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Lack of genotoxicity *in vivo* for food color additive Tartrazine

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ABSTRACT

Tartrazine is approved as a food color additive internationally with INS number 102, in the United States as food color subject to batch certification "Food, Drug, and Cosmetic" (FD&C) Yellow No. 5, and in Europe as food color additive with E number 102. In their evaluation of the color (2013), the European Food Safety Authority (EFSA) expressed concerns of potential genotoxicity, based primarily on one genotoxicity study that was not conducted according to Guidelines. The present *in vivo* genotoxicity study was conducted according to OECD Guidelines in response to EFSA's request for additional data. The animal species and strain, and the tissues examined were selected specifically to address the previously reported findings. The results of this study show clear absence of genotoxic activity for Tartrazine, in the bone marrow micronucleus assay and the Comet assay in the liver, stomach, and colon. These data addressed EFSA's concerns for genotoxicity. The Joint WHO/FAO Committee on Food Additives (JECFA) (2016) also reviewed these data and concluded that there is no genotoxicity concern for Tartrazine. Negative findings in parallel genotoxicity studies on Allura Red AC and Ponceau 4R (published separately) are consistent with lack of genotoxicity for azo dyes used as food colors.

1. Introduction

Tartrazine (chemical name: trisodium salt of 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-4-[4-sulfophenyl-azo]-1H-pyrazole-3-carboxylic acid) is a color additive permitted for use in foods and beverages, dietary supplements, pharmaceuticals, and other consumer products worldwide. In the United States, it is approved as a "Food, Drug, and Cosmetic" (FD&C) color additive listed by the name FD&C Yellow No. 5 in the Code of Federal Regulations (21 CFR 74.705) and is subject to batch certification. In the European Union (EU), it is approved as a color additive with E number 102 (E102), and internationally it is an approved food color listed in the Codex Alimentarius with International Numbering System (INS) number 102 (INS 102). Tartrazine was most recently evaluated for its safety as a food additive by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at their 2016 meeting (JECFA, 2016). It was previously evaluated by European bodies, including the EU Scientific Committee for Food (SCF) in 1975 and 1984 and by the European Food Safety Authority (EFSA) in 2009 and 2013 (EFSA, 2009; European Food Safety Authority, 2013). Until recently, JECFA and SCF had established an Acceptable Daily Intake (ADI) of 0–7 mg/kg body weight (bw)/day for Tatrazine, based on a NOAEL dose equivalent to 750 mg/kg bw/day derived from a chronic toxicity study in rats. As of its last Scientific Opinion of 2013, EFSA has maintained the previously established ADI. In the most recent safety evaluation in 2016, JECFA increased the ADI to 0–10 mg/

Abbreviations: CP, Cyclophosphamide; EFSA, European Food Safety Authority; EU, European Union; FD&C, Food, Drug, and Cosmetic; GLP, Good Laboratory Practices; INS, International Numbering System; JECFA, Joint FAO/WHO Expert Committee on Food Additives; MMS, Methyl methanesulfonate; MnPCE, micronucleated PCE; NCE, normochromatic erythrocytes; OECD, Organisation for Economic Co-operation and Development; PCE, polychromatic erythrocytes; SCF, Scientific Committee for Food; US FDA, United States Food and Drug Administration.

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kg bw/day (JECFA, 2016), on the basis of absence of any convincing evidence of adverse effects up to the highest dose levels tested (≥1000 mg/kg bw/day) in long-term and in reproductive and developmental studies.

An absence of mutagenic potential for Tartrazine has been reported in several *in vitro* studies in *Salmonella typhimurium* (Brown and Dietrich, 1983; Das and Mukherjee, 2004; Ishidate et al., 1984; Izbirak et al., 1990; Pollastrini et al., 1990; Prival et al., 1988) and *Escherichia coli* (Karpliuk et al., 1984; Pollastrini et al., 1990) and in the DNA repair assay in rat hepatocytes (Kornbrust and Barfknecht, 1985). As an azo dye, Tartrazine was subject to mutagenicity concern associated with the possible generation of free amines *in vivo* by azo-reduction. However, Tartrazine and other azo dyes form sulphonated naphthylamines as metabolic products, for which an absence of mutagenicity was previously shown *in vitro* (Jung et al., 1992), including testing under metabolic conditions appropriately modified to ensure the capacity of the system to form free aromatic amines by azo-reduction (Prival et al., 1988; Prival and Mitchell, 1982). Such conditions include the use of flavin mononucleotide (FMN) rather than riboflavin to reduce the azo compounds to free amines, and hamster liver S9 rather than rat liver S9 for metabolic activation (Fujita and Sasaki, 1993, 1995; National Toxicology Program, 2000). Generally, sulphonated aromatic amines lack genotoxic potential in contrast to the genotoxicity of their unsulphonated analogues (Jung et al., 1992).

Positive results for genotoxicity have been reported *in vitro* in chromosomal aberration assays in a mouse fibroblast cell line (Patterson and Butler, 1982) and a Chinese hamster fibroblast cell line (Ishidate et al., 1984), in the Comet assay in human lymphocytes (Soares et al., 2015), in the micronucleus assay in onion (*Allium cepa*) root tip cells (Roychoudhury and Giri, 1989), and in wing and eye mosaic tests in *Drosophila melanogaster* (Tripathy et al., 1989). Mixed results have been reported *in vivo* in sister chromatid exchange and chromosomal aberration assays in rats and in mice (Das and Mukherjee, 2004; Durnev et al., 1995; Farag et al., 2001; Giri et al., 1990). In addition, positive results were reported in the Comet assay in the stomach and colon in mice (Sasaki et al., 2002). However, no evidence of DNA damage was found using the micronucleus assay in cells of colon tissue in a study that was performed to probe the effects presented by Sasaki et al. (Poul et al., 2009). The authors of the latter study suggested that the DNA damage reported in the colon of mice with the Comet assay (Sasaki et al., 2002) was transient and might be partly explained by local cytotoxicity that would not result in stable genotoxic lesions. The lack of genotoxic potential is also supported by absence of carcinogenic activity in long term carcinogenicity studies in mice and rats (Borzelleca and Hallagan, 1988a, b). The studies that have reported indications of positive genotoxicity present limitations, such as using non-standard protocols, or show lack of dose-dependence. The suggested genotoxicity for Tartrazine is primarily derived from the Comet assay in mice, reported by one research group (Sasaki et al., 2002) that has also reported similar results for other azo dyes (Sasaki et al., 2002; Shimada et al., 2010; Tsuda et al., 2001). In the Sasaki et al. (2002) study, the DNA damage reported for Tartrazine in the stomach and colon was not dose-dependent and no similar effect was detected in any of the other tissues evaluated including brain, lung, kidney, urinary bladder and bone marrow. The biological significance of these results is unclear in light of prior negative carcinogenicity studies at repeat dose levels in the same species.

The genotoxicity study described herein was conducted to clarify the findings in the Sasaki et al. (2002) study. The test species and other conditions of the present study were selected specifically to reproduce the test conditions in the Sasaki et al. study.

2. Materials and methods

2.1. Compliance with applicable regulations and guidelines

The study described herein was conducted at BioReliance Laboratories Ltd, Rockville, Maryland (Pant, 2016). The study was performed under Good Laboratory Practices (GLP) according to the United States Food and Drug Administration (US FDA) Good Laboratory Practices 21 CFR Part 58, and the Organisation for Economic Co-operation and Development (OECD) Principles on Good Laboratory Practice ENV/MC/CHEM (98) 17 (Revised in 1997, issued January 1998). All procedures involving animals were consistent with the recommended specifications in the most current version of The Guide for the Care and Use of Laboratory Animals adopted by BioReliance (National Research Council, 2011). The Comet assay was conducted according to OECD Guideline 489 (OECD, 2016a) and the micronucleus assay was conducted according to OECD Guideline 474 (OECD, 1997, 2016b).

2.2. Study design

Tartrazine was tested for genotoxicity in male Hsd:ICR (CD-1) mice at three dose levels, 25, 500, and 2000 mg/kg body weight via oral gavage administration on three consecutive days (0, 24 and 45 h). These dose levels were selected without a prior range-finder experiment for determination of the maximum tolerated dose (MTD) because systemic absorption of Tartrazine from the oral route is known to be limited (<10%) and systemic toxicity is not expected from oral administration for this reason. Therefore, testing at a MTD was not determined necessary based on recommendations for non-toxic substances in the related OECD Testing Guidelines (TG 474 and 489) but instead the limit dose of 2000 mg/kg bw/day was applied. Two additional dose levels were tested, also according to the Guidelines. Because this study was conducted to address concerns expressed by EFSA, the dose levels tested were spaced broadly to reach as closely as possible the low dose levels that were tested in the Sasaki et al. (2002) study which was the basis of EFSA's concerns. In addition, because the oral route of administration is relevant to the context of human exposure from food intake, demonstration of systemic exposure was not determined necessary. The test species was selected on the basis of previously reported evidence of genotoxicity in this species in the Sasaki et al. (2002) study. The liver was selected as the site of metabolism to evaluate the potential genotoxicity of metabolites and as a tissue that reflects bioavailability of a test substance. Stomach and colon were selected as the sites of first contact to assess genotoxicity of Tartrazine in the GI tract prior to metabolic activation. Lastly, the Comet assay was performed in whole cells isolated from relevant tissues, and not in isolated nuclei as described in the Sasaki et al. study.

2.3. Test substance and control substances

A US FDA certified batch of Tartrazine (Chemical Name: disodium salt of 6-hydroxy-5-[(2-methoxy-5-methyl-4sulfophenyl) azo]-2-naphthalenesulfonic acid; CAS Number 25956-17-6, batch number 5257824, Lot AV7101), a red powder with a purity of >85%, was supplied by Sensient Colors, LLC (St Louis, MO, USA). Formulations with Tartrazine were prepared fresh prior to dosing by mixing with deionized water at volumes required to produce the appropriate doses. Methyl methanesulfonate (MMS; CAS Number 66-27-3; Sigma-Aldrich) was used as positive control for the Comet assay at a dosing formulation of 4 mg/mL in saline (0.9% sodium chloride for injection). Cyclophosphamide (CP, 50 mg/kg bw; CAS