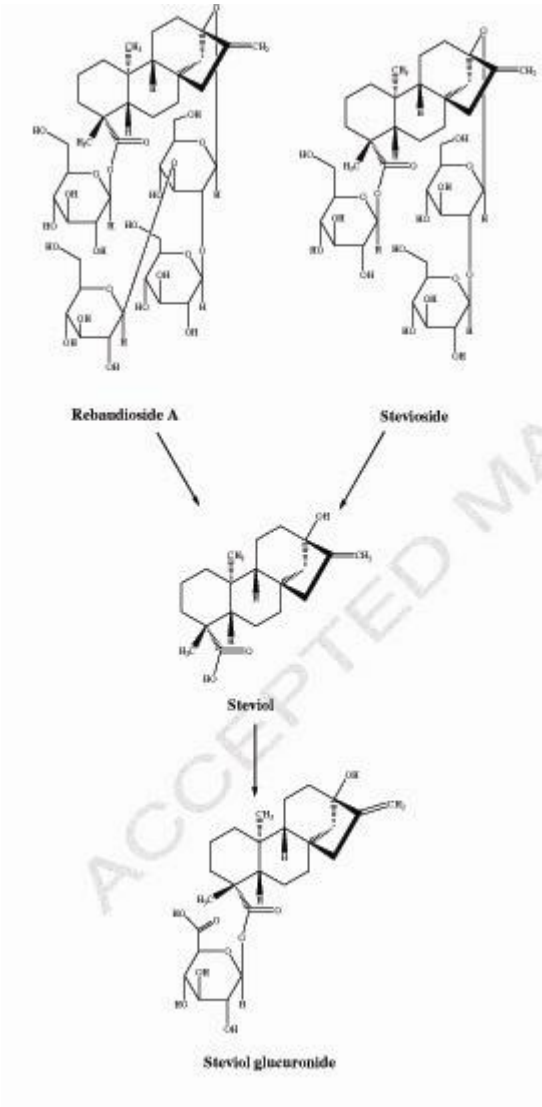

Figure 3.1 Components of steviol glycoside metabolism (126).

Search Strategy and Study Selection

This review of safety data regarding high purity rebaudioside A (rebiana), the subject of two GRAS notifications,[4] was conducted for the Center for Science in the Public Interest (CSPI). Much of the recent

[4] GRAS notification numbers 252 and 253. We have not obtained a copy of No. 252 and so cannot comment on any data that may have accompanied that notification. Furthermore at least one other company, Wisdom Natural Brands, may have self-affirmed its stevia product as GRAS, and not notified the FDA.

research was published in a Food and Chemical Toxicology supplement on rebaudioside A.[5] All the rebiana used in the studies published in that supplement met all current specifications for steviol glycosides set by the JECFA (123, 124). The research described in this supplement was peer reviewed and said to be conducted in compliance with Good Laboratory Practices (GLP) and Good Clinical Practices (GCP) requirements. Information was also obtained via personal correspondence with the FDA.

Data Extraction

Data used in this review was taken directly from the cited sources. In several cases, information was confirmed with the authors of the original text or with experts in the field.

Comparative Metabolism

Humans

Investigators[6] used a review of the literature on the metabolism of stevioside and rebaudioside A by intestinal microbiota to try to establish if toxicity studies on stevioside are relevant for assessing the toxicity of rebaudioside A (127). Gardana *et al.* provided a comprehensive study on steviol glycoside hydrolysis in the human gut (128). The study was carried out under anaerobic conditions with mixed bacterial cultures from fecal samples of healthy human volunteers. Stevioside was completely hydrolyzed to steviol after 10 hours of incubation, with steviolbioside as an intermediate. Steviolbioside formation peaked at 2-4 hours and steviol was

[5]Authors of the supplement are affiliated with manufacturers or potential users of rebaudioside A (see Appendix A).

[6] Investigators refer to the scientists from Cargill, the Coca Cola Company, or contractors who conducted and/or reviewed the studies submitted for the GRAS notifications.

first detected at 3-4 hours of incubation. Rebaudioside A was completely hydrolyzed to steviol after 24 hours of incubation, with steviolbioside as an intermediate. Steviolbioside was detected at 6-7 hours and peaked at 12-15 hours of incubation. Steviol was unchanged by incubation with intestinal microflora after 72 hours of incubation (128). Because stevioside and rebaudioside A are metabolized at different rates, toxicity assessments of stevioside cannot definitively be extrapolated to assess the risk of rebaudioside A.

Stevioside has a steviol-16,17-epoxide metabolite (Fig. 3.2) when incubated for 48 hours with rat intestinal microflora (130). Epoxides are concerning because they are highly reactive with nucleophiles, such as DNA. The creation of the steviol-16,17-expoxide metabolite seen in the Hutapea study could not be replicated by Gardana *et al.* and Koyama *et al.* (128, 131). Renwick *et al.* speculated that the HPLC-UV instrument used to detect the epoxide metabolite in the Hutapea study was not highly specific. However, given the possibility of epoxide formation from steviol and/or its glycosides based on their structures (Fig. 3.1), the creation of an epoxide metabolite in the human system needs to be further investigated.

Human metabolism studies reported similar metabolic and elimination pathways (Fig. 3.3) but not identical pharmacokinetics for rebaudioside A and stevioside (126). Healthy, adult, male subjects received a single oral dose of 5 mg/kg of 98.7% pure rebaudioside A and 4.2 mg/kg of 96.6% pure stevioside (each ~1.6 mg/kg of steviol equivalents).

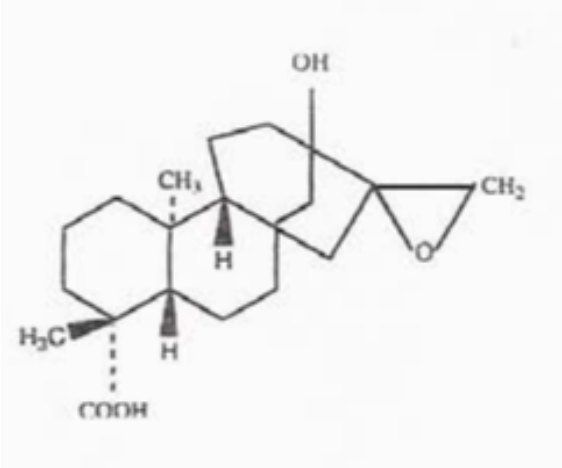

Figure 3.2 steviol-16,17α-epoxide (129)

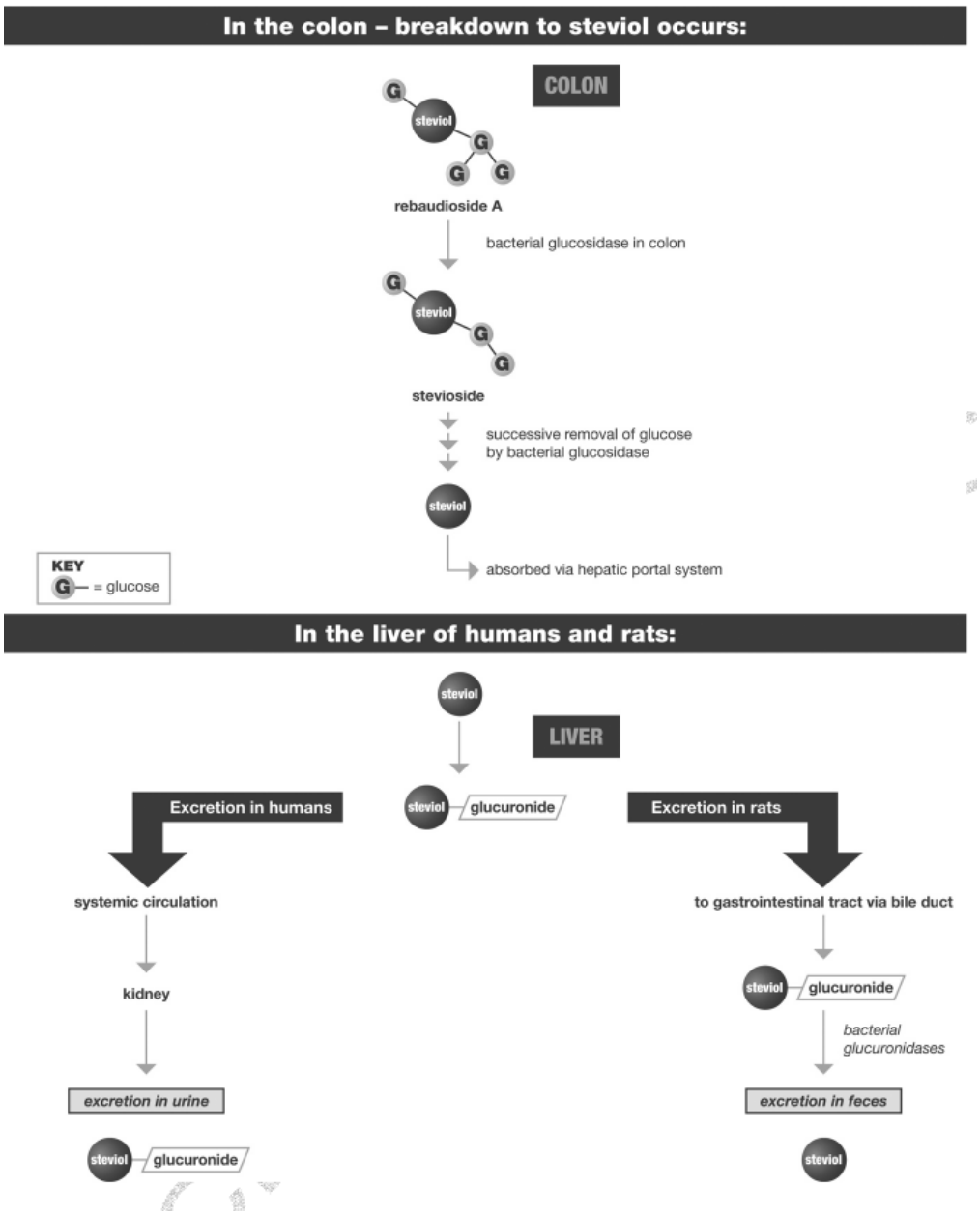

Figure 3.3 Summary of rebaudioside A metabolism in the rat and human (124).

Plasma, urine, and fecal samples were collected during a pre-dose period and up to 72 hours post-dose. Both glycosides were hydrolyzed in the gastrointestinal (GI) tract into steviol, which was absorbed and conjugated to a glucuronide. Steviol glucuronide was predominantly excreted in the urine and accounted for 59% and 62% of the rebaudioside A and stevioside, respectively.

Steviol excreted in the urine only accounted for 0.04% and 0.02% rebaudioside A and stevioside, respectively. Steviol glucuronide was not detectable in the feces, but steviol in the feces accounted for 4.8% and 5.2% of rebaudioside A and stevioside, respectively. The half-life ($t_{1/2}$) for both glycosides was approximately 14 hours (Tables 3.1 and 3.2). However, only 64.2% of rebaudioside A and 67.22% of stevioside was accounted for in the urine and feces after 72 hours of dosing. Plasma steviol glycosides were not measured in this study (126).

Table 3.1 Mean pharmacokinetic parameters for steviol in men (126).

		Treatment			
Parameter	Units	N	Rebaudioside A	N	Stevioside
C_{max}	(ng/mL)	1	227 (NA)	1	121 (NA)
T_{max}	(hr)	1	72.0 (NA)	1	6.00 (NA)
AUC_{0-t}	(ng*hr/mL)	0	NA (NA)	0	NA (NA)
AUC_{0-inf}	(ng*hr/mL)	0	NA (NA)	0	NA (NA)
$t_{1/2}$	(hr)	0	NA (NA)	0	NA (NA)
$?_z$	(1/hr)	0	NA (NA)	0	NA (NA)
$Ae_u(0-72)$	(mg)	8	0.0510 (0.0877)	8	0.0238 (0.0675)
CL_R	(L/hr)	0	NA (NA)	0	NA (NA)
$Ae_f(0-72)$	(mg)	6	5.88 (6.95)	7	6.50 (7.08)

The values given are mean ± SD. NA = Not applicable.

A low transport of rebaudioside A and stevioside were reported in Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line often used to detect absorption rates of drugs, with apparent permeability coefficients of 0.11×10^{-6} and 0.16×10^{-6} cm/s, respectively (132). They reported that steviol was transported much more efficiently than the two glycosides, with an apparent permeability coefficient for absorptive transport of 38.6×10^{-6} cm/s (132). Though it is

apparent that the majority of the stevioside and rebaudioside A are hydrolyzed into steviol, which is absorbed by the GI tract, it is possible that transport and/or absorption of rebaudioside A and stevioside occurs. It is also likely that steviol metabolites are not being excreted within 72 hours, and that may account for the remainder of the dose that was not measured in the feces or urine. The unaccounted-for fraction of rebaudioside A and stevioside after 72 hours (~5 half-lives) of dosing needs to be further investigated. If harmful metabolites are being formed after absorption, it is important to understand their toxicokinetics in order to properly assess their potential toxicological relevance. A more complete study would measure plasma concentrations and excretion of unhydrolyzed steviol glycosides.

Table 3.2 Mean pharmacokinetic parameters for steviol glucuronide in men (126).

			Treatment		
Parameter	Units	N	Rebaudioside A	N	Stevioside
C_{max}	(ng/mL)	8	1588 (700)	8	2222 (1078)
T_{max}	(hr)	8	12.0 (6.02, 24.0)	8	8.00 (6.00, 12.0)
AUC_{0-t}	(ng*hr/mL)	8	33904 (15139)	8	39928 (20129)
AUC_{0-inf}	(ng*hr/mL)	4	46197 (18604)	4	53211 (23782)
$t_{1/2}$	(hr)	4	14.8 (3.32)	4	14.0 (5.61)
$?_z$	(1/hr)	4	0.0483 (0.00908)	4	0.0551 (0.0221)
Ae_u(0-72)	(mg)	8	106 (24.0)	8	112 (36.8)7)
CL_R	(L/hr)	8	3.73 (2.01)	8	3.36 (2.51)
Ae_f(0-72)	(mg)	6	0 (0)	7	0 (0)

The values given are mean ± SD. T_{max} is presented as Median (Min, Max).

In the human metabolism study, rebaudioside A and stevioside had different pharmacokinetic results for certain parameters when steviol and steviol glucuronide were measured (Tables 3.1

and 3.2). For instance, there was a longer T_{max} and lower C_{max} of steviol glucuronide and steviol when the patients were administered rebaudioside A compared to stevioside (126). Stevioside toxicity studies may be a good way to predict the toxicity of rebaudioside A, but they cannot be used in place of directly testing rebaudioside A itself. The toxicity of stevioside and rebaudioside A should be studied individually since each will potentially be used as ingredients in human foods.

Rats

Roberts *et al.* investigated the metabolism of stevioside, rebaudioside A, and steviol in Sprague-Dawley rats in order to determine the toxicokinetic and metabolic similarities between stevioside and rebaudioside A (133). The three compounds were radiolabeled with ^{14}C in the $=CH_2$ group of the steviol moiety (Fig. 3.1). The rats were given a single oral dose of 5 mg/kg bw (mg per kg body weight) rebaudioside A, 4.2 mg/kg bw stevioside, and 1.6 mg/kg bw steviol (molar equivalents) (133). Even though the investigators concluded that the pharmacokinetics of stevioside and rebaudioside A in rats are similar, while that of steviol is different, it appears that most of the pharmacokinetic parameters are quite different for all three compounds in rats of the same sex (Table 3.3). The main radioactive component in plasma was always steviol after rats were dosed with ^{14}C-stevioside, ^{14}C-rebaudioside A, and ^{14}C-steviol. Steviol glucuronide and two unidentified metabolites were also found in the plasma in lower concentrations than steviol. The absorption through the gut from rebaudioside A treatment was 71% for males, 82% for females; from stevioside treatment was 78% for males and 81% for females; from steviol treatment was 97% for males and 99% for females. Steviol was excreted predominantly in the feces and was the primary metabolized component of the parent glycoside. Unlike the human

studies, limited urinary elimination was reported. Steviol glucuronide was the primary form of the metabolized glycoside found in the bile of cannulated rats. Steviol glucuronide from the bile proceeds to the GI tract where it is deconjugated back to steviol. Steviol is then either re-circulated back to the liver or is excreted in the feces (Fig. 3.3) (133). The investigators proposed that because of the pharmacokinetic similarities between stevioside and rebaudioside A, information from stevioside safety studies can be used to extrapolate safety data on rebaudioside A. However, the pharmacokinetic parameters in rats are different enough that toxicity data from stevioside may not be reliably extrapolated to rebaudioside A. Independent toxicity studies on rebaudioside A would be needed to make any conclusive statements about its safety.

Table 3.3 Pharmacokinetic parameters in rats following administration of single oral doses of ^{14}C rebaudioside A, ^{14}C-stevioside, and ^{14}C-steviol (133).

	Administered Substance					
Parameters For Total Radioactivity	Rebaudioside A		Stevioside		Steviol	
	Male	Female	Male	Female	Male	Female
C_{max} (ng equiv./g)	90	177	101	279	114	264
T_{max} (h)	2	8	4	8	0.25	0.25
AUC_{72} (ng equiv.h/g)	645	3329	1617	4287	1251	1604
$AUC^{?}$ (ng equiv.h/g)	630	3349	1607[a]	4359	1926[a]	1926[a]
k (h^{1})	0.1462	0.0721	0.0795[a]	0.0460	0.0437[a]	0.0427[a]
$T_{1/2}$ (h)	5	10	9[a]	15	16[a]	16

[a] Not all of the criteria for reliability were met (see the methods section)

C_{max} – maximum observed plasma concentration

T_{max} – time of maximum observed plasma concentration

AUC_{72} – AUC calculated from zero to 72h after administration

$AUC_{?}$ – AUC extrapolated to infinity using the terminal slope

The difference in excretion pathways between humans and rats is explained by the different molecular weight thresholds for human and rat biliary excretion of organic anions such as steviol glucuronide (127). Steviol and steviol glucuronide are subject to enterohepatic re-circulation in the rat (127, 133). Roberts *et al.* proposed that the rat is an ideal model for studying steviol glycoside toxicity in humans due to their similarities in steviol glycoside metabolism (133). However, due to the differences in steviol glycoside metabolism between humans and rats (Fig. 3.3) the rat does *not* appear to be an ideal model for studying steviol glycoside toxicity. Steviol glycosides are hydrolyzed to their aglycone by similar microflora in the guts of the two species, but steviol and its glucuronide conjugate undergo enterohepatic circulation in the rat. The rat's primary path of elimination is through the feces, while in humans steviol is conjugated to glucuronide and predominantly eliminated in the urine. While studies in rats are certainly useful, rat studies may lead to inappropriate conclusions because of the differences in metabolism between rats and humans.

Dietary Intake Assessment

To project rebaudioside A intakes, a substitution method was used that takes into account actual intake data of high consumption artificial sweeteners (expressed as sucrose equivalents) (134). The study determined that this method was conservative enough for purposes of toxicity assessments. They found that the highest predicted intake of rebaudioside A would be in children and diabetics, but predicted that dietary exposure would always be less than 6 mg/kg bw/day. The estimates were calculated from published intake data of existing artificial sweeteners which had varying age ranges for children. In order to be conservative, the study used data from the age group of each study that showed the highest intake. The predicted daily

intake of rebaudioside A in average consumers was 1.3, 2.1 and 3.4 mg/kg bw in the general population, children, and children with diabetes, respectively. The predicted daily intake of rebaudioside A in high consumers was 3.4, 5.6, and 4.5 mg/kg bw in the general population, children, and children with diabetes, respectively (134).

Hemodynamic Effects

According to a 4-week study, 1,000 mg/day rebaudioside A did not significantly alter resting, seated systolic blood pressure, diastolic blood pressure, mean arterial pressure, heart rate, or 24-hour ambulatory blood pressure responses in patients with low-normal to normal blood pressure compared with a placebo (135). 1,000 mg/day is 7-10 times the predicted average daily intake and 2-4 times the daily intake for high-intake consumers. A secondary analysis noted small changes in diastolic blood pressure and mean arterial pressure (135). The investigators asserted that those findings are clinically insignificant.

Glucose Homeostasis

In one study, rebaudioside A does not affect glucose homeostasis or resting blood pressure in patients with type 2 diabetes mellitus (136). The patients in this study were dosed for 16 weeks with 1,000 mg/day rebaudioside A. The investigators found no hypoglycemia in the rebaudioside A group compared to the placebo. However, there was a small but significant increase in alanine transaminase (ALT) levels in the rebaudioside A group (1.7 U/L) and a decrease in the placebo group (-1.5 U/L) (136). The investigators suggest that the elevation in ALT levels was likely due to random variation and claim it has no clinical significance since mean levels of ALT stayed within normal range. No explanation of "clinically significant" or

"normal range" was provided by the investigators. Further investigation would be necessary to determine one of the many possible causes of the elevated ALT levels.

Genotoxic Effects

According to a literature review by Brusick *et al.* on the genotoxicity of steviol and stevioside, two of 16 studies showed genotoxic activity for stevioside and four of 15 studies (Brusick *et al.* did not include Pezzuto *et al.*, 1985, and TM677 results by Matsui *et al.*, 1996 (137, 138)) showed genotoxic activity for steviol (see Tables 3.4 and 3.5, respectively) (129). Rebaudioside A was not found to cause mutations, chromosome damage, or DNA strand breakage in several *in vitro* and *in vivo* studies (137, 139-141). Examples of (mostly positive) genotoxicity studies using stevioside include:

- Stevioside was positive in *Salmonella typhimurium* (*S. typhimurium)* strain TA98 at 50 mg/plate for 99% pure stevioside. The results showed a 4-fold increase in revertants without S9 extract and a 2-fold increase with S9. That study used stevioside pre-incubated with and without β-glucosidase. The treated and untreated samples showed roughly the same mutagenic results. Those results demonstrate that at 50 mg/plate, stevioside (without β-glucosidase or S9), steviol (stevioside + β-glucosidase), stevioside metabolite(s) (stevioside +S9), and steviol metabolite(s) (stevioside + β-glucosidase + S9) are all mutagenic in TA98 (142).
- Stevioside was not mutagenic in TA98 at a concentration of 50 mg/plate. However, they used S9 extract from rats, mice, hamsters, and guinea pigs (143); Suttajit *et al.* showed the strongest results without S9 extract (142).

- Stevioside did not cause chromosomal aberrations in human lymphocytes incubated with 1, 5, and 10 mg/ml stevioside for 24 hours (142).
- The comet assay showed DNA breakage in blood, spleen, liver, and brain cells in Wistar rats exposed to 400 mg/kg of stevioside in drinking water. The strongest effects of stevioside were found in the liver cells (144).
- Stevioside was not mutagenic in *S. typhimurium* strains TA97, TA98, TA100, TA102, and TA104 with or without S9 at doses up to 5 mg/plate or in strains *S. typhimurium* TA1535, TA1537, and *E. coli* WP2 *uvrA/*pKM101 with S9. Stevioside was also not mutagenic in *S. typhimurium* strain TM677 with or without S9 at 10 mg/ml. Stevioside also gave negative results in the *umu test* with or without S9 and was negative in the spore and streak *rec-assays* with or without S9 at 10 mg/disk (138).

Metabolically-activated steviol was found to cause dose-related positive responses in several mutagenicity tests. These results indicate that a steviol derivative is likely responsible for its mutagenic activity, but the metabolite has not been identified (129). The mutagenicity of steviol metabolites needs to be further investigated.

- Steviol was positive in a plasmid mutagenesis study (145).
- Steviol was mutagenic in *S. typhimurium* strain TM677, caused chromosome aberrations in cultured Chinese hamster lung (CHL) cells, and was mutagenic in CHL cells in the presence of S9. In the same study, steviol produced a weak positive response with or without S9 in the *umu* test (138).

- Steviol was not mutagenic in *S. typhimurium* strains TA97, TA98, TA100, TA102 and TA104 with or without S9 at doses up to 5 mg/plate or in *S. typhimurium* strains TA1535, TA1537, and *E. coli* WP2 *uvrA/*pKM101 with S9. In the same study, steviol was also negative for spore and streak *rec-assays* and did not induce micronuclei in bone marrow erythrocytes of mice (138).
- A forward mutation assay using *S. typhimurium* strain TM677 found mutagenicity using 100 μg/ml steviol when assayed with S9 extract (137).
- Steviol was not mutagenic in TA98 or TA100 at doses of 1-20 mg/plate. This study also showed that steviol does not cause chromosome aberrations at 0.1 and 0.2 mg/ml (142).

Subchronic Toxicity

Curry *et al*. performed subchronic tests of rebaudioside A in a 13-week study on Han-Wistar rats (146). After a 4-week palatability study, investigators dosed rats with 12,500, 25,000, and 50,000 ppm rebaudioside A (dosing equivalents provided in Table 3.6). In the 13-week study, mean body weight gain was significantly less in the first four days for males and females in the 25,000 ppm and 50,000 ppm treatment groups compared to controls. Males in all treatment groups had significantly less mean body weight gain than control groups for the length of the study. Females showed similar results but only in the 25,000 ppm and 50,000 ppm treatment groups (146). Investigators concluded that the reduced weight gain was not an adverse effect due to the following considerations: (1) the effect of rebaudioside A on food conversion efficiency was minimal; (2) rebaudioside A affects food consumption and body weight gain due to palatability issues; (3) reduced food consumption was consistently associated with treatment

Table 3.4 Summary of genetic toxicity tests for stevioside (studies showing genotoxicity are highlighted with a box; either LED or HNED is indicated in third column)

Test	Response	LED/HNED*	Conditions	Comment	Citation
Reverse mutation in S. typhimurium, E. coli, B subtilis	Negative in all strains	Data not available	Tests conducted both with and without S9	Company sponsored testing program	Tama Biochemical C., Ltd. Safety of Stevia (Tama Report 1-20, 1981) cited in Medon et al., 1982
SCEs in human fetal cells, in vitro	Negative	Data not available	Test conditions not available	(see above)	(see above)
Chromosome aberrations in cultured rat cells, in vitro	Negative	Data not available	Test conditions not available	(see above)	(see above)
Forward mutation in S. typhimurium TM677	Negative	Data not available	Tested both with and without S9 induced by Arochlor 1254	SOT Abstract for 1982 meeting presentation	Medon et al., 1982, SOT Abstract publications
Reverse mutation in Ames strains TA98 and TA100	TA100 reported negative. TA98 reported positive, without S9	TA100 = 50 mg/plate (HNED) TA98 = 50 mg/plate (LED)	Pre-incubation method used S9 produced from rats induced with combination of Phenobarbital and 5,6-benzoflavone	Stevioside was 99% pure TA98 showed a 4-fold increase; however, a 1% impurity would be 500 ug/plate at the high concentration.	Suttajit et al., 1993
Chromosome aberrations in human lymphocytes, in vitro	Negative	10 mg/ ml	Study conducted with and without S9 from rats induced by phenobarbital and 5,6 benzoflavone	No data were provided for this study in the publication	(see above)
Reverse mutation in Ames strains plus E.coli WP_2 uvrA/pKM101	Negative in all strains tested	5 mg/plate for all strains and treatment conditions	Pre-incubation method used, Standard Ames strains plus TA102 and TA104	Stevioside purity was 83%, No toxicity was seen in the test at the highest concentration tested	Matsui et al., 1996a
Umu test	Negative	5 mg/ml	Performed according to the methods of Oda et al., 1985	S9 used was from rats treated with a combination of Phenobarbital and 5,6 benzoflavone	(see above)
Rec-assay	Negative	10 mg/paper disk	Performed	Used S9 from	(see above)

			according to the methods of Hirano et al., 1982	rats treated with Arochlor 1254	
Chromosome aberrations in CHL cells, in vitro	Negative	12 mg/ml	Treatments were for 6, 24 and 48 hours without S9 and for 6 hours with S9, maximum concentrations set at >50% toxicity	Used S9 from rats treated with Arochlor 1254	(see above)
Reverse mutation in Ames strains TA98 and TA100	Negative results in both strains for all treatment conditions	50 mg/plate	Pre-incubation method used, all S9s induced by a combination of Phenobarbital and 5,6-benzoflavone	Compared S9s from rat, mouse, hamster and guinea pig.	Klongpanichpak, et al., 1997.
Mouse lymphoma forward mutation assay	Negative	5000 µg/ml	Micro-titer method used, 3 hr exposures with and without S9 plus 24 hr treatment without S9	No toxicity observed at the maximum concentration under either treatment condition	Oh et al., 1999
Mouse micronucleus assay, in vivo	Negative	250 mg/kg	Single dose with 24 harvests of bone marrow and hepatocytes	ICR mice treated at only one dose, no toxicity reported	(see above)
Comet assay, in vivo	Negative results in all tissues examined	2000 mg/kg oral administration to ddY mice	Tissues examined for DNA damage at 3 and 24 hours post exposure	Organs included glandular stomach, colon, liver, kidney, bladder, lung, brain and bone marrow	Sasaki et al., 2002
Comet assay, in vivo	Negative results in all tissues examined	2000 mg/kg administration to BD F_1	Tissues examined for DNA damage at 3 and 24 hours post exposure	Organs included stomach, colon and liver	Sekihashi et al., 2002
Comet assay, in vivo	Positive in all tissues examined	4 mg/ml in drinking water	Blood cells examined weekly, spleen, liver and brain tissues examined at exposure termination	Wistar rats given stevia extract for 45 days in their drinking water. No DNA effects were seen before week five.	Nunes et al., 2007

- LED = Lowest concentration tested that shows a clearly positive response according to the criteria of the specific test.
- HNED = Highest concentration tested for a study with negative results

Table 3.5 Summary of genetic toxicity tests for steviol (studies showing genotoxicity are highlighted with a box; either LED or HNED is indicated in third column)

Test	Response	LED/HNED*	Conditions	Comment	Citation
Reverse mutation in Ames strains TA98 and TA100	Negative	20 mg/plate	Pre-incubation modification using S9 from rats induced by a combination of Phenobarbital and 5,6-benzoflavone	Steviol was prepared by periodae oxidation of stevioside followed by acid hydrolysis and recrystallization	Suttajit et al., 1993
Chromosome aberrations, in vitro	Negative	200 µg/ml	Studies conducted in human lymphocyte cultures with and without S9	No actual data provided to support author's conclusions	(see above)
Reverse mutation in Ames strains plus E. coli WP_2 uvrA/pKM101	Negative	5000 µg/plate	Pre-incubation modification using S9 from rats induced by Kanechlor KC-400	Negative in all standard strains plus strains TA102 and TA104	Matsui et al., 1996a
Umu test	Positive	2500 µg/plate	Performed according to methods of Oda et al., 1985 with S9 from rats induced by a combination of Phenobarbital and 5,6-benzoflavone	Approximate 2-fold increase at the high concentration considered a weak positive	(see above)
Rec-assay	Negative	10 mg/paper disk	Performed according to methods of Hirano et al., 1982	Used S9 from rats induced by PCBs	(see above)
Chromosome aberrations, in vitro	Positive	1000 µg/ml	Studies conducted in CHL cells, Cells sampled at 6, 24 and 48 hours without S9 and at 6 hours with S9	Used S9 from rats induced by PCBs Positive response only with S9	(see above)
Gene mutation in mammalian cells, in vitro	Positive	400 µg/ml	Studies conducted in CHL cells and assessed by resistance to diphtheria toxin	Used S9 from rats induced by PCBs Positive response only with S9 at highly toxic treatments (3% survival)	(see above)
Mouse micronucleus assay, in vivo	Negative	500 mg/kg	MS/Ae mouse strain used, compound administered i.p. with 24 and 48 hour harvests	Toxicity seen at 1000 mg/kg	(see above)
Reverse mutation in Ames strains TA98 and TA100	Negative	2000 µg/plate	Pre-incubation method using S9 from animals induced by a combination of Phenobarbital and 5,6-benzoflavone	Authors compared S9s from rat, mouse, hamster and guinea pig All tests negative	Klongpanichhpak et al., 1997
Gene mutation in mammalian cells, in vitro	Negative	341 µg/ml	Study conducted in mouse lymphoma cells L5178Y at the TK gene (with and without S9)	Toxicity did not exceed a RTG of 40%	Oh et al., 1999

Mouse micronucleus assay, in vivo	Negative	200 mg/kg	Single oral dose with only a 24 hour harvest of liver hepatocytes	No harvest at 48 hours	(see above)
Micronucleus assay, in vivo	Negative	4 gm/kg for hamsters and 8 gm/kg for rats and mice	Study conducted using single oral dose in mice, rats, hamsters (both sexes), bone marrow cells harvested at 24, 30, 48 and 72 hours post exposure	Toxicity seen in all species at high dose with females appearing to be more sensitive	Temcharoen et al., 2000
Comet assay, in vivo	Negative	2 gm/kg	Mice were exposed by a single oral dose and tissues collected at 3 and 24 hours post exposure	Stomach, colon, liver, kidney, and testis tissues evaluated for DNA damage	Sekihashi et al., 2002
Comet assay, in vitro	Negative	500 µg/ml	Studies conducted in TK6 and WTK1 cell cultures both with and without S9		(see above)
Plasmid mutagenesis	Positive	Not reported	Induction of xgprt mutants in plasmid pSV2-gpt in the presence of S9	Mutants analyzed and shown to be small deletions which was offered as an explanation why steviol was not mutagenic in the Ames strains	Matsui et al., 1989

- LED = Lowest concentration tested that shows a clearly positive response according the criteria of the specific test.
- HNED = Highest concentration tested for a study with negative results

groups that demonstrated reduced weight gain; and (4) toxicity was not observed over the dose-range in the 13-week study (147). Also, based on WHO guidance from 1987, the body weight gain reductions observed in both studies would not be considered an adverse effect (148).

Large, but inconsistent, reductions in bile acids occurred in both studies across all treatment groups. However, liver enzyme activities (as measured in serum) and hepatic histopathology were within normal limits and did not differ significantly from controls. Mean plasma urea and creatinine concentration in several treatment groups in both studies were slightly increased from controls, but levels always remained within reference limits (146). Because of low urine

volume, high urine specific gravity, and no change in other urinalysis parameters, investigators concluded that these results were probably not a sign of renal failure but of dehydration, possibly from the osmotic effects of high doses of rebaudioside A. Macroscopic and microscopic evaluation of the kidneys showed no alterations. Absolute epididymal weights of high-dose males and the absolute weights of the ovaries of high-dose females were significantly lower than controls. Spermatogenesis was unaffected by treatment and testicular atrophy was not detected. Microscopic histopathology did not detect any other effects on the testes. The NOAEL (no observed adverse effect level) for rebaudioside A in Han-Wistar rats for the 13-week study was determined to be 50,000 ppm for the 13-week study (4,161 and 4,645 mg/kg bw/day in males and females, respectively) (146). This is ~2,000-fold greater than the ADI of 2.0 mg/kg bw/day of steviol glycosides established by JECFA and ~1,000-fold greater than the predicted human exposure (123).

Table 3.6 Average achieved rebaudioside A dose in treated rats (13-week study; mg/kg bw/day) (146).

	Males: Week 1	Males: Week 13	Females: Week 1	Females: Week 13
12,500 ppm	1,506	698	1,410	980
25,000 ppm	3,040	1,473	2,841	1,914
50,000 ppm	5,828	3,147	5,512	3,704

Carcinogenicity

Studies in rats have failed to produce any evidence of carcinogenicity of stevioside, though rebaudioside A, the subject of the GRAS notification, itself has not been tested (124). The following is a summary of the carcinogencity studies:

- Fischer 344 rats administered 5% stevioside in their diet in a relatively brief 36-week study showed no increased development of pre-neoplastic or neoplastic lesions in the urinary bladders with and without an initiating dose of the bladder carcinogen N-nitrosobutyl-N-(4-hydroxybutyl) amine (149).
- No neoplastic or pre-neoplastic lesions were found in Wistar rats in a 24-month chronic toxicity and carcinogenicity study with 85% pure stevioside (600 mg/kg bw/day) (150).
- A 24-month carcinogenicity study did not find an increase in non-neoplastic or neoplastic lesions in Fischer 344 rats exposed to 2.5% and 5% of 95.6% pure stevioside in the diet (151). JECFA used the 970 mg/kg bw/day dose (2.5% dose in male rats) used in this study to set the temporary ADI of 2 mg/kg bw/day (124).

It is important to note that the FDA normally asks for tests in *two* rodent species, usually rats and mice, in a compound with such a high predicted exposure level (121). Also, all three of the aforementioned studies were done with stevioside, not rebaudioside A. It is possible that differences in metabolism and toxicokinetics would result in different risks of carcinogenicity using the two steviol glycosides.

Reproductive Toxicity

Older studies reported anti-fertility effects, as well as decreases in the weights of the testes, seminal vesicle, and cauda epididymides, and a reduction in spermatozoa concentration, in rats administered crude stevia extracts (152-154). However, other studies with purified stevioside (not rebaudioside A) failed to produce these reproductive effects (155-157).

One study found no treatment-related effects of rebaudioside A on mating performance, fertility, gestation lengths, and estrous cycle in the F_0 and F_1 generation of rats in a two-generation study (158). After a preliminary short-term study to determine appropriate dosage levels, those investigators dosed F_0 and F_1 generation rats with 0, 7,500, 12,500, and 25,000 ppm rebaudioside A via the diet (dosing equivalents provided in Table 3.7). Female rats in the 12,500 ppm and 25,000 ppm groups and male rats in the 25,000 ppm group of the F_1 generation and male and female rats in the 25,000 ppm group of the F_2 generation showed significant decreases in body weight gains compared to controls(158). The investigators considered those effects to be toxicologically insignificant due to the lack of adverse effects on those animals' survival, condition of their offspring, their pre-weaning reflex development, weight gain after 25 days, and timing of sexual maturation. The investigators presume that the weight-gain effects were due to the nature of a diet supplemented with high levels of intense sweeteners that normally leads to reduced consumption and nutritional content (148, 158). Investigators found higher adjusted mean liver weights in females in the 12,500 ppm and 25,000 ppm F_0 and F_1 generations. There were no changes in the relative weights of the thymus glands in treated groups compared to the control group, but there was a reduction in relative and absolute weights of the spleens in several animals of the F_1 and F_2 treatment groups. However, the investigators did not find any clinically significant alterations in blood count or the histopathology of other immune system organs. There was no change on the ability of the females of the F_0 and F_1 generations to litter and rear their offspring to weaning. There was also no effect on litter size, sex ratio, and pre- and post-natal survival of offspring (158).

Curry *et al.* concluded that steviol glycosides do not pose a reproductive or developmental hazard. They found the NOAEL for rebaudioside A for Han-Wistar rats to be 25,000 ppm (2,048-2,273 mg/kg bw/day) for reproductive effects and survival, growth, and general condition of F_1 and F_2 offspring (158).

Table 3.7 Average achieved rebaudioside A dose in treated rats (mg/kg bw/day) (158).

	F_0 Males	F_0 Pre-paired Females	F_0 Gestating Females	F_0 Lactating Females	F_1 Males	F_1 Pre-paired Females	F_1 Gestating Females	F_1 Lactating Females
7,500 ppm	586	699	648-713	715-1,379	734	798	562-625	976-1,406
12,500 ppm	975	1,115	1,119-1,169	1,204-2,388	1,254	1,364	911-1,058	1,752-2,394
25,000 ppm	2,048	2,273	2,263-2,381	2,602-5,019	2,567	2,768	2,036-2,212	3,289-4,893

Discussion

Investigators demonstrated that rebaudioside A has no adverse hemodynamic effects in people with normal to low-normal blood pressure dosed with 1,000 mg/day for 4 weeks. Investigators also found no clinically significant effects of rebaudioside A treatment on patients with type 2 diabetes mellitus. The only concerning finding of the glucose homeostasis study was an increase in ALT levels. This finding is not of great concern since it did not lead to any adverse effects, but further investigation would be necessary to determine the cause of the increase and the long-term effects of rebaudioside A on ALT levels.

In both the rat and human metabolism studies, investigators demonstrated that rebaudioside A and stevioside have similar metabolic pathways within each species. However, rebaudioside A's extra glucose moiety causes differences in the two compounds' pharmacokinetic parameters

(Tables 3.1, 3.2, and 3.3). Because of those differences, toxicity data for stevioside cannot be assumed to be an appropriate basis for assessing the safety of rebaudioside A. Separate toxicity studies on rebaudioside A itself are necessary to make definitive conclusions about its safety. Investigators concluded from the metabolism studies that the rat is an ideal model for steviol glycoside human toxicity studies. Both species hydrolyze the glycosides into steviol by the gut microflora, but after absorption the metabolic pathways differ (Fig. 3.3). Since steviol glycoside metabolism in rats and humans is not identical, the rat may not be an ideal model for evaluating human toxicity.

Hutapea *et al.* reported a steviol-16,17-epoxide stevioside metabolite (130). Given the structures of stevioside and rebaudioside A, an epoxide is a likely metabolite. The possibility of a steviol glycoside forming an epoxide metabolite needs to be investigated carefully, because epoxides may react with DNA and cause mutations.

Genotoxicity studies raised significant concerns. Suttajit *et al.* reported positive results for reverse mutations in the *S. typhimurium* strain TA98 with and without S9 extract at a 50 mg/plate dose of stevioside (142). Results by Klongpanichpak *et al.* have been used to discredit the work of Suttajit, but Klongpanichpak used S9 extract while the mutagenic results from Suttajit were highest without S9 (143). The ability of stevioside and rebaudioside A to cause reverse mutations as indicated by TA98 needs to be further investigated because such mutations suggest the possibility of carcinogenesis. Stevioside also caused DNA breakage in blood, spleen, liver, and brain cells in rats (144). The mutagenicity of this compound requires further, careful investigation.

Steviol was positive in an *umu* test, mutagenic in a forward-mutation assay, and caused chromosome aberrations and gene mutations in mammalian cells and plasmid mutagenesis (138, 145). Pezzuto *et al.* found that steviol is both toxic and mutagenic in the TM677 assay using S9 extract (137). Matsui's studies were all conducted with S9. These results indicate that steviol has a mutagenic metabolite that has yet to be identified. These finding are very important because rebaudioside A is hydrolyzed into steviol before it is absorbed by the GI tract. The mutagenic steviol intermediate needs to be identified and further studied and should have been before rebaudioside A was labeled as GRAS. Overall, because of the warning flags raised by several studies, it is critical that further genotoxicity testing be conducted to clarify the potential risks.

Carcinogenicity studies showed stevioside to be noncarcinogenic in rats (149-151). However, as noted above, the rat is not an ideal model for evaluating steviol glycoside toxicity and carcinogenicity risks in humans because of the differences in metabolism in the two species. Carcinogenicity studies in another rodent species (ideally one whose metabolism of steviol glycosides and steviol was closer to that of humans) would be necessary to confirm the carcinogenicity data of stevioside. Also, based on the differences in pharmacokinetics between rebaudioside A and stevioside, rebaudioside A itself should be tested in two rodent species.

Studies also report mutagenic activity, chromosomal damage, and DNA breakage for stevioside and steviol (137, 138, 142, 144, 145). In addition, based on a maximum estimated intake level of steviol glycosides of 1.7 mg/kg bw/day (steviol equivalent), steviol glycosides meet the

requirements to be categorized as a concern level III chemical (121). The FDA recommends carcinogenicity studies in *two* rodent species (usually mice and rats) for concern level III food additives. Importantly, bioassays of chemicals with a variety of structures that *did not* find carcinogenicity in rats *did* find carcinogenicity in mice (see Appendix B). A lifetime carcinogenicity study in mice of rebaudioside A should have been conducted before it (or other steviol glycosides) can be classified as a GRAS additive that likely would be consumed by tens of millions of people.

In conclusion, the FDA should ensure that the genetic toxicity studies that produced either positive or conflicting results be repeated. Studies that look at potential DNA adducts related to the potential reactive metabolites (C-13 carbonium ion or the epoxide) of steviol would be a strong addition to the genotoxicity data. Finally, the FDA should require carcinogenicity and toxicology studies on rebaudioside A using B6C3F1 mice and F344 rats according to the standard NTP bioassay for carcinogenesis before accepting rebaudioside A as a GRAS substance or approving it as a food additive. Ideally, all those studies would be conducted by an independent party, such as the National Toxicology Program of the National Institute of Environmental Health Sciences.

Chapter 4: A critical review of the risk characterization of artificial food dyes[7]

Abstract

Food dyes, synthesized originally from coal tar and now petroleum, have long been controversial because of safety concerns. Many dyes have been banned because of their adverse effects on laboratory animals or inadequate testing. This review evaluates the risk assessment data available for all of the nine currently US-approved dyes and finds health concerns of varying degrees for each dye. Red 3 causes cancer in animals, and there is evidence that several other dyes also are carcinogenic. Three dyes (Red 40, Yellow 5, and Yellow 6) have been found to be contaminated with benzidine or other carcinogens. At least four dyes (Blue 1, Red 40, Yellow 5, and Yellow 6) cause hypersensitivity reactions. Numerous microbiological and rodent studies of Yellow 5 were positive for genotoxicity. Toxicity tests on two dyes (Citrus Red 2 and Orange B) also suggest safety concerns, but Citrus Red 2 is used at low levels and only on some Florida oranges and Orange B has not been used for several years. The inadequacy of much of the testing and the evidence for carcinogenicity, genotoxicity, and hypersensitivity, coupled with the fact that dyes do not improve the safety or nutritional quality of food, indicate that the benefits of adding the dyes to the food supply do not out-weigh the concerns of their potential harmful effects. All of the currently used dyes should be removed from the food supply and replaced, if at all, by safer colorings. It is recommended that regulatory authorities require better and independent toxicity testing and conduct a rigorous benefit versus potential harm analysis before approving use of dyes as food additives.

[7] This chapter is from a paper that has been accepted for publication and is awaiting revisions. Modifications have been made to this chapter from the published version.

Introduction

Synthetic dyes have been used to artificially color foods in industrialized nations for at least a century, and they are used in thousands of foods sold in the United States (159). Foods are artificially colored to make unattractive mixtures of basic ingredients and food additives acceptable to consumers.[8] Added colors can also mask the absence of brightly colored natural ingredients such as fruit.

Dyes are complex organic chemicals that were originally derived from coal tar, but now are made from petroleum. Industrial food producers use synthetic dyes because they are cheaper, more stable, and brighter than most natural colorings. However, they raise significant health concerns. Over the past century, more food dyes have been found to be risky than any other category of food additive.[9] At this time, consumers' growing preference for natural foods is leading some companies to either avoid colorings or to switch to safe natural colorings, such as beta-carotene (a precursor to vitamin A), paprika, and beet juice. That trend is stronger in Europe than the United States, but some US companies recognize that an ''All Natural'' label can attract customers and may be moving in that direction.

[8] For a list of all approved synthetic and natural colorings, see FDA, 2007

[9] [Banned dyes include: Green 1: liver cancer (animals); Orange 1 and Orange 2: organ damage (animals); Orange B (ban never finalized): contained low levels of a cancer-causing contaminant (it was used only in sausage casings, but is no longer used in the US); Red 1: liver cancer (animals); Red 2: possible carcinogen; Red 4: high levels damaged adrenal cortex of dog; Red 32: damages internal organs and may be a weak carcinogen (since 1956, it continues to be used as Citrus Red 2 only to color oranges at 2 parts per million); Sudan 1: toxic and carcinogenic (animals); Violet 1: cancer (animals) (was used to stamp the US Department of Agriculture's (USDA) inspection mark on beef carcasses); Yellow 1 and Yellow 2: high dosages caused intestinal lesions (animals); Yellow 3: high dosages caused heart damage (animals); Yellow 4: high dosages caused heart damage (animals). Note, though, that in some cases companies did not bother to go to the expense of re-testing chemicals, which may not have accounted for significant sales, that might have been harmful only at high dosages and not at the lower dosages consumed in foods. http://cspinet.org/reports/chemcuisine.htm.]

Just three dyes — Red 40, Yellow 5, and Yellow 6 — account for 90% of all dyes used. US Food and Drug Administration (FDA) data show a dramatic five-fold increase in consumption of dyes since 1955 (Fig. 4.1) as people in the United States have increasingly relied on processed foods, such as soft drinks, breakfast cereals, candies, snack foods, baked goods, frozen desserts, and even pickles and salad dressings that are colored with dyes (160). This paper includes a brief discussion of the laws regulating food dye use, followed by toxicological evaluations of four dyes: Red 3, Red 40, Yellow 5, and Yellow 6. Evaluations of the other food dyes — Blue 1, Blue 2, Citrus Red 2, Green 3, and Orange B — are provided in Appendix C.

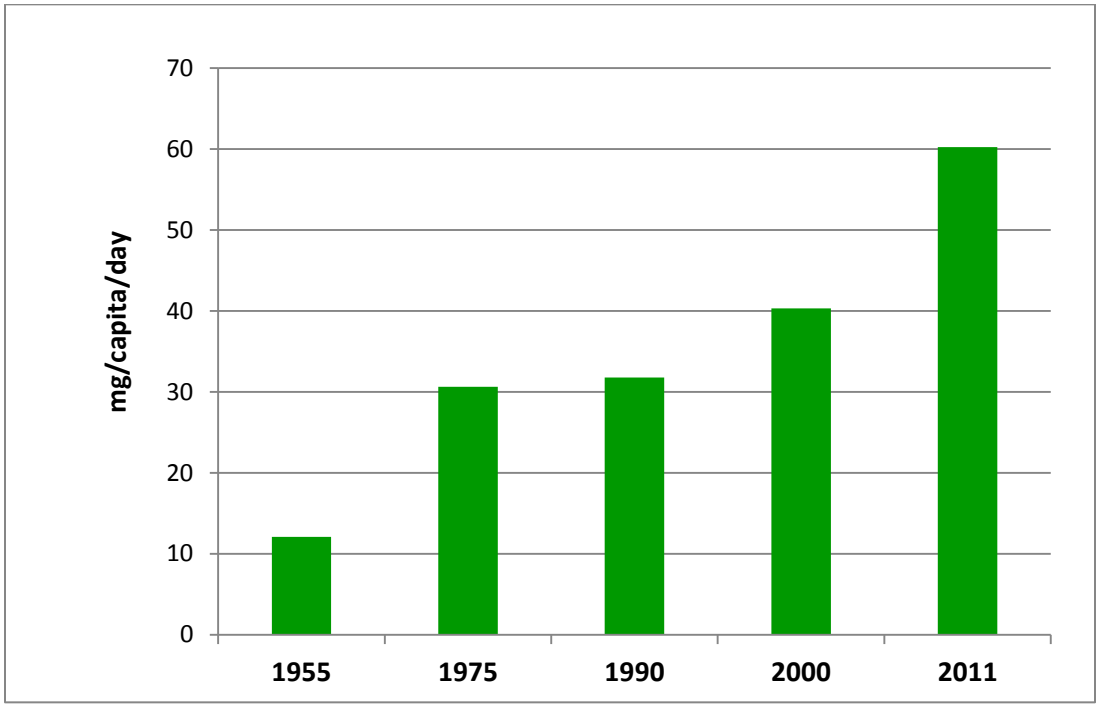

Figure 4.1. Food dyes marketed per capita per day (mg).

Methods

Eligibility criteria

Information about the nine dyes currently approved by the FDA was obtained from published, peer-reviewed studies, as well as unpublished studies and other writings that were used in the risk assessment and approval process. Studies were not restricted by date, language, or source. Studies judged to be of inadequate and/or unnecessary for this review were excluded.

Search strategy and study selection

Articles and information were found using the following five methods: (1) searches of the US National Library of Medicine's PUBMED; (2) publicly available government documents; (3) Internet searches; (4) news articles; and (5) personal correspondence and memoranda in the Center for Science in the Public Interest's (CSPI) files. Relevant articles were identified in PUBMED without restriction on date or source for experimental studies. The primary search strategy was entering each dye as a key word in multiple formats including: ''FD&C Yellow #5'', ''Tartrazine'', ''Yellow 5'', and ''FD&C Yellow No. 5''. Other key words included: ''toxicity'', ''hypersensitivity'', ''chronic'', ''metabolism'', and ''carcinogenicity''. Government documents, predominantly those published in the Federal Register, were obtained from http://www.federalregister.gov or http://www.heinonline.org (subscription required). Other documents were collected from requests of the authors. Information from websites used the most updated webpage versions; access dates are provided in references. News articles were not restricted by region or date and were obtained from personal collections of the authors or through archival searches, including http://www.nexis.com (subscription required). Personal correspondence and memoranda were between the authors and another party via phone, mail or

email, or from meetings. Original letters and emails are saved and are in possession of the authors.

Data extraction

Data used in this review were taken directly from the cited sources. In several cases, information was confirmed with the authors of the original text or with experts in the field.

Food Dyes and the Law

Prior to 1960, US law required that dyes be absolutely ‘‘harmless,’’ regardless of dose — a virtual impossibility (161). Congress passed the 1960 Color Additives Amendment in order to loosen requirements on food dye use, while retaining, along with the FDA, special concerns about the safety of food dyes. James T. O’Reilly, an adjunct professor at the University of Cincinnati College of Law, observed that ‘‘Congress felt that … colors deserved greater regulation because of their lesser net benefit to society than such items as food preservatives and common spices’’ (162). For instance,

- Congress required that each batch of food dyes, but not other colorings (such as from carrots or grape skins), be tested and certified to contain only acceptable levels of contaminants, such as lead and benzidine. Food additives, such as preservatives or flavorings, are not subject to such testing.
- Congress did not permit companies to declare that any dyes are ‘‘generally recognized as safe’’ (GRAS), and thereby not further regulated by the FDA. In contrast, companies are permitted to declare flavorings, emulsifiers, and other such ingredients to be GRAS, even in the absence of toxicity testing.

- The FDA's definition of safety for color additives states that ''safe means that there is convincing evidence that establishes with reasonable certainty that no harm will result from the intended use of the color additive'' (163). The term ''convincing evidence'' is a stronger standard of proof than that used for noncolor additives.

Members of Congress have emphasized that the safety standard for artificial colorings should be particularly high because the colorings do not offer any health benefit to offset even small risks. Representative Ted Weiss (D-NY) said, ''It doesn't make any difference how much or how little (of a carcinogenic additive) a particular substance contains, especially when you've got a color additive that has no nutrient value and no therapeutic value.''(164). Representative King (It is unclear which Rep. King was quoted in the case: Rep. Cecil King (D-CA) or Rep. David King (D-UT).) said, ''The colors which go into our foods and cosmetics are in no way essential to the public interest or the national security …. Consumers will easily get along without (carcinogenic colors).'' (165). Unfortunately, as evidenced by the continual approval of dyes for which there is evidence of carcinogenicity, enforcement of the 1960 law has been inadequate.

The FDA has also established legal limits for cancer-causing contaminants in dyes. Those tolerances are intended to ensure that a dye will not pose a lifetime risk of greater than one cancer in one million people (166). FDA chemists test each batch of dye to confirm that those tolerances are not exceeded. Unfortunately, the FDA's process suffers from several problems. For one thing, those tolerances are based on 1990 dye usage, but per-capita usage has increased by about 50% since then. Second, the FDA did not consider the increased risk that dyes pose to children, who are both more sensitive to carcinogens and consume more dyes per unit of body

weight than adults (167). Third, and most importantly, the tests do not look for ‘‘bound’’ carcinogens (those that occur as parts of larger molecules and are freed during digestion), but generally only ‘‘free’’ contaminants (168).

Consumer activists have long sought to persuade the FDA to ban dyes. In the early 1970s, CSPI urged the government to ban Violet 1, which was the coloring used in the United States Department of Agriculture’s (USDA) meat inspection stamp, because it appeared to cause cancer in animal studies (the dye was banned in 1973). Subsequently, in the 1970s and 1980s, Public Citizen’s Health Research Group petitioned and sued the FDA to ban food dyes (169). In 2008, CSPI petitioned the FDA to ban colors because of their adverse effects on children’s behavior.

Even if all color additives were deemed safe, many uses of colorings, both synthetic and natural, still could be considered illegal under the Food, Drug, and Cosmetic Act. Sections 402(b)(3) and (b)(4) of that law stipulate that ‘‘A food shall be deemed to be adulterated …(3) if damage or inferiority has been concealed in any manner; or (4) if any substance has been added thereto or mixed or packed therewith so as to…make it appear better or of greater value than it is.’’ Section 403 of the same law says that a food is misbranded ‘‘if its labeling is false or misleading in any particular’’.

Food colorings added to fruit drinks, frozen desserts, gelatin desserts, salad dressings, child-oriented breakfast cereals and snack foods, and countless other products conceal the absence of fruits, vegetables, or other ingredients and make the food ‘‘appear better or of greater value than it is’’. Defenders of colorings would say that consumers could simply read the list of ingredients

on the back of the package to detect the presence of colorings and/or absence of nutritive ingredients, but it may be unfair to put that burden on consumers. It is worth noting that the use of artificial flavorings must be declared conspicuously as part of the product name on the front labels (170). The FDA could require the same of artificially colored foods. A national poll commissioned by CSPI and conducted by Opinion Research Corporation in January 2010 found that 74% of respondents favored such labeling.

Toxicology review of individual dyes

A summary of study results for all dyes are found in Table 4.1. The currently approved FD&C dyes are Blue 1 (Brilliant Blue), Blue 2 (Indigo Carmine), Citrus Red 2, Green 3 (Fast Green FCF), Orange B, Red 3 (Erythrosine), Red 40 (Allura Red), Yellow 5 (Tartrazine), and Yellow 6 (Sunset Yellow). Blue 1, Red 40,Yellow 5, and Yellow 6 cause allergic reactions. Blue 1 did not cause tumors in rats and one unpublished study reported kidney tumors in mice; however, the latter study did not include an *in utero* exposure. An *in vitro* study showed that Blue 1 inhibited nerve cell development. Blue 2 did not induce tumors in mice, but neither study was long enough nor included an *in utero* exposure. Blue 2 possibly causes brain and bladder tumors in rats. Citrus Red 2 induced bladder and other tumors in mice and bladder tumors in rats. A study on Green 3 without *in utero* exposure did not induce tumors in mice. However, a rat study possibly induced bladder and other tumors. Orange B was found to be toxic in rats but was not carcinogenic in mice. The mouse studies did not include *in utero* exposure. Red 3 was not carcinogenic in mice, but the only study did not include an *in utero* exposure. Red 3 did induce thyroid tumors in rats. Red 40 is often contaminated with aniline. It possibly induces reticuloendothelial (RE) tumors in mice but did not induce tumors in rats. Yellow 5 has been

Table 4.1. Summary of studies on FD&C dyes					
Food dye	Allergic reactions	Carcinogenic contaminants	Tests for cancer*		Other**
			Mouse	Rat	
Blue 1	Yes		No *in utero* studies. One abstract (study not published) reported kidney tumors.	No tumors in the only good study.	Test tube study found inhibition of nerve-cell development.
Blue 2			Both studies were too brief and did not include *in utero* exposure.	Dosage was likely too low; possible brain and bladder tumors.	
Citrus Red 2 (used only on peels of some oranges at 2 ppm)			Bladder and other tumors	Bladder tumors	
Green 3			The only study did not include *in utero* exposure.	Possible bladder and other tumors	
Orange B (no longer used; in 1978 FDA proposed, but never finalized, a ban)			The only two studies did not include *in utero exposure*.	Toxic	
Red 3 (FDA has banned it from cosmetics**,** externally applied drugs, and lakes)			The only study did not include *in utero* exposure.	Thyroid tumors	
Red 40	Yes	Aniline[10]	Possible reticuloen-dothelial tumors of the immune system	No tumors in the only good study	
Yellow 5	Yes	Benzidine, C-mino-BiPhenyl	The only mouse study was too brief, used too few mice, and did not include *in utero* exposure.	No tumors in the only good study	6 of 13 studies showed geno-toxicity. Hyperactivity in children.
Yellow 6	Yes	Benzidine, C-mino-BiPhenyl	Neither study included *in utero* exposure.	Possible adrenal and testicular tumors.	

* Tests should be done on both sexes of two rodent species, use sufficient numbers of animals, include in utero exposure, last at least two years after birth, and use maximally tolerated dosages. Ideally, tests would be conducted by independent labs, but most tests on dyes were conducted by industry.

** In addition, studies have found that mixtures of dyes cause hyperactivity and other behavioral impairments in children.

[10] Not all agencies consider aniline to be carcinogenic.

found to be contaminated with benzidine and C-mino-BiPhenyl. It was not carcinogenic in mice, but the only study was too short, did not use the recommended number of animals (121), and did not include an *in utero* exposure. Yellow 5 also did not induce tumors in rats. Six out of 11 genotoxicity studies were positive. It has also been shown to cause hyperactivity in children. Yellow 6 has been found to be contaminated with benzidine and C-mino-BiPhenyl. Yellow 6 did not induce tumors in mice, but these studies did not include an *in utero* exposure. It possibly causes adrenal and testicular tumors in rats.

FD&C Red 3

FD&C Red 3 (Fig. 4.2), or Erythrosine B, has been used as a food dye since its approval by the USDA in 1907. It is a water-soluble dye with a 58% iodine content (171). It is used in maraschino cherries, sausage casings, oral drugs, baked goods, and candies. The Acceptable Daily Intake (ADI) for Red 3 is 2.5 milligrams per kilogram of body weight per day (mg/ kg bw/day) or 75 mg/day for a 30-kg child (172). Annual production of Red 3 is equivalent to about 1 mg/person/day (per capita production figures are based on FDA data on the amounts of dyes certified per year).

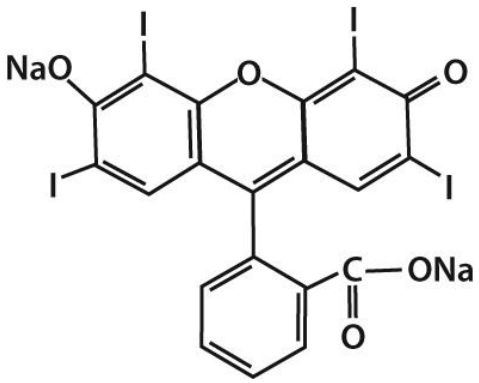

Figure 4.2. FD&C Red No. 3 (Erythrosine B)

Metabolism

Osborne–Mendel rats were administered 0.5–500 mg/ kg bw/day Red 3 by stomach tube. Qualitative analysis demonstrated that the dye excreted in the urine or bile was unchanged (173).

In another study, 14 male rats were given one dose (0.5 mg/kg bw) of Red 3. Approximately 55–72% was excreted unchanged in the feces within 3 days. In two bile-duct cannulated rats, 0.44 and 1.67% of the dye was excreted in the bile, indicating that a small amount is absorbed. No color was recovered in the urine. Investigators concluded that ‘‘Red 3 is metabolized to some extent in the tissue’’ (174). Rats administered Red 3 twice weekly for 3 months at doses (according to an industry petition) of 5, 10, 15, and 50 mg/200–250 g bw had elevated serum levels of protein-bound and total iodine (175). Butterworth *et al.* also showed that rats administered Red 3 at 0–2% dietary doses over 13 weeks had a dose-related increase in serum levels of protein-bound and total iodine (176).

In a human study, subjects were orally administered 16 mg of Red 3 for 10 days (more than 15 times typical consumption). Subjects had approximately twice as much protein-bound iodine in their serum compared to levels prior to administration. Levels peaked around days 15–20 and did not return to normal until about 3 months after the beginning of the study (177).

In vitro effects on neurotransmitters

Red 3 was applied to isolated frog neuromuscular synapses to test its effect on neurotransmitter release using electrophysiological techniques. Concentrations of 10 mmol/l and greater caused an irreversible, dose-dependent increase in acetylcholine release. Investigators concluded that Red 3 may alter the function of more complex systems, but any conclusions regarding its effects on mammalian behavior would be premature given the *in vitro* nature of the study (178).

Genotoxicity

Of twelve genotoxicity studies on Red 3, four were positive, including one *in vivo* study, which demonstrated the genotoxic potential of the dye (179-181). (Table 4.2 shows numbers of positive and negative genotoxicity tests for each food dye studied.) Of particular concern is that the positive results were in studies using mammalian cells or an *in vivo* method (comet assay), while most of the negative results came from prokaryotic systems. The genotoxicity studies on Red 3 are summarized in Table 4.3.

Table 4.2. Numbers of positive and negative genotoxicity studies of FD&C food dyes

FD&C Color (generic name)	Total Number of Positive Studies	Positive *in vivo* Studies*	Negative Studies
Blue No. 1 (Brilliant Blue)	2	0	7
Blue No. 2 (Indigo Carmine)	1	0	10
Green No. 3 (Fast Green)	3	0	6
Red No. 3 (Erythrosine)	4	1	8
Red No. 40 (Allura Red)	3	3	7
Yellow No. 5 (Tartrazine)	6	2	7
Yellow No. 6 (Sunset Yellow)	2	1	8

* The numbers of "Positive *in vivo* studies" are included in "Total Number of Positive Studies."

Chronic toxicity/carcinogenicity

Chronic toxicity studies focusing on the effects of Red 3 on hematology, thyroxine, and protein-bound iodide in Osborne–Mendel rats did not find any adverse effects. Twenty-five rats/sex/group were fed 0 (the only group with 50 rats/sex), 0.5, 1, 2, or 4% Red 3 for 86 weeks or intubated twice weekly with 0, 100, 235, 750, or 1500 mg/kg Red 3 for 85 weeks. The study did not include an *in utero* phase. At the end of the treatment periods, the rats were fed the control diet until the studies reached the 2-year mark. The studies found no adverse effects in gross or microscopic pathology and no changes in thyroxine-iodide levels. The levels of protein-bound iodide increased, and it was determined that this was due to increased dye levels in the serum (182).

The Certified Color Manufacturers Association (CCMA) contracted with Borzelleca *et al.* to conduct a chronic toxicity/carcinogenicity study in Charles River CD-1 mice. The maximum duration of exposure of the mice to 0 (two control groups), 0.3, 1, or 3% Red 3 was 24 months (no *in utero* exposure). All groups consisted of 60 males and 60 females. Investigators reported no statistically significant compound-related effects on behavior, morbidity, mortality, hematology, or general physical observations. A statistically significant increase in the incidence of lymphocytic lymphoma was observed in male mice in the 0.3% low-dose group. However, that effect was not considered compound-related, because there was no dose–response relationship, and the incidence of lymphomas in the high-dose group was similar to that in the controls. The NOAELs (no observed adverse effects levels) were deemed to be 3% (4759 mg/kg bw/day) in males and 1% (1834 mg/kg bw/day) in females (183).

Table 4.3. Summary of genotoxicity studies on Red No. 3.					
Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	100 mg/kg in glandular stomach and colon; >100 mg/kg in urinary bladder	Positive after 3 hours; negative after 24 hours	(181)
S. Typhimurium TA1535, TA100	Base pair	Yes and No	1-10 mg/plate	Negative	(171)
S. Typhimurium TA1537, TA98, TA1538	Frameshift	Yes and No	1-10 mg/plate	Negative	
Mouse lymphoma assay ($L5178Y/TK^{+/-}$)	Gene mutation	No	100-600 μg/ml	Negative	
Mouse micronucleus Assay					
Rec-assay	Chromosomal breakage/spindle damage	Yes	24, 80, 240 mg/kg	Negative	
	DNA damage	NA	100-10,000 μg/ml	Negative	
E. coli WP2 *uvrA*	Base substitution	Yes and No	0.5 mg/ml	Negative	(184)
In vitro chromosome aberrations in Chinese Hamster fibroblast cells	Chromosome aberrations	No	0.6 mg/ml	Positive	(179)
Yeast strain D7	Mitotic gene conversion	NA	0-10 mg/ml	Positive	(180)
Yeast strain XV185-14C	Reverse mutation in eukaryotes	NA	0-10 mg/ml	Positive	
Yeast strain D5	Mitotic recombination	NA	0-5 mg/ml	Negative	

Borzelleca *et al.* also performed two CCMA-sponsored chronic toxicity/carcinogenicity studies in Charles River CD rats. Unlike the mouse study, these studies included an *in utero* phase. In the F_0 generation of both studies, 60 rats/sex/group were fed 0 (two control groups), 0.1, 0.5, or 1% (original study) and 0 or 4% (high-dose study) Red 3. Random offspring were selected for the F_1 generation and 70 rats/sex/group were given the same dietary levels as the F_0 generation. The maximum exposure was 30 months. Investigators reported no compound-related effects on fertility, gestation, parturition, lactation, pup survival through weaning, or numbers of live and stillborn pups. The most notable effects of the chronic feeding phase were statistically significant increases in the incidences of thyroid follicular cell adenomas in male rats in the 4% treatment group (15 adenomas in the 4% group compared to one in the control groups) and non-significant increases in these tumors in female rats in the 0.5, 1, and 4% treatment groups. High-dose (4%) male rats also showed a statistically significant increase in non-neoplastic proliferative changes of the thyroid. The changes included follicular cell hypertrophy and hyperplasia and follicular cystic hyperplasia. Also, 94% of male rats in the 4% treatment group showed proliferative changes of thyroid follicular cells. Based on the results of the two studies, investigators asserted that Red 3 had NOAELs of 0.5 and 1% in male and female rats, respectively (185).

Reproductive toxicity

In each generation of a 3-generation study on Red 3 in Sprague–Dawley rats, 25 rats/sex/group received 0, 0.25, 1, or 4% of the dye in their chow. The only significant finding was a statistically significant reduction in body weights of parents and pups in all generations at the 4%

dietary level, which could have been due to the large consumption of a non-nutritive compound. No compound-related adverse effects on reproductive indices and no gross anomalies were seen. Investigators concluded that the NOAEL for rats was 0.25%, or approximately 149 and 255 mg/kg bw/day for males and females, respectively. That NOAEL was based on the reduced body weight in the 4% group and reduced body-weight gain during gestation in females in the 1% and 4% groups (186).

FDA efforts to ban Red 3

Red 3 is genotoxic in *in vivo* and *in vitro* assays and is an animal carcinogen. Petitioners seeking Red 3 approval submitted CCMA-sponsored studies after provisional listings in 1960.[11] The CCMA studies showed no safety concern, and in 1969 the FDA permanently approved the dye for use in ingested drugs and foods (188). However, in 1984, FDA's Acting Commissioner, Mark Novitch, said that Red 3 was ''of greatest public health concern …. The agency should not knowingly allow continued exposure (at high levels in the case of FD&C Red No. 3) of the public to a provisionally listed color additive that has clearly been shown to induce cancer while questions of mechanism are explored'' (169). Around the same time, Secretary of Agriculture John R. Block was pressing his counterpart, at the Department of Health and Human Services, Secretary Margaret Heckler, not to ban the dye (189). He wrote, 'Some segments of the agricultural community are quite dependent on Red Dye #3 in the processing and marketing of

[11] Food colorings that were in use when the Color Additives Amendment of 1960 (21 USC 1 379e) was passed were 'provisionally listed' pending further testing by industry. Some colorings were subsequently permanently listed, while some were eliminated from the food supply because their safety was not demonstrated, in some cases because industry did not care to market them 187. FDA/CFSAN. Background document for the food advisory committee: certified color additives in food and possible association with attention deficit disorder in children. 2011(http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/FoodAdvisoryCommittee/UCM248549.pdf):accessed February 4, 2012.

certain commodities, especially canned fruits. I have assured the affected industry that their concerns would be made known to you, as well as my own concern …'. In 1989, at the behest of growers and packers, the House of Representatives told the FDA not to ban the dye until it had done further review of the scientific studies (190, 191). Red 3 petitioners claimed that the color acts as a secondary rather than primary carcinogen and therefore was exempt from the Delaney Clause. However, in 1990, the FDA concluded that Red 3 was not proven to be a secondary carcinogen and that ''FD&C Red 3 is an animal carcinogen'' (192). In 1990, the FDA terminated the provisional listing of Red 3 for use in cosmetics and externally applied drugs; all uses of Red 3 lakes (lakes are water-insoluble forms of dyes and typically contain aluminum) were also banned (192). At the time, the FDA estimated that the lifetime risk of thyroid tumors imposed by Red 3 ''was at most 1 in 100,000'' (188). Based on today's population, that would indicate that Red 3 is causing cancer in about 3000 people.

Notwithstanding its 1990 finding that Red 3 is an animal carcinogen, the agency still permits Red 3 in ingested drugs and foods, though in 1990 it was reported to have said it would ''take steps'' to ban those uses, too (193). As of 2012, the FDA still had not acted.

Conclusions

The harm that Red 3, an acknowledged animal carcinogen, is likely causing far outweighs the effort entailed in banning the dye. It is worth noting that Red 3 has been seen as invaluable by some makers of maraschino cherries, but other brands are dyed with Red 40 or have no added coloring and some brands (Del Monte, Giant) of canned fruit cocktail contain cherries colored with natural colorings. However, the natural colorings used, carmine or cochineal extract, can

cause severe allergic reactions. About 5 million pounds of Red 3 have been used since the FDA's acting commissioner stated that the dye should not be used.

FD&C Red 40

Red 40 (Fig. 4.3) or Allura Red, is approved for use in beverages, bakery goods, dessert powders, candies, cereals, foods, drugs, and cosmetics and, in terms of pounds consumed, is by far the most-used dye (Table 4.4). Red 40 has an ADI of 7 mg/kg bw/day (194). That ADI translates into 210 mg for a 30-kg child. Companies produce the equivalent of about 25 mg of the dye per person per day, with many children, to whom colorful cereals, candies, snack foods, and dairy products are marketed, may consume several times as much.

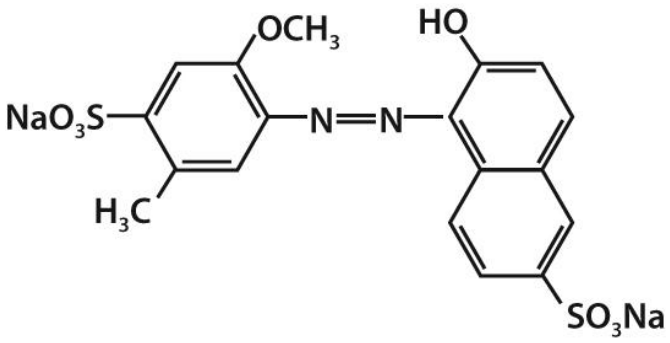

Figure 4.3. FD&C Red 40 (Allura Red)

Metabolism

In an unpublished report, rats were fed a diet with 5.19% Red 40. While 0.1% was excreted in the urine, 29% of the dye was excreted intact in the feces. The parent dye appears to be broken down by gut flora via azo-reduction into two metabolites, cresidine-4-sulfonic acid and 1-amino-2-naphthol-6-sulfonic acid (195). In another study, rats and dogs were pretreated daily for 3 days with unlabeled Red 40 followed by a tracer of ^{36}S-Red 40 for up to 72 hours. Within 72 hours, 92–95% and 76– 92% of the radioactivity in feces and 5.7–19.8% and 2.7–3.6% in urine

was recovered from dogs and rats, respectively. There was significant retention of radioactivity in the guts of animals (195).

Table 4.4. Food dye certification by the FDA in fiscal year 2011		
Food dye	Pounds of total dye (includes lakes)	Percentage of Total
Blue 1 21 CFR 74.101	706,997	4.7
Blue 2 21 CFR 74.102	556,643	3.7
Citrus Red 2 21 CFR 74.302	2,734	0.0
Green 3 21 CFR 74.203	16,746	0.1
Orange B 21 CFR 74.250	0	0
Red 3 21 CFR 74.303	219,560	1.5
Red 40 21 CFR 74.340	5,487,226	36.4
Yellow 5 21 CFR 74.705	4,221,745	28.0
Yellow 6 21 CFR 74.706	3,862,135	25.6
TOTAL	**15,073,786**	**100**

Genotoxicity

Red 40 was negative in seven genotoxicity assays, but positive in the *in vivo* comet assay in the glandular stomach, lungs, and colon of mice (181). That indicates that Red 40 can cause DNA damage *in vivo*. Details of the genotoxicity assays on Red 40 are provided in Table 4.5.

Table 4.5. Summary of genotoxicity studies on Red No. 40.					
Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	10 mg/kg in colon; 100 mg/kg in glandular stomach; 1,000 mg/kg in lungs	Positive	(181)
Comet Assay	DNA damage	NA	2,000 mg/kg to pregnant mice 10 mg/kg in male mice	Positive in colon Positive in colon	(181)
E. coli WP2 *uvrA*	Base substitution	Yes and No	10 mg/ml	Negative	(197)
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	50-500 μg/plate	Negative	(198)
S. Typhimurium TA98, and TA1537	Frameshift	Yes and No	50-500 μg/plate	Negative	
S. Typhimurium TA1535 and TA100	Base pair	Yes and No	0.2-400 μg/plate	Negative	(199)
S. Typhimurium TA98, and TA1537	Frameshift	Yes and No	0.2-400 μg/plate	Negative	
S. Typhimurium TA 1535 and TA 1538	Base pair (TA1535) and Frameshift (TA1538)	Yes and No	1, 10, 50, 100 and 250 μg/plate	Negative	(200)
Yeast strains D-3 and D-5	Mitotic recombination	Yes and No	10 mg/ml	Negative	

Hypersensitivity

Fifty-two patients suffering from urticaria (hives) and angioedema for more than 4 weeks were placed on a 3-week elimination diet (free of synthetic dyes and other food ingredients or

additives that might be allergenic). Red 40 administered orally in doses of 1 or 10 mg induced a hypersensitivity reaction in 15% of the patients who were generally symptom-free at the time of provocation (196).

Chronic toxicity/carcinogenicity

In the 1970s, Hazleton Laboratories conducted chronic toxicity/carcinogenicity feeding studies of Red 40 in rats and mice, both of which included an *in utero* phase. Using Sprague–Dawley rats, the F_0 generation included 30 rats/sex/group that were administered 0, 0.37, 1.39, and 5.19% of Red 40 in their chow 1 week prior to mating, during mating, gestation, and lactation. F_1 rats, a group of 50 rats/ sex/group chosen at random from surviving F_0 offspring were exposed for 118 to 121 weeks. The F_0 and F_1 generations were exposed to the same dose. No compound-related effects of concern were reported. The studies indicated a NOAEL of 5.19% (2829 mg/kg bw/day) for males and 1.39% (901 mg/ kg bw/day) for females (201).

Hazleton Laboratories also performed two chronic toxicity studies of Red 40 in CD-1 mice. In the first study, 50 mice/sex/group (F_0) were administered 0, 0.37, 1.39, or 5.19% Red 40 in their chow 1 week prior to breeding through the gestation and lactation periods. The F_1 generation was randomly selected from surviving pups, and the chronic feeding study used 50 mice/sex/group. The dosages were the same in the F_0 and F_1 generations. At 42 weeks, a total of six RE tumors occurred in the males and females (zero in controls, one each in the low- andmid-dose groups, and four in the high-dose groups). That led the investigators to kill and examine 36% of the animals, reducing each group to 30 mice/sex/group. The remaining F_1 mice were fed Red 40 for a total of 104 weeks. By the end of the study, the investigators concluded that Red 40

did not accelerate the appearance of RE tumors (202). However, M. Adrian Gross, a senior FDA pathologist, concluded that there was clear evidence to support an acceleration effect on RE tumors, because there was a decreased latency period without a corresponding increase in overall tumor incidence (203).

A second mouse study was conducted to address the possibility that Red 40 accelerated the appearance of RE tumors, a sign of carcinogenicity (202, 204). Although the second study used the same dosage groups as the first, the studies differed in several respects. First, the initial study used Ham/ICR (CD-1) mice, while the second used CD-1 outbred mice. Second, the F_0 generation in the second study used 70 mice/sex/ group, and the F_1 generation consisted of 100 mice/ sex/group. Third, the second study did not include a 42-week interim killing. Fourth, the second study used two control groups instead of one. Finally, the mice in the second study were exposed to Red 40 for 109 weeks — 5 weeks longer than the first study (202).

The second study, according to the investigators, did not show an early appearance of or increase in RE tumors. However, the difference in RE death rates between the two control groups was statistically significant at the P=0.008 level (205). Only the high dose males and females experienced a significant increase in relative and absolute thyroid weight. The investigators set a NOAEL of 5.19% in mice or 7300 and 8300 mg/kg bw/day for males and females, respectively (202).

Limitations of the mouse studies

The first mouse study suggested a reduced latency period for RE tumors and small numbers of RE system tumors were seen in all treatment groups prior to the 42-week killing, the highest incidence being in the high-dose group. The FDA recommended killing 36% of the mice to gain information about the possible acceleration of occurrence of RE tumors, and the killings were done at week 42 of the 2-year study. However, that left a relatively small number of mice available at the end of the study and reduced the ability to analyze tumor incidence (205).

To better understand the results of the first mouse study, in 1976 the FDA created a working group of scientists from the FDA, National Cancer Institute (NCI), and the National Center for Toxicological Research to monitor the rat and mouse studies being performed for Allied Chemical. Midway through the second mouse study, the working group concluded that the first study did not indicate a risk of carcinogenesis. Following controversy over that conclusion, FDA Commissioner Donald Kennedy appointed four non-governmental statisticians, including Harvard's Frederick Mosteller and Stephen Lagakos, to review the statistical methods used to analyze the studies. Those statisticians were independent and not a part of the FDA working group (206).

Two problems found with the mouse studies included caging and litter effects (205). In the second study, the mice housed in the upper row of racks experienced a higher incidence of RE tumors than the mice in lower cages, according to the FDA consultants (207). The incidence of RE tumors was significantly correlated to the row ($P=0.0005$) and position ($P=0.02$) of the racks (207). The working group also noted that it was impossible to know if mice were being housed with siblings (litter effect), which might have had an influence on tumor incidence (206).

Confounders such as potential caging and litter effects strongly decrease the credibility of a study.

Also, there was a large variation in RE tumor rates between the two studies. That difference could have been due to the different strains of mice used in the two studies, but does raise questions about how to interpret the studies.

Regarding the statistical analyses of the two mouse studies, Lagakos and Mosteller commented that the difference in RE tumor rates between the two studies limited the conclusiveness of the results. They argued that the statistical methods used by the FDA Working Group were not oriented to detecting an acceleration effect (205). Their analysis concluded that both studies suggested a decreased latency period for, and increased incidence of, RE tumors (207).

Carcinogenic contaminants

As discussed below with regard to Yellow 5 and Yellow 6, Red 40 has been found to contain cancer-causing and other contaminants. Health Canada scientists, using a test method that could detect bound and free contaminants, identified small amounts of aniline, pcresidine, and 1-naphthylamine in the dye (208). p-cresidine is ‘‘reasonably anticipated to be a human carcinogen’’, according to the US National Toxicology Program (NTP), and ‘‘possibly carcinogenic to humans’’, according to the International Agency for Research on Cancer (IARC) (209, 210). The NCI and the FDA considered aniline to be weakly carcinogenic to rats, though other agencies have not determined that aniline and 1-naphthylamine pose a risk to humans (209, 211, 212).

Reproductive toxicity/teratogenicity

To investigate the potential embryotoxicity and teratogenicity of Red 40, pregnant female rats were dosed with 0, 7.5, 15, 30, 100, or 200 mg Red 40/kg bw daily on days 0–19 of gestation through intubation or 0 or 2 mg Red 40/kg bw daily through drinking water on days 0–20 of gestation. No adverse effects on reproduction, embryo-lethality, or feto-toxicity were reported (213).

Conclusions

There is evidence, albeit controversial and inconclusive, that Red 40, the most widely used dye, accelerates the appearance of tumors of the RE system in mice. Also, independent consultants (Lagakos and Mosteller) appointed by the FDA raised concerns about the FDA-appointed Working Group's statistical analysis of the data. Considering the positive results in comet genotoxicity assays, the disputed mouse chronic-toxicity studies, causation of hypersensitivity reactions, possible causation of hyperactivity in children, cancer-causing contaminants, and the non-essentiality of the dye, Red 40 should not be used in foods.

FD&C Yellow 5

FD&C Yellow 5 (Fig. 4.4), also known as Tartrazine, is used in numerous bakery goods, beverages, dessert powders, candies, cereals, gelatin desserts, pet food, and many other foods, as well as pharmaceuticals and cosmetics. After Red 40, it is the most widely used dye (Table 4.4). The ADI for Yellow 5 is 5 mg/kg bw/day, which equates to 150 mg/day for a 30-kg child (214).

Companies produce the equivalent of 15 mg of the dye per person per day, with many children likely consuming at least several times that much.

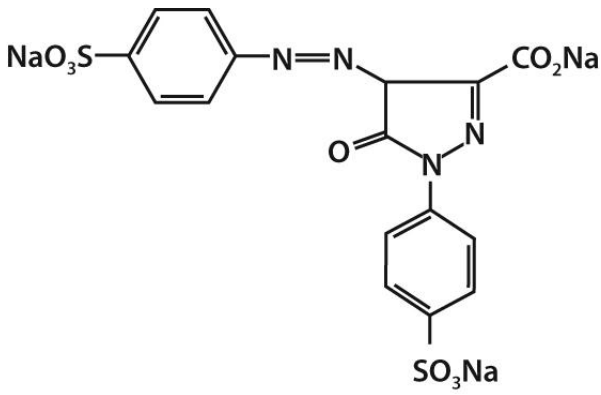

Figure 4.4. FD&C Yellow 5 (Tartrazine)

Metabolism and metabolic effects

Sulfanilic acid is a metabolite that results from the reduction of Yellow 5 at the N=N azo link. However, when Yellow 5 labeled at the phenylazo group with ^{14}C was administered intraperitoneally in rats and rabbits, no radioactive sulfanilic acid was recovered in the urine (215). In the same study, when Yellow 5 was administered orally to rats, rabbits, and humans, sulfanilic acid, but little or no unchanged dye, was recovered in the urine. Those results indicate that the reduction of Yellow 5 occurs via the GI flora. Ryan *et al.* confirmed that Yellow 5 is primarily metabolized by the gut microflora of rats after an oral dose (216).

Apart from the metabolism of the dye, a 50-mg dose of Tartrazine, but not Amaranth (the generic name for the now-banned FD&C Red 2), led to increased or accelerated urinary excretion of zinc in hyperactive children (217). Whether the effect on zinc is a cause of hyperactivity is not known.

Genotoxicity

Potential genotoxicity of Yellow 5 was tested in 13 studies, with six studies, including two *in vivo* studies, showing positive effects (Table 4.6). A 1985 report from the US Department of

Health and Human Services (HSS) criticized two of the genotoxicity studies and disagreed with their conclusions that Yellow 5 induces chromosomal aberrations (179, 218, 219). However, the HHS report stated, 'If chromosome aberrations of the type reported for Tartrazine in cultured cells occurred *in vivo*, they certainly would represent a serious adverse effect.' Sasaki *et al.* subsequently demonstrated that Yellow 5 does induce DNA damage *in vivo* in the comet assay (181).

Chronic toxicity/carcinogenicity

The earliest chronic feeding study reported that Yellow 5 was not carcinogenic or toxic in a 2-year study using Osborne–Mendel weanling rats.[12] The rats were fed 0, 0.5, 1, 2, and 5% Yellow 5 (221). However, that study used only 12 rats of each sex per dosage group. The FDA recommends a minimum of 20 rodents/sex/group for chronic toxicity studies, though many experts consider that far too small a number (121). Also, the rats were not exposed *in utero* (221).

Later, in a feeding study sponsored by CCMA, 70 Charles River CD rats/sex/group were exposed to 0, 0.1, 1, 2, or 5% Yellow 5 starting *in utero* for either 30 months or until only 10 rats/sex/group survived (222). The researchers did not find any compound-related effects on fertility, gestation, parturition, lactation, pup survival, or number of still-born pups. Complete histopathology was performed on all killed animals, and gross necropsies were conducted on

[12] Davis *et al*. also tested three groups of two male and two female beagles for 2 years at dosages of 0, 1, and 2% Yellow 5, but that small number of dogs and the brevity of the test do not permit conclusions about the long-term effects of the dye 221. Davis KJ, Fitzhugh OG, Nelson AA. Chronic rat and dog toxicity studies on tartrazine. Toxicology and Applied Pharmacology1964;6:621-6..

Table 4.6. Summary of genotoxicity studies on Yellow No. 5.					
Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	10 mg/kg >10 mg/kg	Positive (colon) Positive (glandular stomach)	(181)
Cytogenetics Assay	Chromosomal aberrations	NA	NA	Positive	(223)
S. Typhimurium TA94, TA1537, TA98	Frameshift	Yes and No	5 mg/plate	Negative	(179)
S. Typhimurium TA1535, TA100, TA92	Base pair	No	2.5 mg/ml	Positive	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	No	6 mg/ml	Positive	
In vitro Muntiacus muntjac	Chromosomal aberrations	No	3 μg/ml	Positive	(219)
S. Typhimurium TA1537, TA1538, TA98	Frameshift	Yes and No	5 mg/plate	Negative	(224)
S. Typhimurium TA1535, TA100	Base Pair		5 mg/plate	Negative	
S. Typhimurium TA100	Base pair	Yes and No		Negative	(225)
S. Typhimurium TA98	Frameshift	Yes and No		Negative	
Rec assay	DNA damage	Yes and No		Negative	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	Yes and No		Negative	

animals that died spontaneously, but no adverse effects were reported. This group reported a NOAEL of 5% for both male and female rats (222).

Borzelleca and Hallagan also performed a chronic toxicity/carcinogenicity study in CD-1 mice (220). Groups of 60 males and females were fed 0 (two control groups), 0.5, 1.5, or 5% Yellow 5 for 104 weeks. The protocol for this study was similar to Borzelleca and Hallagan's rat study, but the mice were not exposed *in utero*, and were 42 days old at the start of the study — a serious drawback, because infant animals are likely to be more susceptible to toxic or carcinogenic effects than older animals. The investigators claimed that a sufficient number of mice survived until the end of the study (24 months), however half of the groups did not meet the FDA recommendation that in a carcinogenicity study at least 25 mice/sex/group should survive until study termination (see italic numbers in Table 4.7) (121, 220). In any case, the investigators did not report any significant compound-related effects and concluded that the NOAEL for this study was 5% for both male and female mice (indeed, the lack of any effect at the highest dosage level suggests that a higher dosage should have been used in the chronic feeding studies) (220).

Carcinogenic contaminants

Yellow 5, the second most widely used dye (Table 4.4), may contain up to 13% of other organic and inorganic chemicals (214). Yellow 5 may be contaminated with several carcinogens, including benzidine and C-mino-biphenyl. The FDA limits free benzidine to 1 part per billion (ppb), though analytical methods can only detect 5 ppb. Importantly, FDA tests have found that some batches of dye contained as much as 83 ppb of free and bound benzidine, with the latter being liberated in the GI tract (226). The FDA does not test for bound benzidine when it

Table 4.7. Mouse survival at termination of a 24-month study(220)		
Dose Level (%)	Survival*	
0 (control 1)	30/50	**Males**
0 (control 2)	28/60	
0.5	31/60	
1.5	**21/60**	
5.0	29/60	
0 (control 1)	**20/60**	**Females**
0 (control 2)	**24/60**	
0.5	**18/60**	
1.5	**24/60**	
5.0	33/60	
*No. surviving at termination of study/no. at initiation; boldface indicates inadequate numbers of mice surviving.		

certifies the purity of dyes. The FDA's 1985 risk assessment (using projections for 1990 consumption levels) calculated a risk for Yellow 5 of 4 cancers in 10 million people, which is slightly smaller than the ''concern'' level of 1 in 1 million (212). However, that risk assessment failed to consider the: (1) greater sensitivity of children to carcinogens (227); (2) greater consumption of Yellow 5 by children than the general population; (3) substantial increase in per capita consumption of Yellow5 since 1990; (4) possibility that some batches of dye contain large amounts of bound benzidine and other carcinogenic contaminants; and (5) the presence of similar contaminants in Yellow 6. FDA scientists found that one company eliminated benzidine contamination in 1992, suggesting that other companies could do (or might have done) the same (228). However, with more chemicals being imported from China, India, and other countries, it is important that dyes routinely be tested for bound contaminants.

Hypersensitivity

It is generally accepted that Yellow 5 has hypersensitivity effects. In the 1970s, several cases of Tartrazine sensitivity were reported, most frequently in the form of urticaria and asthma (229). Neuman *et al.* reported that 26% of patients with a variety of allergic disorders had a positive allergic reaction 10–15 minutes after ingesting 50 mg of the dye. Those reactions included heat-wave, general weakness, blurred vision, increased nasopharyngeal secretions, a feeling of suffocation, palpitations, pruritus, angioedema, and urticaria. An association between aspirin intolerance and Tartrazine sensitivity has been demonstrated in several studies (230). Stenius and Lemola separately administered aspirin and Yellow 5 to 96 patients and found that about half of the patients with positive reactions to aspirin also had positive reactions to Yellow 5, and about three-fifths of the positive Yellow 5 cases also had positive aspirin reactions (231). In a double-blind crossover study, Settipane *et al.* found that 0.22 mg of Yellow 5 (much less than is used in most dyed foods) caused a positive reaction in 8% of patients with chronic urticaria and 20% of patients with aspirin intolerance (232).

In 1986, the Joint Council of Allergy and Immunology, which was established by two major medical organizations, told the FDA that listing Yellow 5 on the label was not sufficiently protective, because reactions could be life-threatening, and urged the agency to ban Yellow 5.

Conclusions

Six out of 13 mutagenicity studies indicated potential health hazards, but Yellow 5 did not appear to be carcinogenic in rats. The chronic feeding study in mice was inadequate and cannot be used to support the dye's safety. In addition, Yellow 5 may be contaminated with significant

levels of carcinogens. Tartrazine (the only dye to be tested on its own in hyperactivity studies, instead of in mixtures) has caused hyperactivity in children (233, 234). Yellow 5 can cause sometimes-severe hypersensitivity reactions. Since Yellow 5 poses some risks, has not been adequately tested in mice, and is a cosmetic ingredient that serves no nutritional or safety purpose, it should not be allowed in the food supply.

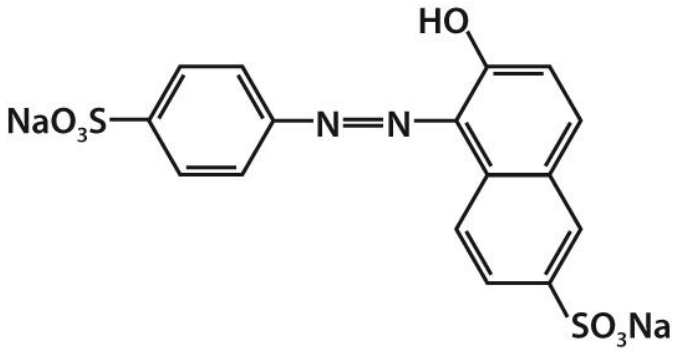

Figure 4.5. FD&C Yellow 6 (Sunset Yellow)

FD&C Yellow 6

FD&C Yellow 6 (Fig. 4.5), or Sunset Yellow, is a water-soluble, sulfonated, azo dye used to color baked goods, cereals, beverages, dessert powders, candies, gelatin desserts, sausage, and numerous other foods, as well as cosmetics and drugs. Yellow 6 has an ADI of 3.75 mg/kg bw/day, or 112.5 mg for a 30-kg child (235). Current average per capita production of Yellow 6 is equivalent to about 14 mg/day, making it the third most widely used dye (Table 4.4). Considering that the FDA estimates that an average ‘‘high user’’ consumes about five times as much dye as an average user over their lifetimes, some children may be consuming amounts above the ADI (160, 236).

Metabolism and metabolic effects

Several metabolites were found in the urine of rabbits given a single 0.5 mg/kg oral dose of Yellow 6. Yellow 6 is reduced at the azo linkage primarily in the gut by intestinal microflora to

produce sulfanilic acid and 1-amino-2-naphthol-6-sulfonic acid, as well as the n-acetylated form of sulfanilic acid, p-acetamidobenzene-sulfonic acid. Intact Yellow 6 in the feces accounted for about 2% of the dose (174). Those findings were confirmed by Honohan *et al.* who dosed five rats with 2.7 mg of ^{14}C-Yellow 6 orally and found only 1–2% of the dose in the form of intact dye in the feces after 24 hours (237). In another rat study, after asingle oral dose of 100 mg, only 0.8% of intact dye was excreted in the feces, with the rest being the metabolites indicated above. Only 3.6% of the intact dye was absorbed by rats administered 50 mg of Yellow 6 orally (238).

Apart from the metabolism of the dye, a 50-mg dose of Sunset Yellow (like Tartrazine) led to increased or accelerated urinary excretion of zinc in hyperactive children. Whether the effect on zinc is a cause of hyperactivity is not known (217).

Genotoxicity

Yellow 6 was negative in eight genotoxicity assays, but induced forward mutations and chromosome aberrations in two other assays (223, 239). As shown in Table 4.8, Yellow 6 did not induce DNA damage in a comet assay or cause frameshift, base pair, or forward mutations, chromosomal aberrations, or mitotic gene conversion.

Chronic toxicity/carcinogenicity

The NTP conducted chronic bioassays using 50 animals/sex/group in F344 rats and B6C3F1 mice. Each group was fed a diet containing 0, 1.25, or 2.5% Yellow 6 for 103 weeks. The control groups consisted of 90 rats or 50 mice of each sex. There was no *in utero* exposure in

Table 4.8. Summary of genotoxicity studies on Yellow No. 6.					
Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	2,000 mg/kg	Negative (stomach colon, liver, kidney, bladder, lung, or brain)	(181)
Cytogenetics Assay	Chromosomal aberrations	--	--	Positive	(223)
S. Typhimurium TA98	Frameshift	Yes and No	300 µg/plate	Negative	(241)
S. Typhimurium TA100	Base Pair	Yes and No	300 µg/plate	Negative	
Bone marrow micronucleus assay	Chromosomal damage	NA	2,000 mg/kg	Negative	(242)
L5178Y $TK^{+/-}$ mouse lymphoma assay	Forward mutation	Yes	1 mg/ml	Positive	(239)
S. Typhimurium TA1537, TA1538, TA98	Frameshift	Yes and No	5 mg/plate; also tested 1 mg/plate sulfanilic acid	Negative	(224)
S. Typhimurium TA1535, TA100	Base pair	Yes and No	5 mg/plate; also tested 1 mg/plate sulfanilic acid	Negative	
Saccharomyces cerevisiae BZ 34	Mitotic gene conversion	No	5 mg/ml	Negative	(243)
E. coli WP2 *uvrA*	Base substitution	Yes and No	10 mg/ml	Negative	(184)

either study, and both studies were terminated at two years instead of 30 months or the lifetimes of the animals, significantly reducing the sensitivity of the studies. The rat study did not find any

statistically significant dye-related neoplastic or non-neoplastic lesions in any of the groups. Low dose, but not high-dose, male mice had a significantly higher incidence of hepatocellular carcinomas and adenomas compared to controls. Partly because of the lack of a dose-response relationship in the mice, NTP concluded that Yellow 6 was ‘‘not clearly related’’ to a higher rate of carcinogenicity. However, the high rate in the low-dose group certainly raises questions that could only be answered with a new study (240).

In 1982, Bio/dynamics Inc., under contract to CCMA, conducted two multigeneration, long-term feeding studies in Charles River Sprague–Dawley rats at doses of 0 (two control groups), 0.75, 1.5, and 3% in the first study and 0 (one control group), 0.75, 1.5, and 5% in the second study. The first study was conducted for 30 and 28.5 months for males and females, respectively, and the second study lasted for 25.6 and 27.8 months for males and females, respectively. In the F_1 generation, females in the 3% group in the first study and males in the 5% group in the second study had increased mortality. At termination of both studies, there was an increase in mean absolute and relative kidney weights in females in the 3% and 5% groups, as well as an increase in the mean relative and absolute thyroid weights in males and females in the 5% groups. Females in the 3% group and both males and females in the 5% groups had statistically significant increased incidences of adrenal medullary adenomas compared to controls. Also, males in the 3% group had an increased incidence of testicular interstitial cell adenomas compared to pooled controls. Notwithstanding those findings, the investigators concluded that the studies did not find any evidence of carcinogenicity (244).

After examining the results of the Bio/dynamics study, the FDA argued that the increased incidence of the tumors was not related to Yellow 6 because of the: (1) lack of dose-response in the 3% and 5% dosage groups (though that is comparing two different studies); (2) lack of precancerous lesions; (3) similar morphology of adrenal medullary lesions in control and treated animals; (4) lack of a difference in the latency periods before tumors occurred; (5) fact that the tumors seen are common spontaneous tumors in older rats; and (6) lack of other studies finding an association between Yellow 6 and this type of tumor (236).

Bio/dynamics, again under contract to CCMA, performed a chronic toxicity/carcinogenicity study in Charles River CD-1 COBS mice, with 60 mice/sex/group. The study used dosages of 0 (two control groups), 0.5, 1.5, and 5% Yellow 6 in the animals' chow. The study was terminated at only 20 months for the males and 23 months for the females. Another deficiency was that the mice were not exposed *in utero*. Males in the 5% group had significantly higher mortality rates at the end of the study compared to controls. The laboratory concluded that the study did not indicate any concern about carcinogenicity in mice (245).

In the 1960s, the FDA completed a 7-year feeding study on a small number of beagle dogs. This study was neither large nor long enough to detect carcinogenicity. However, Kent J. Davis, an FDA veterinarian, attributed ''tears, eye lid encrustations, pannus [corneal inflammation], and corneal opacity approaching blindness'' to ingestion of Yellow 6. He concluded that, because of the eye lesions, ''it is apparent that immediate decertification of this color is necessary in order to protect the public health at the recommended level of present safety standards''. His recommendation was not followed (246).

Carcinogenic contaminants

Yellow 6 may be contaminated with several carcinogens, including benzidine and C-mino-biphenyl. The FDA set a limit of 1 ppb of free benzidine, but Peiperl *et al.* reported that some batches of dye contained a hundred or even a thousand times as much benzidine bound up in other chemical moieties, which is likely liberated in the colon (228). The FDA does not test for bound benzidine in the aliquots taken from batches of dyes submitted for certification. The FDA's 1986 risk assessment (using estimates for 1990 consumption levels) estimated a risk of three cancers in 10 million people, which is smaller than the official ''concern'' level of 1 in 1 million (235). However, that assessment failed to consider the: (1) greater sensitivity of children(227); (2) greater consumption of Yellow 6 by children than the general population; (3) substantial increase in per capita consumption of Yellow 6 since 1990; (4) possibility that some batches of dye contain bound forms of benzidine and other contaminants (227); and (5) presence of similar contaminants in Yellow 5. FDA scientists found that in 1992 one company eliminated benzidine contamination of Yellow 5, suggesting that other companies could do the same for Yellow 6 (228). However, a Health Canada study found that Sunset Yellow FCF (Yellow 6) was still contaminated with benzidine in 1998 (247). With more and more chemicals being imported, it is important that dyes routinely be tested for bound contaminants.

Hypersensitivity

Human hypersensitivity to Yellow 6 was reported as early as 1949 (248). Since then, several cases of hypersensitivity to the color have been reported:

- A 15-year-old pregnant girl experienced anaphylactic shock after receiving an enema that contained Yellow 5 and Yellow 6. The patient was tested via the skinprick technique for sensitivity to all of the soluble components in the enema. Positive results were observed for both Yellow 5 and Yellow 6 (249).
- A 43-year-old physician was hospitalized for stomach cramps four times over a 2-year period. Double-blind tests confirmed that the cramps were caused by a hypersensitivity to Yellow 6 (250).
- A 53-year-old woman visited the doctor for severe skin lesions. Two days after receiving treatment she was hospitalized for distaste for food, as well as indigestion, retching, belching, severe abdominal pain, and vomiting. When the drugs (administered orally) were stopped early, the symptoms subsided, and when the drugs were administered again the symptoms reappeared. A challenge test confirmed that Yellow 6 was the causative agent (251).

A study by Michaelsson and Juhlin involved 52 patients with, and a control group of 33 patients without, recurrent urticaria. All subjects were put on a dye-free diet and were free of antihistamines prior to administration of the possible allergen. The researchers tested the effects of several food dyes (including Yellow 6) and preservatives, as well as aspirin, sulfanilic acid (a metabolite of Yellow 6), and a placebo. A dose of 0.1 mg (initial dose for asthma patients) or 1 mg of Yellow 6 was administered to patients with slight or no urticaria symptoms. If no reaction was observed after the initial dose, a higher dose of 2, 5, or 10 mg was administered to the latter group of patients 1 hour after each previous dose. Symptoms of a hypersensitivity reaction included urticaria, angioedema of lips, eyes, or face, reddening of the eyes, sweating, increased

tear secretion, nasal congestion, sneezing, rhinitis (runny nose), hoarseness, wheezing, and a variety of subjective symptoms. Of the 33 control patients, only two with a history of rhinitis showed signs of rhinitis when administered Yellow 5 and Yellow 6. Of the 27 patients with recurrent urticaria who were challenged with Yellow 6, 10 developed urticaria and six experienced subjective symptoms; 11 were negative for symptoms. Eight out of nine patients with positive reactions to Yellow 6 also experienced a positive reaction to aspirin (people sensitive to Yellow 5 also are often sensitive to aspirin) (250).

Michaelsson *et al.* tested seven patients having allergic vascular purpura with oral provocation by 5 mg Yellow 6. One patient had a strongly positive reaction to the dye. That patient was a 32-year-old woman who suffered for 12 years from recurring purpuric lesions. After the patient was put on a diet free from dyes and benzoates (a preservative that has been linked to allergy-like reactions) for 6 months, she was essentially free from lesions (252).

Conclusions

A NTP study did not detect any problems in chronic feeding studies on rats and mice, though the animals were not exposed *in utero* and the studies were terminated at 2 years. Bio/dynamics concluded that its studies on rats and mice showed that Yellow 6 was not an animal carcinogen, but rats in the two highest dosage groups (3% and 5%) experienced higher incidences of adrenal medullary adenomas. The FDA has given reasons for not considering those tumors significant, but differences between test and control groups should not be rejected on qualitative grounds. A Bio/dynamics mouse study did not report evidence of carcinogenicity, but the study was not as

sensitive as it might have been because the mice were not exposed *in utero*. Yellow 6 may be contaminated with significant levels of recognized carcinogens. Whether or not it causes cancer, Yellow 6 raises other, lesser concerns, such as mild to severe hypersensitivity reactions. Because it provides no health benefit whatsoever, Yellow 6 should be removed from the food supply.

Discussion

Our review of the toxicology of the nine dyes used in the US food supply (many of the dyes are used in other countries, as well), identified concerns about the adequacy of the testing of all the dyes. In addition, research indicates that some of the dyes may cause cancer, hypersensitivity reactions, genotoxicity, and hyperactivity (see Table 4.1).

Most of the studies reviewed in this report suffer from several significant limitations. First, most of the studies were commissioned or conducted by dye manufacturers, so biases could influence the design, conduct, or interpretation of the studies. Ideally, the tests would have been conducted and interpreted by independent scientists. Second, most of the studies lasted no longer than 2 years — some were shorter. Third, many studies did not include an *in utero* phase. Bioassays would be more sensitive if they lasted from conception through 30 months or the natural lives of the rodents (as long as 3 years) (253).

Another consideration of unknown importance is that virtually all the studies evaluated the safety of individual dyes. Many foods, though, contain mixtures of dyes, such as the Blue 1, Blue 2,

Red 40, Yellow 5, and Yellow 6 in Kellogg's Hot Fudge Sundae Pop Tarts. Dyes conceivably could have synergistic effects with one another or with other food additives or ingredients.

One significant limitation of this report is that the authors were restricted to reviewing mostly published studies. Unpublished toxicology studies in the files of the FDA or companies might shed further light on the safety of the dyes.

Neurotoxicity

This report does not explore neurobehavioral toxicity of food dyes in detail but that topic must be touched upon. In the early 1970s, allergist Benjamin Feingold observed that food dyes could cause hyperactivity and other impaired behaviors in child and adult patients. His recommendation that hyperactive children be put on an ''elimination'' diet generated huge publicity and spurred numerous scientific studies over the years.[13] A 2004 meta-analysis concluded that there was a cause-and-effect relationship between food dyes and hyperactivity. The authors stated that dyes ''promote hyperactivity in hyperactive children, as measured on behavioral rating scales'' and that ''society should engage in a broader discussion about whether the aesthetic and commercial rationale for the use of [artificial food colorings] is justified'' (254).

[13] See http://cspinet.org/fooddyes/index.html for more detailed information about food dyes and hyperactivity, especially 'Diet, ADHD & Behavior: a quarter-century review — 2009 Update'. Jacobson MF, Schardt D. (Washington: Center for Science in the Public Interest). http://www.cspinet.org/new/pdf/dyesreschbk.pdf; accessed 2010 Feb 20. Also, see CSPI's 2008 petition to the FDA.

Two major studies on British children found that mixtures involving six dyes (and the food preservative sodium benzoate) impaired the behavior of even non-hyperactive children (255, 256). As a result, the British government told the food and restaurant industries to eliminate the dyes tested by the end of 2009, and the European Parliament passed a law that requires a warning notice on all foods that contain one or more of the dyes tested (257). Between that notice and the fact that dyes were never used as widely in Europe as in the United States, dyes are now rarely used but not eliminated.

Because of those governmental actions and Europeans' aversion to synthetic food ingredients, some products made by McDonald's, Mars, Kraft, PepsiCo, and other major American multinational companies contain dyes in the United States, but natural or no colorings in the United Kingdom. In June 2008, CSPI petitioned the FDA to ban all the widely used food dyes because of their impact on children's behavior.[14] Food dyes and all other food additives should be screened in animals and in *in vitro* systems for potential behavioral effects before they are allowed into the food supply.

Getting unsafe dyes out of food

This review suggests the need for improvements in the FDA's regulation of food dyes and of food additives more generally. Tests of food and color additives are often deficient in terms of duration, number of animals in each dosage group, number of species tested, and dosages used, and fail to consider the cumulative risk of all dyes, rather than of each dye independently. Indeed, the Food, Drug, and Cosmetic Act requires the FDA to consider ''the cumulative effect,

[14] See http://cspinet.org/new/pdf/ petition-food-dyes.pdf

if any, of such additive … taking into account the same or any chemically or pharmacologically related substance …'' [21 USC379e(b)(5)(A)(ii)]. The FDA should routinely require all carcinogenesis studies to include *in utero* exposure, to last 30 months or the natural lives of the animals, and to be sure that the highest dosage used has some observable effect on the animals. Additives should be evaluated, based on their chemical structures, for potential hypersensitivity reactions and should be monitored after introduction into the food supply. The agency should routinely test for the presence of bound carcinogens, which are not detected in the analytical chemistry tests currently used, and hazardous contaminants should be restricted to safe levels. Approvals should be revoked if unnecessary additives are found to cause serious reactions (e.g. urticaria, anaphylactic reactions) or widespread milder reactions (e.g. nausea, vomiting). The law barring the approval of chemicals that cause cancer in animals should be strictly enforced. Ideally, tests would be conducted and evaluated by independent researchers, but such a reform, initially proposed in legislation (S. 925) in 1975 by Senator Gaylord Nelson, has not been adopted.

In the absence of improved regulations, food processors, and restaurants voluntarily should consider reformulating their foods without dyes (and without natural colorings, including annatto, cochineal extract, and carmine, that cause hypersensitivity reactions). Several major multi-national companies have told the authors that they do not use dyes in Europe, because governments have urged them not to, but that they would continue to use dyes in the United States until they were ordered not to or consumers demanded such foods. But there may be a

nascent movement away from dyes. Two chain restaurants, Starbucks and Jason's Deli, and snack manufacturer Frito-Lay will be phasing out dyes in the next several years (258). Also, General Mills has removed dyes from its Trix yogurt.

The FDA, which is charged with protecting the public from unsafe food ingredients, could ban dyes that fail to meet their safety requirements. However, the Food, Drug, and Cosmetic Act makes it even harder for the FDA to revoke previous approvals of food colors than other food additives. To challenge a proposed ban on a food or color additive, companies can request that the FDA hold a formal public hearing and, if the FDA subsequently still wants to ban the substance, companies can go to court. The process for color additives, though, includes another hurdle, because, if a dye is alleged to cause cancer, companies can request that the FDA create an outside advisory committee to review the matter. Compare 21 USC 379e(b)(5)(C) and 21 USC371(e)(2) and (f)(2) for colorings with 21 USC 348409(f) and (h) and 21 CFR 171.130 for other additives. As one legal analyst stated, "Thanks to the foresight and effective lobbying of the cosmetics industry in the 1960s, the proponent of a color additive petition is in an excellent position if the FDA decides to remove [a coloring's] permanent listing. The burdens of proof in a complex process fall on the FDA, and the time required to pass through the procedural maze acts as a disincentive to FDA undertaking any delisting action." (162). Ideally, the law would be changed to provide greater consumer protection from dyes that appear to be unsafe. Rigorous analysis of the benefit to risk ratio should be conducted for each dye before approval is even considered. Meanwhile, though, consumers who wish to avoid dyes should carefully read ingredient statements on product labels; it is more difficult to avoid dyes in restaurant foods.

Part II Conclusions

Artificial sweeteners and food dyes are both highly consumed food additives in the United States. Even though their use is widespread among consumers, evidence of their toxicity should not be ignored by scientists. The final two chapters of this dissertation provided thorough and critical reviews of the toxicity of rebaudioside A and artificial food dyes. The purpose of these publications was to apply knowledge in the field of molecular toxicology to the public interest.

Rebaudioside A is a steviol glycoside purified from the stevia plant. Recently, the FDA approved its GRAS classification and international companies such as Cargill and Coca Cola Co. have starting using it in their beverages. Rebaudioside A was a hopeful new safe zero-calorie sweetener, however, the toxicology studies revealed that it is probably not the "silver bullet" of artificial sweeteners it was once thought to be and the FDA should not have been so quick to give it a GRAS label. The FDA recommends that a compound with such a high potential usage undergo toxicity and carcinogenicity testing in 2 rodents. Rebaudioside A was only tested in rats. This is particularly concerning because rebaudioside A and/or its metabolites were genotoxic in several assays. Investigators who analyzed the data (and were also involved in submitting rebaudioside A's GRAS notification) tried to claim that rats were an adequate model for humans and a mouse study was unnecessary, but metabolism studies showed differences between rats and humans that show the rat is not an ideal model. All the more reason a mouse study should have been conducted as well. Finally, investigators tried to submit studies on stevioside, another steviol glycoside, in lieu of rebaudioside A studies. However, this is unreasonable give the structural and metabolic differences in the two compounds. It is clear

from analyzing the studies submitted to the FDA that rebaudioside A was rushed through the GRAS classification process without being adequately tested. It is particularly important that consumers are aware of the potential toxicity of rebaudioside A because it is advertised as being "all natural", a label that can deceive a non-scientific consumer into believing this means it is 100% safe.

Artificial food dyes are another food additive that were originally thought to be a safe and easy way make food and beverages look more appealing. However, more than any other food additive, individual dyes have been revealed as toxic compounds. The review presented in this dissertation shows that, although the interpretation of the data is disputed, all of the 9 FDA-approved dyes show signs of being toxic, genotoxic, and/or carcinogenic in animals. And most of the studies were inadequate due to no *in utero* exposure, too few animals, or too short of a study duration. Also, batches of some dyes have been contaminated with known carcinogens. Studies have revealed that many people have allergies to Blue 1, Red 40, Yellow 5, and Yellow 6 and because of the ubiquity of food dyes, allergies such as these often go undiagnosed. Finally, compelling evidence exists for food dyes cause hyperactivity in children, particularly those already prone to hyperactivity. The endpoints associated with food dyes consumption are potentially severe. Seeing as food dyes are of no nutritional value and often conceal the lack of quality of food products, food dyes should be eliminated from use in the United States. Several companies and stores have already begun eliminating food dyes from their products (eg. Trader Joe's is a food dye free establishment), demonstrating the feasibility of eliminating these compounds from the food market.

Molecular toxicology and public health are highly intermingled fields. Toxicology is an essential component of risk assessment and provides the scientific underpinning of risk characterization of chemicals used in commerce. Toxicological studies and their interpretation make it possible for the public to make healthy choices in the marketplace, particularly cancer-preventative choices.

Appendix A: Conflicts of interest regarding studies conducted on steviol glycosides

Authors Bisognano, Brown, Brusick, Renwick, Roberts, and Tarka received financial support from Cargill for consulting services. Authors Brown, Brusick, Renwick, Roberts, Tarka, and Wheeler received financial support from Cargill for manuscript preparation. Authors Boileau, Curry, and Fosdick are employed by Cargill, Inc. Authors Carakostas, Clos, DuBois, Prakash, and Wilkensare are employed by The Coca-Cola Company.

Appendix B: Results of cancer bioassays conducted in mice and rats

Chemical	Male Rats	Female Rats	Male Mice	Female Mice	Reference
Primidone	EE	NE	CE	CE	(259)
1,3-Butadiene	NE	NE	CE	CE	(260)
Diphenylhydantoin (Phenytoin)	EE	NE	NE	CE	(261)
N-Methylolacrylamide	NE	NE	CE	CE	(262)
Chloroethane (ethyl chloride)	EE	EE	NA	CE	(263)
Bis (2-chloro-1-methylethyl)ether, technical grade	NE	NE	CE	CE	(264)
5-Chloro-o-toluidine	NE	NE	CE	CE	(265)
4-Chloro-o-toluidine hydrochloride	Neg	Neg	Pos	Pos	(266)
DDE	Neg	Neg	Pos	Pos	(267)
Toxaphene	EE	EE	CE	CE	(268)
1,1,2,2-Tetrachloroethane	EE	EE	CE	CE	(269)
Aldrin	EE	EE	CE	NE	(270)
Heptachlor	EE	EE	CE	CE	(271)
Chlordane	EE	EE	CE	CE	(272)
Propylene glycol mono-t-butyl ether	EE	NE	CE	CE	(273)

EE – equivocal evidence
NE – no evidence
CE – clear evidence
NA – not available
Neg- negative
Pos-positive

Appendix C: Food dyes supplement

FD&C Blue 1

FD&C Blue 1 (Fig. C.1), or Brilliant Blue, is a watersoluble coloring used in baked goods, beverages, dessert powders, candies, cereals, drugs, and other products. Blue 1 received FDA approval for general use in foods and ingested drugs in 1969. In 1982, the FDA permanently approved the color for use in externally applied drugs and general use in cosmetics excluding the area of the eye. The FDA suggests a maximum ADI for Blue 1 of 12 mg/kg bw/day (274). For a 30-kg (66-pound) child, that equates to 360 mg/day. Current average dye usage is equivalent to about 3 mg/person/day (based on the entire population, not just children).

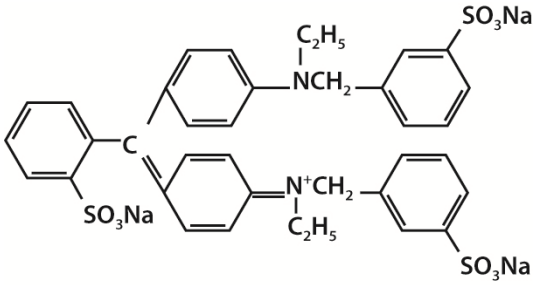

Figure C.1. FD&C Blue 1 (Brilliant Blue)

Metabolism

In a study of rats, Blue 1 was largely excreted unchanged in the feces (96%) within 36 hours after a 200-mg oral administration. None of the dye was excreted in the urine. In the same study, only 0.7 and 2.8% of a 200-mg oral dose was excreted in the bile of two bile-duct cannulated dogs indicating some intestinal absorption. Investigators calculated that the quantity of absorption of the color from the GI tract was about 10 mg out of a 200-mg dose (275). Brown *et al.* reported similar results after administering a single 0.27-mg dose of ^{14}Clabeled Blue 1 to female Sprague–Dawley rats (198). Bile duct-ligated rats excreted the dye in their urine and feces at concentrations of 2.02 and 97.28%, respectively. Given the lower percentage of dye being excreted in the bile, the large amount eliminated through the feces indicates that the dye is

poorly absorbed by the GI tract. In this particular study, total intestinal absorption was estimated to be about 2.05 and 0.27% of the total dose in bile duct-ligated and intact rats, respectively. Analysis of the biliary and urinary excretion showed that 95% of the recovered radioactivity was from unchanged Blue 1 while 5% was an unidentified metabolite or degradation product. Blue 1 does not appear to be broken down by intestinal microbiota in rats, but up to 5% is absorbed via the GI tract (198).

Genotoxicity

Seven studies did not find Blue 1 genotoxic in terms of DNA damage, base pair mutations, base substitutions, or frameshift mutations (Table C.1). However, Blue 1 caused chromosomal aberrations in two studies (223, 276).

Chronic toxicity/carcinogenicity

Hansen *et al.* performed a chronic toxicity study of Blue 1 in rats (another study on dogs was too brief and used too few dogs to provide meaningful results). The rat study lasted 2 years and used 24 Osborne–Mendel rats/sex/group at doses of 0, 0.5, 1, 2, and 5% of the diet. There were no reported compound-related effects in any group on mortality, hematology, or organ weights (heart, liver, spleen, testis, kidney), nor was significant growth inhibition or gross lesions reported. The small numbers of rats in each group renders this study quite insensitive and of marginal value (277).

The highest-quality carcinogenicity/toxicity studies were performed by Borzelleca *et al.* for the CCMA. The 2-year studies used Charles River CD rats and CD-1 mice. The rat study included an *in utero* phase with 60 rats/sex/group. The rats were fed 0 (two control groups), 0.1, 1, and

Table C.1. Summary of genotoxicity studies on Blue No. 1.					
Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	2,000 mg/kg	Negative	(181)
Cytogenetics Assay in Chinese Hamster Cells	Chromosomal Aberrations	No	?	Positive	(223)
S. Typhimurium TA1535 and TA100	Base Pair	Yes and No	10 mg/plate	Negative	(278, 279)
S. Typhimurium TA1538, TA98, and TA1537	Frameshift	Yes and No	10 mg/plate	Negative	
S. Typhimurium TA1538	Frameshift	Yes and No	10 mg/ml	Negative	(184)
E. coli WP2 *uvrA*	Base Substitution	Yes and No	10 mg/ml	Negative	
S. Typhimurium TA92, TA1535, TA100	Base Pair	Yes and No	5 mg/plate	Negative	(179)
S. Typhimurium TA1537, TA94, TA98	Frameshift	Yes and No	5 mg/plate	Negative	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	No	5 mg/ml	Positive	

2% Blue 1 in the chow for about two months prior to mating. Investigators reported no compound-related effects on reproduction. F_1 generation rats were randomly selected and 70 rats/sex/group were used in the lifetime feeding study (same dosage groups, including two controls, as in the F_0 phase). The maximum exposure times for males and females were 116 and 111 weeks from birth, respectively. F_1 females in the 2% group had a significant decrease in terminal mean body weight (15%) and decreased survival compared to controls. No other compound-related effects were noted. The NOAEL was 1072 mg/kg bw/day (2% group) for males and 631 mg/kg bw/day for females (1% group) (280).

The mouse study did not include an *in utero* phase and used 60 mice/sex/group. Mice were administered 0 (two control groups), 0.5, 1.5, and 5% Blue 1 in their food. The maximum exposure time was 104 weeks for both sexes and the NOAEL was determined to be 5%, or 7354 and 8966 mg/kg bw/day for males and females, respectively. No significant compound-related effects were noted in any of the groups (280).

Neurotoxicity

Lau *et al.* investigated the individual and potential synergistic effects of Blue 1 and L-glutamic acid (a close relative of the food additive monosodium glutamate) on neuronal development. Investigators used NB2a neuroblastoma cells that were induced to differentiate and grow neurites in the presence or absence of the two food additives. Neurotoxicity was measured in terms of an inhibition of neurite outgrowth. Individually, Blue 1 was found to have an IC_{50} (half-maximal inhibitory concentration) of 0.0514 mmol/l, while L-glutamic acid was found tohave an IC_{50} of 48.7 mmol/l. When cells were treated with the two additives together, rather than just

seeing an additive effect, the two compounds worked synergistically (Fig. C.2). A 50 : 50 mixture of Lglutamic acid and Blue 1 produced 46.1% neurite growth inhibition, which was significantly different from the expected value of 15.8% if the compounds acted additively. On the other hand, the effect on cell viability from the combination of the two additives was increased only in an additive fashion (281). Without further research it is unknown whether other food dyes might behave similarly.

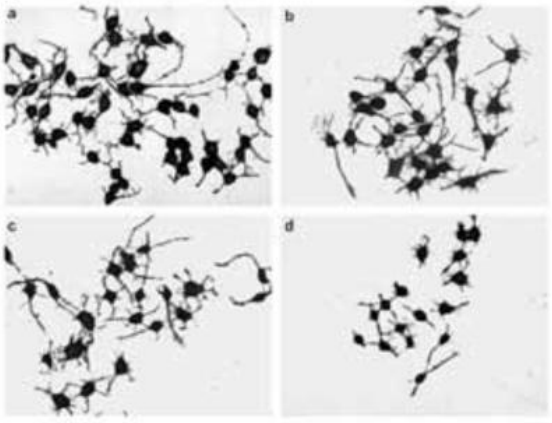

Figure 3. Images of Coomassie Blue-stained differentiating NB2a cells:

(a) in the absence of additives; (b) in the presence of 0.0001 µM Blue 1 to produce approximately 20% neurite inhibition; (c) 1 µM L-glutamic acid to produce approximately 20% neurite inhibition; (d) a mixture of Blue 1 and L-glutamic acid each at 50% of the above concentrations.

Figure used with the permission of Oxford University Press: Lau, K., W. G. McLean, *et al.* (2006). "Synergistic interactions between commonly used food additives in a developmental neurotoxicity test." Toxicological Sciences 90(1): 178-187.

Figure C.2. Synergistic neurite inhibition with addition of Blue 1 and L-glutamic acid (281).

Feingold suggested that food dyes and additives are associated with hyperactivity disorders in children (282). The developmental period of synaptogenesis (brain growth-spurt period) occurs in humans from three months before birth to several years after birth (281). Small amounts of Blue 1 are absorbed by the GI tract in rats, but metabolism studies in children have not been conducted. Blue 1 might possibly have potent effects, and it might take only a small absorbed amount to affect a child's brain development. The blood–brain barrier is not fully developed until 6 months in humans and even after complete development some regions

of the brain are never protected by the blood–brain barrier (283, 284). Further neurotoxicity studies need to be conducted on Blue 1 and other dyes.

Conclusions

The most thorough studies of Blue 1, which were sponsored by industry, did not find evidence of carcinogenicity or other toxicity in rats or mice. On the other hand, in an *in vitro* test, Blue 1 inhibited neurite growth and acted synergistically with Lglutamic acid, suggesting the potential for neurotoxicity. That is particularly worrisome for fetuses and babies under the age of 6 months whose blood–brain barrier is not fully developed. Further research needs to be conducted to establish this dye's safety with greater certainty.

FD&C Blue 2

FD&C Blue 2 (Fig. C.3) is the approved form of Indigo Carmine. In 1983, the FDA permanently listed Blue 2 for use in foods and ingested drugs (285). It is widely used to color beverages, candies, pet foods, and other foods and drugs. Blue 2 has an ADI of 2.5 mg/kg bw/day (286). That ADI is equivalent to 75 mg for a 30-kg child. The FDA certifies an amount of Blue 2 that is equivalent to about 2 mg/person/day.

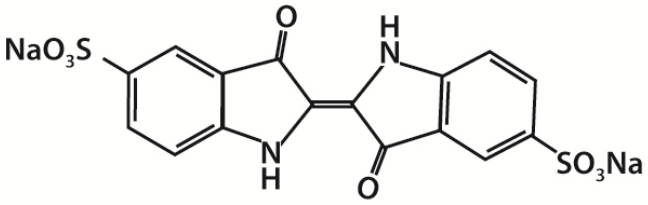

Figure C.3. FD&C Blue 2 (Indigo Carmine)

Metabolism

Studies in rats demonstrated that the majority of Blue 2 and/or its metabolites (including 5-sulfoanthranilic acid, its final breakdown product) are excreted in the feces, with smaller amounts being found in the urine (215, 287). In one bile-duct-cannulated rat given a 20-mg dose of Blue 2, only 0.004% of the dye was excreted in the bile—125 times as much was found in the urine. The authors concluded that the majority of the small amount of dye that is absorbed intact is excreted through the urine and not the bile, and the dye excreted in the feces is mostly from unabsorbed dye (287). Those studies show that 5-sulfoanthranilic acid is absorbed more readily by the GI tract than is the intact dye (287).

Genotoxicity

Details of the genotoxicity studies performed on Blue 2 are provided in Table C.2. All of the 11 tests were negative except for a chromosomal aberration assay (276).

Chronic toxicity/carcinogenicity

Between 1984 and 1986, Borzelleca *et al.* performed several toxicology studies using Blue 2. One was a chronic toxicity/carcinogenicity study in rats. The study included an *in utero* phase in which five groups of 60 male and 60 female Charles River CD albino rats were fed 0 (two different control groups), 0.5, 1, or 2% Blue 2 starting at least 2 months prior to mating. F_1 offspring in each dosage group were randomly selected, and 70 rats/sex/group were continued on the same dosages for 29 months in males and 30 months in females. Administration of the dye did not affect the number of pregnant females per group or pup viability at birth. However, there was possible evidence of carcinogenicity (288).

Table C.2. Summary of genotoxicity studies on Blue No. 2.					
Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	2,000 mg/kg	Negative	(181)
S. Typhimurium TA1535 and TA100 *S.* Typhimurium TA1538, TA98, and TA1537	Base Pair Frameshift	Yes and No Yes and No	10 mg/plate 10 mg/plate	Negative Negative	(278)
S. Typhimurium TA1538 *E. coli* WP2 *uvrA*	Frameshift Base Substitution	Yes and No Yes and No	1 mg/ml 10 mg/ml	Negative Negative	(184)
S. Typhimurium TA92, TA1535, TA100 *S.* Typhimurium TA1537, TA94, TA98 Chromosomal aberration test, CHL cells	Base Pair Frameshift Chromosomal aberrations	Yes and No Yes and No No	5 mg/plate 5 mg/plate 12 mg/ml	Negative Negative Positive	(276)
S. Typhimurium TA1535 and TA100 *S.* Typhimurium TA1538, TA98, and TA1537	Base Pair Frameshift	Yes and No Yes and No	1 mg/plate 1 mg/plate	Negative Negative	(198)
rec-Assay	DNA damage	No	NA	Negative	(289)

- Treated male rats showed a dose-related increase in the incidence of transitional cell neoplasms of the urinary bladder, but the numbers of affected animals were small and the apparent increase was not statistically significant when compared to combined controls (0.8, 1.6, 2.9, and 4.5% of the animals had bladder neoplasms in the control, low-, mid-, and high-dose groups, respectively; there appears to be a dose-related trend but the authors did not do a statistical test) (288).
- Male rats in the 2% group had statistically significant increases in malignant mammary-gland tumors and brain gliomas. However, the investigators concluded that the increased mammary-gland tumors were not related to Blue 2. They also concluded that the gliomas were not consistent with several criteria they said were required to classify a compound as a carcinogen. For instance, neither a dose–effect relationship nor a decrease in survival time was seen. They also reported that the incidence of gliomas in treated animals was consistent with historical controls. (Companies [and the FDA] sometimes make comparisons to historical controls when the test group has more tumors than the concurrent controls.). Based on this study, the investigators estimated that the NOAEL for Blue 2 was 2.0%, or 1282 mg/kg bw/day and 1592 mg/kg bw/day for males and females, respectively (288).

The FDA's Cancer Assessment Committee concluded that the occurrence of urinary bladder transitional cell neoplasms in the male rats, though apparently dose-related, was not related to treatment with Blue 2 because: (1) historical evidence suggests that this form of cancer is not rare in Charles River CD albino rats; (2) the number of neoplasms in the high-dose group was

small; and (3) the number of tumors in the high-dose group was not significantly higher than in the control groups (285).

Regarding the malignant tumors of the mammary gland in the high-dose males, when the Committee combined malignant and benign tumors, there was no longer a statistically significant difference between the controls and high-dose male rats. The Committee concluded that Blue 2 did not cause any significant treatment-related effects in rats (285).

Although there was a significantly higher incidence of brain gliomas in the high-dose male rats, the FDA's Cancer Assessment Committee was still reluctant to conclude that Blue 2 was the cause because: (1) of a lack of gliosis in the high-dose animals; (2) the first two observed gliomas of the brain occurred in controls animals; and (3) data were lacking on the historical incidence of brain gliomas in Charles River albino rats that survive for 30 months. The FDA concluded that 'except for brains of male rats for which the data are equivocal, there is no evidence for carcinogenicity in rats or mice of either sex for all organs examined.' Upon reevaluation of the brain microslides and comparison to controls from a simultaneous study on Green 3, new statistical tests produced P values that were just above 0.05 (the Breslow time-adjusted analysis, produced a P value of 0.053) (285). It is highly questionable to switch a comparison to a different control group after a study is completed. Still, the FDA stated, '… although statistical methods provide insight into the likelihood of being right or wrong in making specific conclusions, they do not provide for certainty as to whether an increase or decrease in tumor incidence is related to treatment.' The Board of Scientific Advisors of the NTP concluded that Blue 2 is safe, citing: (1) no doserelated trend; (2) lack of non-neoplastic cellular changes in

addition to frank neoplasia; (3) no reduction in latency period; (4) no varying progression of brain tumors; (5) the inability of Blue 2 to cross the blood–brain barrier; (6) negative mutagenicity assays; and (7) lack of evidence in structure-activity analysis (285).

Borzelleca *et al.*consulted three outside toxicologists on the carcinogenicity issues. Robert Squire, a prominent industry consultant at the Johns Hopkins University School of Medicine, found a lack of persuasive evidence for compound-related carcinogenicity in the glioma and urinary bladder samples (288, 290). However, Aleksandar Knezevich and Geoffrey Hogan, former vice president of pathology and former vice president of toxicology, respectively, at Bio/dynamics (an industry consulting firm), concluded that the glioma findings 'cannot be dismissed as accidental'. On the other hand, they agreed with the FDA committee that the rates of urinary neoplasms in treated male rats were not clearly different from the controls and were probably not of concern (291).

After Blue 2 was permanently approved in 1983, the Public Citizen Health Research Group (HRG) filed a formal objection on the grounds that the increase in brain tumors in rats fed Blue 2 was statistically significant. The group argued that the decision to approve Blue 2 violated both the Delaney Clause (which bars cancer-causing food and color additives) and the general safety clause since the dye had not been proven safe (292).

In a statement to the HRG in 1982, Dr. William Lijinsky, a cancer specialist at the NCI's Frederick Cancer Research Center, wrote, ' … the incidence of these (brain) tumors in the high dose group versus the controls is highly significant… In my own laboratory this would be

considered prima facie evidence of carcinogenicity of a treatment. This is especially so because this tumor is so rare, and my conclusion is that Blue 2 is a carcinogen, and should be regulated accordingly.' Regarding his own evaluation of the histopathology of brain/spinal cord sections in microslides, Dr. Benjamin A. Jackson, of the FDA's Division of Pathology in the Color and Cosmetics Evaluation Branch, wrote, '… the possibility cannot be outrightly excluded that the compound (Blue 2) itself, its metabolite(s) or a secondary effect induced by the high dose of the color may have acted to increase the number of brain tumors seen in this study.' (292). An administrative law judge found that a lack of certain biological factors, such as gliosis, invasiveness of tumors, a clear dose-response relationship, and an increased latency, outweighed the statistically significant incidence of brain gliomas in the rats. The FDA commissioner then concluded that the evidence supported the notion that Blue 2 was not an animal carcinogen and that the permanent listing of Blue 2 was appropriate (292).

HRG challenged the FDA's decision, contending that the rats may not have been exposed to the maximum tolerated dosage (MTD). According to the FDA's testing guidelines, the highest dosage used in a study 'should be sufficiently high to induce toxic responses in test animals, and should not cause fatalities high enough to prevent meaningful evaluation of the data from the study.' Chronic-study doses '… should be based on results from subchronic studies and other related test substance information.' (121). HRG questioned whether the MTD was used in the chronic toxicity rat study because: (1) no subchronic study was conducted to establish the MTD (the FDA found it acceptable to rely on the results of a previous 1966 study by Hansen); (2) adult rats in the study did not show alterations typical of animals given the MTD according to the FDA Redbook (FDA's guide for the testing of additives); (3) 5% was used as the MTD for the chronic

mouse study discussed below (as opposed to 2% in the rat study); and (4) the Hansen study used a high dose of 5%, which led to an increase in the overall number of tumors compared to other groups. HRG argued that allowing a 2% MTD was contradictory to the FDA's own guidelines (293). Notwithstanding those arguments, the court ruled in favor of the FDA (294).

In another study, 30 Charles River CD-1 mice/sex/group were fed 0.2, 0.4, 0.8, or 1.6% Blue 2 for 84 weeks (295). Controls consisted of 60 males and 60 females. The overall death rates in treated mice did not differ significantly from that in the controls. The most common neoplasms seen in both the control and treated mice were generalized lymphoblastomas and pulmonary adenomas. The incidence of lymphoblastomas was not associated with the feeding of Blue 2. There was a significant increase in the incidence of pulmonary adenomas in the lowest-dose treatment group in males compared to controls. That increase was not seen in higher-dosage males or in females and, therefore, was not considered by the authors to pose a risk to humans. In this study the NOAEL was determined to be 0.4% of the diet or approximately 600 mg/kg/day. With a safety factor of 100, that translates into an intake of about 360 mg/day for a 60 kg person (295). This study was flawed because of its brevity — Charles River CD-1 mice often live to well over 2 years — because the mice were not exposed *in utero*, and because the numbers of mice exposed to each dosage were small (253).

Borzelleca *et al.* also conducted a carcinogenicity/toxicity study of Blue 2 in mice. That study did not include an *in utero* phase. Blue 2 was fed to 60 Charles River CD-1 mice/sex in 0 (two control groups), 0.5, 1.5, and 5% groups. The study lasted 22 months for males and 23 months for females —longer than the Hooson study discussed above, but still shy of 2 years, let alone

the lifetime of the mice. The investigators concluded that Blue 2 did not cause any significant effects on behavior, morbidity, mortality, hematology, or physical observation and considered the NOAEL to be 5%, or 8259 mg/kg bw/day in male CD-1 mice and 9456 mg/kg bw/day in female CD-1 mice (296).

Reproductive toxicity/teratogenicity

Borzelleca *et al.* conducted a three-generation reproductive study of Blue 2 in Charles River CD rats. Groups of 10 males and 20 females were fed the dye at levels of 0, 2.5, 25, 75, or 250 mg/kg bw/day. Retinoic acid, a known teratogen in rats, was used as a positive control. Treated parents and pups were normal in terms of general appearance and behavior. The compound was not teratogenic and did not affect fertility, length of gestation, viability, or lactation indices. The compound did not cause anatomical abnormalities in the uteri or ovaries of females given caesarian sections. There were also no compound related effects on organ weights and gross and microscopic pathological lesions (297).

Borzelleca *et al.* tested the potential teratogenicity of Blue 2 in Charles River CD rats and Dutch Belted rabbits. Twenty pregnant rats/group received 0.5% methacol (a vehicle control), 7.5 mg/kg/day retinoic acid (a positive control), or 25, 75, or 250 mg/kg/day Blue 2. Ten pregnant rabbits/group followed the same regimen as the rats, except that 150 mg/kg/day thalidomide was used as a positive control in place of retinoic acid. Investigators reported no compound related adverse effects on maternal appearance, behavior, body weight, or mortality. There were also no adverse effects on fetal body weight, viability, or abnormalities. The NOAEL for Blue 2, on the basis of this study, was determined to be 250 mg/kg/day in rats and rabbits (298).

Conclusions

Two chronic toxicity/carcinogenicity studies of Blue 2 in mice did not find any problems, but they were flawed because they did not include an *in utero* phase and were shorter than 2 years. More worrisome was a chronic toxicity/carcinogenicity study in rats that found that males in the 2% group had statistically significant increases in brain gliomas and malignant mammary gland tumors. The FDA found reasons to excuse that evidence of carcinogenesis and neoplasia and approved the continued use of the dye.

Given the statistically significant occurrence of tumors, particularly brain gliomas, in male rats, Blue 2 cannot be considered safe for human consumption. The evidence on Blue 2 certainly does not meet the legal standard for safety: 'that there is convincing evidence that establishes with reasonable certainty that no harm will result from the intended use of the color additive …'[emphasis added] (163). Since Blue 2 is a non-nutritive color additive that does not provide any health benefit, and there is hardly 'convincing evidence' of safety, it should not be permitted in the food supply.

Citrus Red 2

Citrus Red 2 (Fig. C.4) is an azo dye approved only to color the skins of Florida oranges not used for processing. Amounts are permitted up to 2 parts per million (ppm) in the whole fruit (299). Only about 1500 pounds of this dye are certified annually, but that is enough to color about two billion oranges.

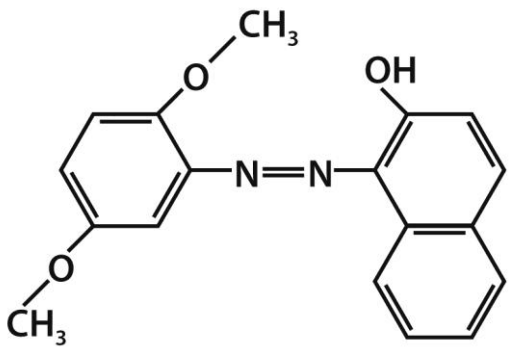

Figure C.4. Citrus Red 2

Metabolism

Radomski *et al.* administered a single oral dose of Citrus Red 2 to rats, dogs, and rabbits. Rats given a single oral dose of 2–20 mg excreted 5–7% of intact dye in their feces over 48 hours. Similar to water-soluble azo dyes, this water-insoluble dye is broken down in the GI tract by intestinal bacteria. One breakdown product is 1-amino-2-naphthol, which has been shown to cause bladder cancer in mice (300). At single doses higher than 5 mg, the dye accumulated in the fat of rats. Small amounts of 1-amino-2-naphthyl sulfate were found in the urine of rats, demonstrating that the 1-amino-2-naphthyl metabolite is absorbed, sulfonated, and then Excreted (238).

Chronic toxicity/carcinogenicity

In one study, 50 mice/sex/group were fed Citrus Red 2 at levels of 0, 0.01, 0.03, 0.1, 0.3, 1, or 3% of their diet. The study lasted up to 80 weeks, an inadequate duration. The study was

discontinued in the 0.3, 1, and 3% groups due to increased morbidity and mortality. Mice in the 0.1% group also experienced increased mortality, and females showed degeneration of the liver (301).

The same researchers conducted a study with 50 mice/sex injected subcutaneously with 10% Citrus Red 2 for 35 weeks, followed by injections every 3 weeks for 15 weeks. The control group received only vehicle injections. Female mice showed an increase in total malignant tumors, which appeared earlier than tumors in the control group. The most common malignant tumors were adenocarcinomas of the lung and lymphosarcomas. There were no injection-site tumors (301).

Hazleton Laboratories conducted a chronic feeding study in rats. The toxicological data were evaluated by the director of FDA's Division of Pharmacology, A. J. Lehman, who concluded that the synthetic dye is toxic. In this study, 40 rats/sex/group were fed Citrus Red 2 at doses of 0, 0.05, 0.1, 0.5, 1, 3, and 5%. Rats in the two highest dosage groups were sacrificed after 31 weeks because of severe toxicity. The remainder of the rats remained in the study for 104 weeks. Rats in the 0.5% and 1% groups experienced differences from controls in gross appearance, growth, organ weights, and gross and microscopic pathology. At the 0.1% levels, rats showed differences in organ weights, incidence of edema-like swelling, a possible trend toward an increased incidence of fatty metamorphosis (fat droplets in the cytoplasm of cells), and a significant difference in weight gain in females. Researchers did not report an increase in the occurrence of tumors. The NOEL (no observed effect level) was judged to be 0.05% (500 ppm) (302).

Dacre administered Citrus Red 2 for 24 months to 20 mice and 20 albino rats per dosage group (303). The dye was given at dosages of 0, 0.05, and 0.25% beginning immediately after weaning, without *in utero* exposure. This study found hyperplasia and a thickening of the urinary bladder wall in both treatment groups in rats and mice. Of greater concern, 2 out of 20 mice that were examined developed benign papillomas and one male mouse developed a malignant papilloma in the urinary bladder, and four out of 28 rats that were examined developed benign papillomas. About the same number of pathological changes were seen in the low- and high-dosage groups in both species and sexes. No problems were seen in control animals (303).

An internal FDA memo expressed concern about the carcinoma seen in Dacre's mouse study because benign tumors and hyperplasia also were seen (246). FDA veterinarian Kent J. Davis wrote, '… this becomes a level of meaningful significance to cancer research workers.' He added, Citrus Red 2 then becomes an intolerable human health hazard if only from the amounts consumed from fingers after peeling oranges treated with this dye. (Some additional dye may be ingested with peel or orange.) The continued certification and use of this color may also be a violation …of the Federal Food, Drug, and Cosmetic Act as amended which prohibits use of any carcinogenic color additive for uses which may result in ingestion of part of such additive.

Conclusions

Citrus Red 2 is toxic to rats and mice at modest levels and, according to an FDA scientist and the IARC, is a bladder carcinogen (304). The FAO/WHO Expert Committee on Food Additives stated bluntly, 'This color should not be used as a food additive' (305).

FD&C Green 3

FD&C Green 3 (Fig. C.5), or Fast Green FCF, is a synthetic dye approved for use in food, drugs, personal care products, and cosmetics except for in the area of the eye. It is one of the least-used dyes (Table 4.4), but may be found in candies, beverages, dessert powders, ice cream, sorbet, and other foods, as well as in ingested drugs, lipsticks, and externally applied cosmetics (306). The ADI for Green 3 is 2.5 mg/kg bw/day, or 75 mg/day for a 30-kg child (307). Current usage is equivalent to only 0.1 mg/person/day.

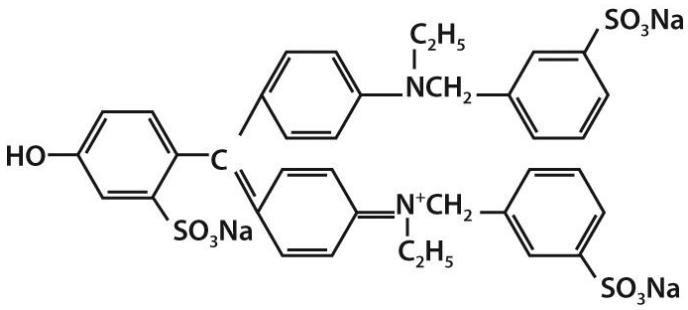

Figure C.5. FD&C Green 3 (Fast Green FCF)

Metabolism

Hess and Fitzhugh studied the metabolism of Green 3 in rats and dogs. Three female and 3 male Osborne–Mendel rats were orally administered a single 200-mg dose of Green 3. An average of 94% of the dye was excreted intact in the feces. No recovery from the urine was reported. Male and female bile duct-cannulated dogs were orally administered a single 200-mg dose of Green 3. None of the color was found in the urine and about 2% of the dye was recovered in the bile of two of three dogs. Hess and Fitzhugh calculated the absorption of the dye from the GI tract of rats and dogs to be y5% (275).

Genotoxicity

As Table C.3 indicates Green 3 was mutagenic in the *S. Typhimurium* strain TA100 Ames Assay at 10 mg/plate. That assay tests for base-pair mutations, and Green 3 only yielded positive results when tested as a mixture of several batches of dye of varying purity (179). Green 3 was also positive for mutagenicity in a Fischer rat embryo cell transformation assay (308). That particular assay tests for malignant cell transformation, an indicator of carcinogenic potential. Green 3 was positive at 1 mg/ml but, surprisingly, produced negative results at higher concentrations. In summary, three of nine studies indicated mutagenicity, but the data overall do not necessarily indicate a human health risk.

Chronic toxicity/carcinogenicity

In 1977, the FDA required that additional chronic toxicity studies be conducted before Green 3 could become a permanently listed food coloring (309). To fulfill that requirement, the CCMA sponsored chronic feeding studies in mice and rats.

In the first study, Green 3 was administered to 60 Charles River albino rats/sex/group at dosage levels of 0 (two control groups), 1.25, 2.5, and 5% for at least 2 months prior to mating. After reproduction, 2, 3, or 4 pups/sex/litter/group were randomly selected for the long-term study. The same dosage levels used in the *in utero* phase were administered to 70 rats/sex/group for approximately 30 months. No significant effects were noted during the *in utero* phase except

Table C.3. Summary of genotoxicity studies on Green No. 3.					
Assay	Mutation Type	S9 Activation	Dose	Results	Reference
Comet Assay	DNA damage	NA	2,000 mg/kg	Negative	(181)
S. Typhimurium TA100	Base Pair	Yes	10 mg/plate	Positive (in crude sample)	(179)
S. Typhimurium TA92, TA1535	Base Pair	Yes and No	10 mg/plate	Negative	
S. Typhimurium TA1537, TA94, TA98	Frameshift	Yes and No	10 mg/plate	Negative	
Chromosomal aberration test, CHL cells	Chromosomal aberrations	No	4 mg/ml	Positive	
Diploid yeast *Saccharomyces Cerevisiea* (BZ 34)	Mitotic gene conversion	No	5 mg/ml	Negative	(243)
Fischer rat embryo cell transformation	Malignant cell transformation (indicator of carcinogenic potential)	No	1 μg/ml	Postive (Negative at 10 and 100 μg/ml)	(310)
S. Typhimurium TA1535 and TA100	Base Pair	Yes and No	50 μg/plate	Negative	(198)
S. Typhimurium TA1538, TA98, and TA1537	Frameshift	Yes and No	50 μg/plate	Negative	

that pup mortality was increased in the mid- and high-dose groups of the F_1 generation. In the F_1 generation, a significant decrease in survivorship was seen in all treated groups of males and females, but there was no dose–response trend, making that decreased survivorship difficult to

interpret. Urinalysis, hematologic parameters, physical observations, and ophthalmology did not indicate any adverse effects of Green 3 (311).

Histopathological examination revealed that the high-dose group of male rats had increased incidences of urinary bladder transitional cell/urothelial neoplasms, testes Leydig's cell tumors (usually rare and benign in humans), and liver neoplastic nodules. Statistical analyses found that the increased incidences were significant for the urinary bladder transitional cell/urothelial neoplasms (P=0.04, Bio/Dynamics analysis) and testes Leydig's cell tumors (P=0.04, FDA analysis), when compared to combinedcontrols (311). Mark Nicolich, a statistician working at the company that conducted the study, stated, 'Therefore, there is statistical evidence that the high dose of the test material increases the occurrence of certain types of tumors in rats' (312). Nevertheless, FDA scientists concluded that the tumors in the testes were not compound-related because they are common in aged rats (but the concurrent control groups should control for that) and because the numbers of tumors in the low-dose and high-dose groups were comparable (though it is possible that the maximum rate of tumors occurred in the low-dose group). Regarding the urinary bladder neoplasms, the original report submitted by the petitioners stated that the high-dose male rats had a significantly increased incidence of those benign tumors. However, in the final submission, the petitioners submitted an addendum claiming, without any specific justification, lack of statistical significance. The FDA pathologists concluded that neither the incidence nor the severity of the transitional cell hyperplasia of the urinary bladder was treatment related (306).

In the CCMA-sponsored chronic toxicity/carcinogenicity study on Charles River CD-1 mice, 60 mice/ sex/group were fed 0 (two control groups), 0.5, 1.5, or 5% Green 3 in their diet for 24 months. The mice were not exposed to Green 3 *in utero*. No gross or microscopic neoplastic and non-neoplastic observations related to administration of the color were observed. Statistical analysis concluded that Green 3 did not have any negative effect on time-to-tumor, survivorship, or tumor incidence in mice (312).

Conclusions

Green 3 did not increase tumor rates in CD-1 mice, though the only study did not include *in utero* exposure. Green 3 caused significant increases in bladder transitional cell/urothelial neoplasms and testes Leydig's tumors in high-dose male rats. Despite a last-minute assertion by the testing laboratory that the bladder neoplasms were no longer statistically significant and the FDA's dismissal (based on qualitative considerations, not statistical analyses) of the significance of the testes tumors, Green 3must remain suspect until further testing demonstrates that it is safe. Evidence of safety is not 'convincing,' as FDA regulations require (163).

Orange B

Orange B is an azo dye (Fig. C.6) that is approved by the FDA for use only in frankfurter and sausage casings up to 150 ppm in the finished food (313). Batches of Orange B have not been certified for use in the past decade or longer.

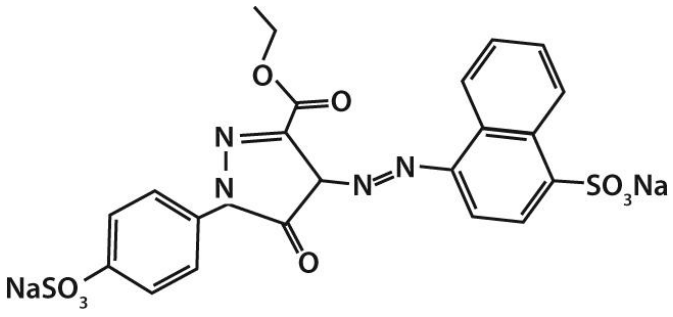

Figure C.6. Orange B

Metabolism

Orange B is poorly absorbed in rats. The color is reduced in the gut to form naphthionic acid. That metabolite appears in both the feces and the urine, indicating that some of the metabolite is absorbed (314).

Chronic toxicity/Carcinogenicity

Orange B was fed to 50 Sprague–Dawley rats/sex/group at doses of 0, 0.5, 1, 2 or 5% for 2 years (an *in utero* phase was not included). By the end of the second year, all of the rats in the 2% group and most in the remaining groups (including the control groups) were dead. Male and female rats in the two highest-dose groups showed lymphoid atrophy of the spleen and bile-duct proliferation. All examined animals in the highest-dose group experienced moderate chronic nephritis, but increased tumor rates were not reported. Investigators gave Orange B a NOAEL of 0.5% for rats (314).

Orange B was fed to 50 C3H mice/sex/group and 50 C57BR/cd mice/sex/group at doses of 0, 1, or 5% dietary supplement for their lifespans (the mice were not exposed *in utero*). There was no effect on tumor development or lifespan. The growth rate of the C3H mouse was depressed in the 5% groups. Investigators assigned a NOEL of 1% to mice (314).

Conclusions

In 1978, the FDA proposed banning Orange B, but, presumably because companies stopped using it, the FDA never bothered to finalize the ban; it should do so now (315).

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